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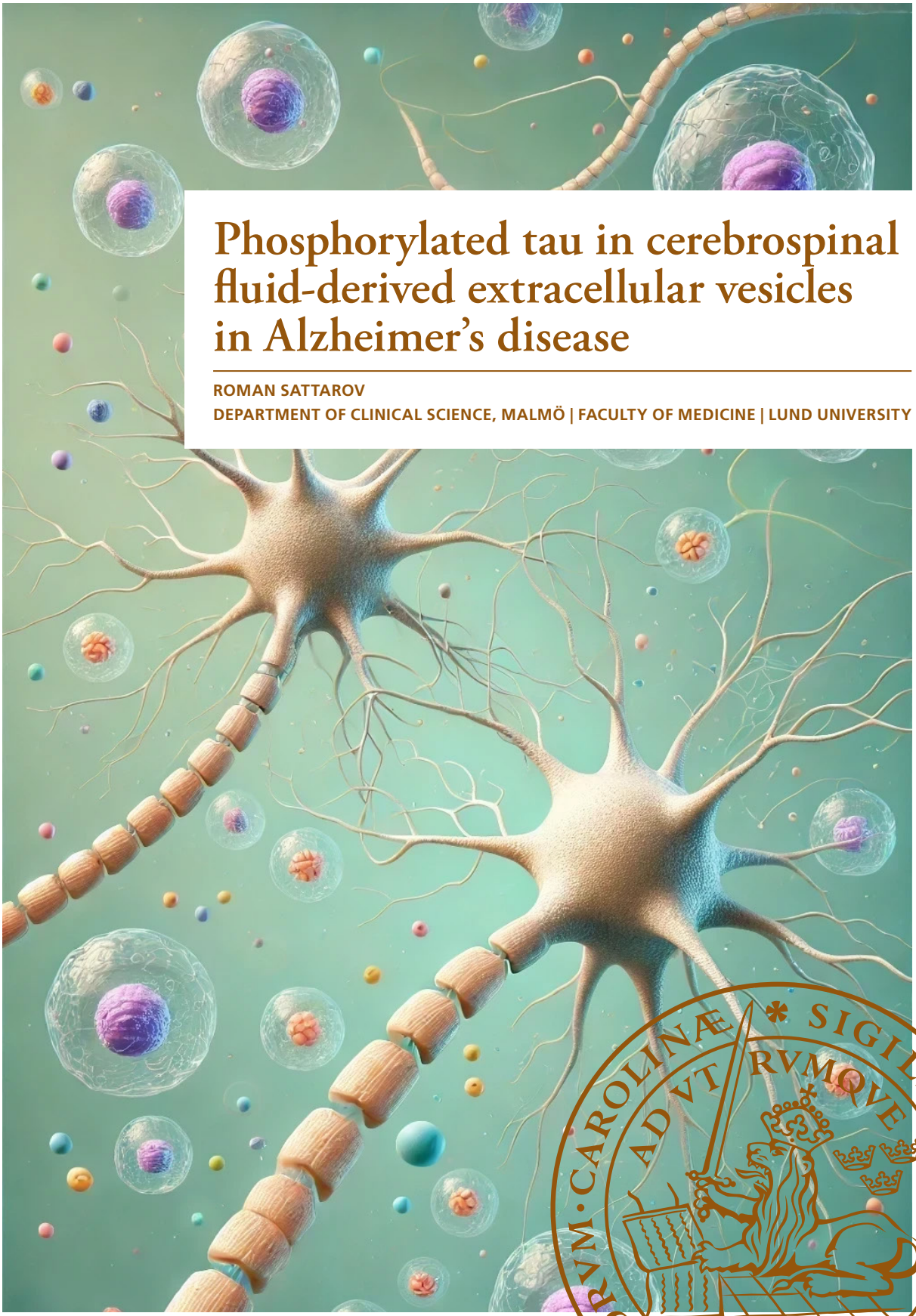

Phosphorylated tau in cerebrospinal fluid-derived extracellular vesicles in Alzheimer's disease

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Title and subtitle: Phosphorylated tau in cerebrospinal fluid-derived extracellular vesicles in Alzheimer's disease.

Abstract:

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder characterized by the accumulation of β-amyloid (Aβ) peptides and phosphorylated tau (P-tau) proteins in the brain, leading to cognitive decline and memory impairment. Despite extensive research, the exact mechanisms of AD pathogenesis remain elusive, and reliable biomarkers for early diagnosis and disease monitoring are still needed. Extracellular vesicles (EVs), including exosomes (~50–150 nm diameter) and microvesicles (~0.1–1.0 μm diameter), are considered multifunctional molecular complexes that act as mediators of intercellular communication, with roles in maintaining homeostasis and facilitating the exchange of information in both physiological and pathological conditions. Furthermore, EVs have emerged as potential biomarkers due to their ability to carry proteins related to the key pathologies in AD, such as P-tau and Aβ. EVs may facilitate the spread of these proteins between cells, potentially propagating pathological changes throughout the brain. While numerous studies have examined plasma-derived EVs in AD, limited research has focused on cerebrospinal fluid (CSF)-derived EVs, which may more closely reflect brain changes.

Therefore, this study aims to isolate EVs from CSF using a novel acoustic trapping method and to characterize their content for AD-related biomarkers. We included CSF samples from 20 patients with AD and 20 cognitively unimpaired (CU) individuals. The setup (AcouTrap 2, AcouSort AB) was employed to isolate EVs from minimal volumes (75 μL) of CSF, and then isolation of EVs was confirmed through transmission electron microscopy (TEM) and the presence of EV-specific markers such as CD9, CD63, CD81 and ATP1A3 (a marker specific for brain-derived EVs) and nanoparticle tracking analysis (NTA) for size and concentration. For Meso Scale Discovery immunoassays, the EVs were lysed and their content in the form of levels of two variants of P-tau: P-tau181 and P-tau217 were measured.

Our findings demonstrate that EVs can be efficiently isolated from CSF using acoustic isolation, providing a sample suitable for further analysis, without other fragments. Although there were no significant differences in counts or size between EVs isolated from AD and CU CSF, the results revealed that AD patients exhibited higher levels of P-tau181 and lower levels of P-tau217 in CSF-derived EVs compared to CU individuals. This differential presence of P-tau variants opens for future studies of tau biology in AD. It suggests that the ratio of P-tau181 to P-tau217 within EVs could serve as a novel biomarker for AD.

Additionally, this study is the first to utilize acoustic trapping for EV isolation from CSF, which may have advantages over traditional methods such as ultracentrifugation, including higher throughput, reduced sample requirements, and enhanced purity. This innovative approach could significantly impact clinical practices by providing a more practical and reliable method for EV-based biomarker discovery. Overall, this research shows distinct differences in P-tau variants within EVs from AD patients compared to CU individuals, highlighting the potential of EVs as biomarkers for AD and suggesting a new way to investigate AD pathophysiology.

Keywords: Alzheimer's disease, extracellular vesicles, P-tau181, P-tau217, acoustic trapping

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Original Papers

This thesis is based on the following original manuscript:

Roman Sattarov, Megan Havers, Camilla Orbjörn, Erik Stomrud, Shorena Janelidze, Thomas Laurell, Niklas Mattsson-Carlgren **Phosphorylated tau in cerebrospinal fluid-derived extracellular vesicles in Alzheimer's disease.** The manuscript was submitted to Scientific Reports.

Papers outside the thesis

Roman Sattarov, Håkan Toresson, Camilla Orbjörn, Niklas Mattsson-Carlgren **Direct Conversion of Fibroblast into Neurons for Alzheimer's Disease Research: A Systematic Review** J Alzheimers Dis 2023 95(3):805-828.

Roman Sattarov, Camilla Orbjörn, Malin Parmar, Sara Linse, Thomas Roos, Håkan Toresson, Niklas Mattsson-Carlgren Induced. **Neurons as a Model to Study Alzheimer's Disease to study Biomarkers such as β-Amyloid and pTau181 and 217**. Manuscript in preparation.

Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder characterized by the accumulation of β-amyloid (Aβ) peptides and phosphorylated tau (P-tau) proteins in the brain, leading to cognitive decline and memory impairment. Despite extensive research, the exact mechanisms of AD pathogenesis remain elusive, and reliable biomarkers for early diagnosis and disease monitoring are still needed. Extracellular vesicles (EVs), including exosomes (~50–150 nm diameter) and microvesicles (~0.1–1.0 μm diameter), are considered multifunctional molecular complexes that act as mediators of intercellular communication, with roles in maintaining homeostasis and facilitating the exchange of information in both physiological and pathological conditions. Furthermore, EVs have emerged as potential biomarkers due to their ability to carry proteins related to the key pathologies in AD, such as P-tau and Aβ. EVs may facilitate the spread of these proteins between cells, potentially propagating pathological changes throughout the brain. While numerous studies have examined plasma-derived EVs in AD, limited research has focused on cerebrospinal fluid (CSF)-derived EVs, which may more closely reflect brain changes.

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discovery. Overall, this research shows distinct differences in P-tau variants within EVs from AD patients compared to CU individuals, highlighting the potential of EVs as biomarkers for AD and suggesting a new way to investigate AD pathophysiology.

Lay summary

Alzheimer's disease (AD) is a progressive and age-related disorder, that causes devastating memory loss, leaving them highly dependable on third parties. Furthermore, a rapidly rising incidence worldwide due to increasing life expectancy results in high burdens to healthcare and caregivers. With a limited understanding of what causes the disease and few treatments available to the public (mainly in the US), finding ways to treat and unravel the pathogenesis of this disease has become more urgent than ever. One of the new developing areas of research involves the tiny particles found in the brain's fluid, known as cerebrospinal fluid (CSF). These particles are called extracellular vesicles (EVs), which can carry important information about what is happening in the brain, especially cell-to-cell communication. Therefore, our study focuses on these EVs to uncover new insights into AD.

One can imagine EVs as tiny “delivery trucks” in the brain, that are released by all brain cells, which transport various cargo that is essential for cells. It includes the delivery of proteins, lipids, and genetic materials, from one brain cell to another. In AD, two particular proteins, β-amyloid (Aβ) and phosphorylated tau (P-tau), are known to accumulate abnormally and cause neuronal damage. Therefore, by examining the cargo of these tiny “EV trucks”, we hope to understand more about how AD develops and progresses.

Our research utilizes a novel method called acoustic trapping to isolate EVs from small-volume samples of CSF. Unlike traditional methods, like ultracentrifugation, are time-consuming and require larger sample volumes, making them less practical for clinical use. Meanwhile, acoustic trapping is faster, gentler, and requires much smaller samples. It is important from a clinical perspective as CSF is precious and its harvest causes inconvenience to patients. In our study, we used CSF samples from 20 patients with AD and 20 cognitively unimpaired (CU) individuals. Using acoustic trapping, we isolated EVs from these samples and examined their cargo, focusing on two variants of the P-tau biomarkers: P-tau181 and P-tau217. Think of biomarkers as biological flags that help doctors and researchers identify and track the progression of diseases. These two forms of P-tau are linked to AD and therefore can provide insight into disease processes. Our findings are intriguing, as we demonstrated that EVs from AD patients have higher levels of P-tau181 and lower levels of P-tau217 when compared to those from CU individuals. Moreover, our study is the first to demonstrate that acoustic trapping can effectively isolate EVs from such small CSF samples. This method not only simplifies the process but also ensures that the isolated EVs are of high purity, making the analysis more reliable. It's a significant step forward in the practical application of EV research in clinical settings and research for potentially shedding light on how AD spreads in the brain.

Abbreviations

AD	Alzheimer's Disease
AICD	APP intracellular domain
APOE	apolipoprotein E
APP	Amyloid precursor protein
ATP1A3	transporting subunit alpha 3
Aβ	β-Amyloid
CSF	Cerebrospinal Fluid
CTFα	α-C terminal fragment
CU	Cognitively Unimpaired
EVs	Extracellular Vesicles
ε3	Allele 4
MSD	Meso Scale Discovery
MMSE	Mini-Mental State Examination
NTA	Nanoparticle Tracking Analysis
P-Tau	Phosphorylated Tau
PSEN	Presenilin
sAPPα	Soluble amyloid precursor protein α
TEM	Transmission Electron Microscopy

Introduction

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder, that accounts for 60-80% of all dementia cases. With a rapidly increasing prevalence, with 50 million people living with AD, this number is expected to rise to 75 million by 2030, mainly due to increased life expectancy, putting a huge burden on the economy due to the health care cost (Prince et al., 2015). Early signs of AD are a progressive cognitive decline, which includes memory impairment, behaviour changes, and a decline of social skills. Neuropathologically, the hallmarks of AD are the accumulation of amyloid-β (Aβ) in plaques and tau in neurofibrillary tangles in the brain. These changes are typically associated with neuronal loss and cognitive impairment (Scheltens et al., 2021). Aβ accumulation is established before cognitive impairment (Insel, Hansson and Mattsson-Carlgren, 2021), while aggregation of neurofibrillary tangles and loss of neurons and synapses appear to accrue later, in parallel with the progression of cognitive decline(Serrano-Pozo et al., 2011). When symptoms start before age 65, the syndrome is called early-onset AD (EOAD), which represents ∼5% of AD cases(Mantzavinos and Alexiou, 2017; Palmqvist et al., 2017).

Hallmark and pathology of AD

The rate of amyloid can be accelerated with certain genetic "blueprints", which means genes can escalate disease progression, but not necessarily mean one would develop it. In a small fraction of patients (<1%), who often have EOAD, the disease is caused by autosomal dominant mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2) genes, causing familial forms of AD (FAD) (Breijyeh and Karaman, 2020). However, mutations in APP, PSEN1, and PSEN2 explain 5–10% of the occurrence of early-onset AD (Cacace, Sleegers and Van Broeckhoven, 2016; Van Cauwenberghe, Van Broeckhoven and Sleegers, 2016). Genes, which are part of γ-secretase, are responsible for snipping off APP proteins in the membrane, (**Fig. 1**), resulting in Aβ form that is more prone to form tables in extracellular space and aggregation of which causes neuronal toxicity, (**Fig. 2**). Meanwhile, in late disease progression, tau hyperphosphorylation causes tau to misfold in neurofibrillary tangles (NFTs) within neurons, (**Fig. 2**).

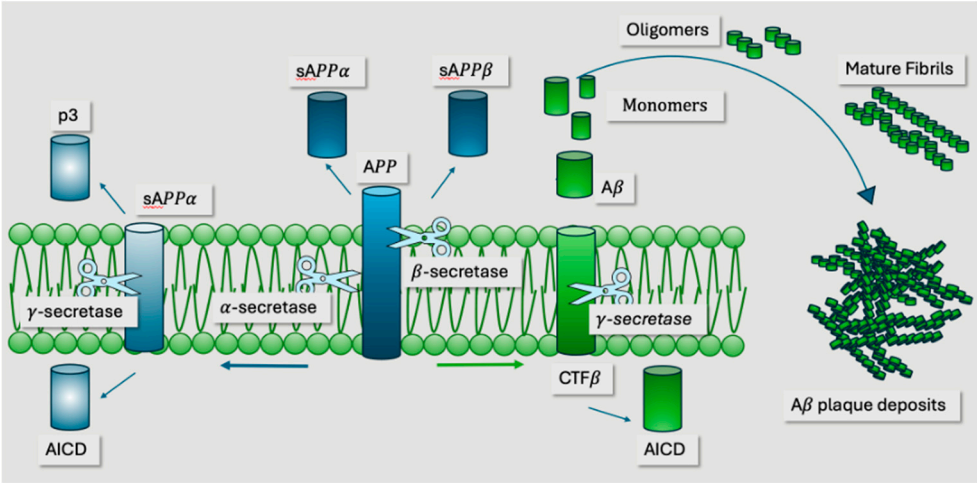

Figure 1. Amyloid precursor protein (APP) proteolytic pathways. APP breakdown in both the non-amyloidogenic and the amyloidogenic pathways. Non-amyloidogenic processing of APP refers to the sequential processing of APP by membrane-bound α-secretases, which cleave within the Aβ domain to generate the membrane-tethered α-C terminal fragment (CTFα) and the N-terminal fragment Soluble amyloid precursor protein α (sAPPα). CTFα is subsequently cleaved by γ-secretases to generate extracellular P3 and the APP intracellular domain (AICD). Amyloidogenic processing of APP is carried out by the sequential action of membrane-bound β- and γ-secretases. β-Secretase cleaves APP into the membrane-tethered C-terminal fragments β CTFβ and N-terminal sAPPβ. CTFβ is subsequently cleaved by γ-secretases into the extracellular Aβ and AICD. A pathway for the conversion of Aβ monomers to higher-order oligomers, protofibrils, and fibrils. Aβ monomers can form higher order assemblies ranging from low molecular weight oligomers, including dimers, trimers, tetramers, and pentamers, to mid-range molecular weight oligomers including hexamers, monomers, and dodecamers to protofibrils and fibrils.

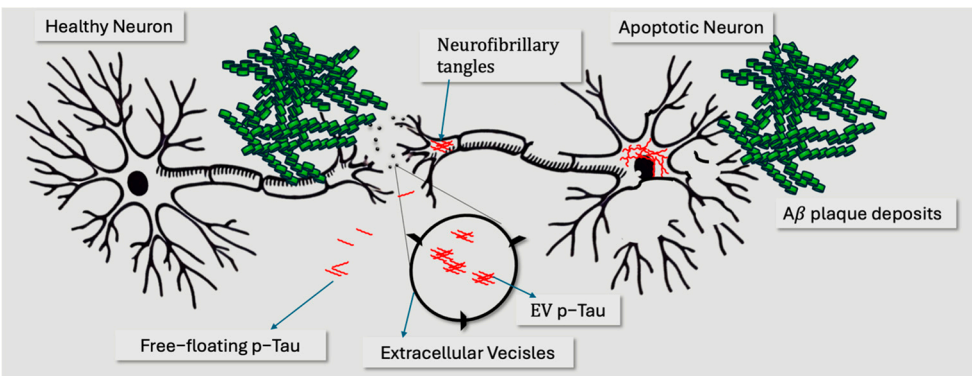

Figure 2. Illustration of a loss of neurons due to cell death in parallel with the Aβ aggregation process and tau tangles accumulation tau propagation. Cell death through apoptosis or necrosis is triggered by the accumulation AD related proteins. This leads to the loss of neurons due to cell death in parallel with the Aβ aggregation process, tau phosphorylation, and the formation of misfolded neurofibrillary tangles (NFTs). Additionally, tau propagation through EVs and extracellular pathways contributes to the spread of neurodegeneration across different regions of the brain.

However, most patients (>99%) have sporadic AD (SAD) which typically occurs past the age of 65, often referred to as late-onset AD (LOAD) (Tanzi, 2012). A combination of risk factors, including age, gender, genetics, high blood pressure (Sáiz-Vazquez et al., 2023), mid-life (51-60 of age) high glucose levels (Zhang et al., 2023a), and environmental factors all contribute to SAD (Selkoe, 2002). Furthermore, a less popular, but promising hypothesis on the potential role of viral(Readhead et al., 2018; Da Mesquita and Rua, 2024) and bacterial infections (Da Mesquita and Rua, 2024) on AD, where Aβ underlined as an antimicrobial peptide (Prosswimmer, Heng and Daggett, 2024).

However, the strongest genetic factor is the ε4 allele of the apolipoprotein E (*APOE*) gene. The pathobiological function of the ε4 allele is complex, but it may affect the clearance pathway of Aβ, as well as potentially also affect tau-mediated neurodegeneration and neuroinflammation (Troutwine et al., 2022). The risk for AD increases and the age of onset of AD declines with an increased number of ε4 alleles.

Advances in the treatment of AD

Currently, there is still no cure for AD. Furthermore, multiple phase II and III studies of Aβ and tau removal treatments have failed (Doody et al., 2014; Salloway et al., 2014; Egan et al., 2019; Wang et al., 2021; Imbimbo et al., 2023), but a few recent studies have demonstrated potentially beneficial effects of anti-Aβ immunotherapies. So far, Aducanumab, Donanemab, and Lecanemab are only three disease-modifying AD treatments that have been approved by the US Food and Drug Administration (FDA) (Cavazzoni, 2021; FDA, 2023; 2024). However, even with the promising Donanemab, participants who carried *APOE4* benefited less from treatment than those who did not have the allele. Furthermore, Donanemab is significantly less effective in patients with high levels of tau (Reardon, 2023). Meanwhile, Lecanemab has been shown to be substantially less effective in women. This is especially significant because women have a twofold increased risk of AD compared to men. Lecanemab did not slow cognitive decline in *APOE4* carriers; rather, it enhanced the decline in study participants with homogeneous ε4 allele (Kurkinen, 2023).

Refining understanding of the disease may accelerate the development of efficient therapies, by providing a better understanding of the mechanisms and processes of ageing the main factor of AD. The open question stands regarding mechanisms that drive LOAD-related gene mutations to cause Aβ or tau pathological changes. Multiple studies point out that tau pathology can develop in LOAD independently of Aβ pathways (Raj et al., 2015; Jack et al., 2019).

Furthermore, several studies pointed out the multiple processes that have been identified as significant for biomarker mediation and AD progression, including immune activation, that may contribute to neuroinflammation in AD (Van Eldik et

al., 2016), exosome metabolism, studies showing the disturbance of lipid metabolism by *APOE* polymorphisms, (Liu and Zhang, 2014; Yang et al., 2023), endosomal vesicle recycling, as it plays a vital role in many cellular processes including communication with the extracellular environment, nutrient uptake, and cell signalling (Zadka et al., 2023), and autophagy, a lysosome-dependent and homeostatic process, dysfunction of autophagy is suggested to lead to the accretion of neurotoxic proteins in the AD brain (Uddin et al., 2018), decreased glucose metabolism, dysregulated glucose, and lipid metabolism are key features of AD, with changes shown to precede classical clinical symptoms by many years. (Dewanjee et al., 2022). Notably, the production and secretion of Extracellular vesicles (EVs) are linked to all these processes (Hessvik and Llorente, 2018; Arbo et al., 2020; Heiston et al., 2022).

Overview of the biogenesis and secretion of EVs

EVs, which include exosomes and microvesicles, are small lipid bilayer structures formed and released by a variety of cells, including neurons, microglia, and astrocytes (Kalluri and LeBleu, 2020). EVs are generated by a specific intracellular pathway that involves the invagination of early endosomes to generate small intraluminal vesicles during early endosome maturation (Kalluri and LeBleu, 2020). Then mature endosomes become multivesicular bodies, which are processed in several pathways and these EVs are categorized into two main classes based on their biogenesis: exosomes and microvesicles. Microvesicles are vesicular structures approximately 0.1–1.0 μm in size, formed by outward blebbing of the plasma membrane, (**Fig. 3**). In contrast, exosomes are generally smaller, around 50–150 nm in diameter, and originate within the endosomal system (Bongiovanni et al., 2021). EVs function as multifunctional molecular complexes that facilitate intercellular communication(Doyle and Wang, 2019).

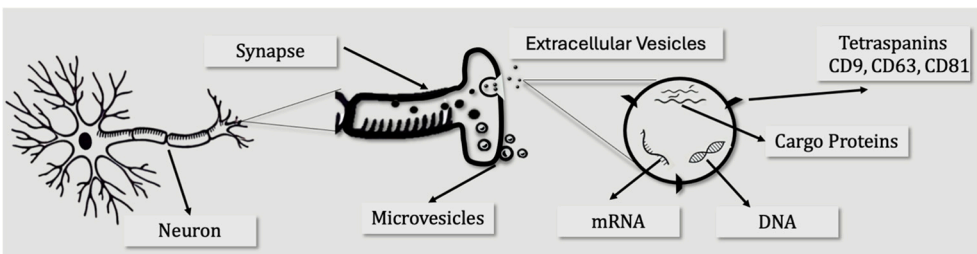

Figure 3. Illustration of extracellular vesicles (EVs) and their secretion pathway. Microvesicles fuse with the plasma membrane to release exosomes into the extracellular space at a synapse. EVs release form by direct budding at the plasma membrane leading to direct release into the extracellular space. EV's cargo may contain, micro ribonucleic acids (mRNAs), deoxyribonucleic acids (DNAs), and proteins. EVs are typically identified by the presence of these 'surface markers' on the particles namely the tetraspanins CD63, CD9, and CD8.

EVs are emerging as a novel form of message exchange via conveying cargo to target cells and maintaining homeostasis in both normal and pathological conditions. In the brain, EVs carry bioactive molecules such as proteins, non-coding RNAs, and lipids, (**Fig. 3**), linked to neuronal function and neurotransmission, thereby supporting communication between neural cells, synaptic plasticity, and neuronal activity. Furthermore, proteins such as tetraspanins and specific proteins as cargo, that EVs carry from their specific cell of origin (Kalluri and LeBleu, 2020).

However, due to limitations in technology, little is known about which proteins EVs carry and which types of brain cells release these harmful vesicles. Numerous studies consistently highlight the crucial roles of EVs in AD pathogenesis.

Tau and AD pathology

Tau itself is important in neurite outgrowth and axonal development (Lee et al., 1998) in a regular healthy brain environment. However, it is well documented that it also plays a significant role in tau-associated neurodegeneration, such as tau secretion, toxicity, and uptake are moderated by disease-relevant tau changes such as phosphorylation and aggregation of tau in distal dendrites (Lee et al., 2012), cleavage of tau (Guillozet-Bongaarts et al., 2005; Zilka et al., 2010), and oligomerization (Rayman, 2023).

In a mouse model of AD, it has been shown that the spread of tau occurred by the release of EVs containing protein and that depleting microglia reduced the propagation of tau. The study has shown that inhibiting EV release reduces tau propagation in both cell and mouse models (Asai et al., 2015). Furthermore, the study by (Guix et al., 2018) suggests that EV- isolated from plasma, contained more Tau fragments than full-size tau when compared to free-floating in the extracellular medium. This is relevant to AD pathology, as fragmented tau has an increased tendency to aggregate compared to full-length Tau (Lyu et al., 2021). Furthermore, tau is strongly associated with the main AD risk factor aging, as the levels of the tau fragment increase with aging (Matsumoto et al., 2015; Aquino Nunez et al., 2022). While it is not clear how a protein such as tau can move from cell to cell in AD (Gibbons, Lee and Trojanowski, 2019), previous reports have suggested that this may involve EVs (Saman et al., 2012a; Wang et al., 2017).

Aβ and tau in EVs

Tau is secreted by exosomes, and the extracellular appearance of tau and independent of neuronal death, as it is rather part of physiological tau transfer from one neuron to another (Lee et al., 2012). Saman et al., 2012 have shown that EVs associated with phosphorylated tau, particularly p-tau181 are present in CSF samples of AD patients. Furthermore, EVs were significantly enriched relative to

total tau markers in EVs fractions in early AD patients. A pattern was observed in early AD, being marked in patients with Braak stage III neurofibrillary pathology but progressively less so in later stages of AD and was absent from non-AD controls diagnosed with vascular or Lewy body dementia. This indicates that the initial elevation of P-tau levels in AD is not associated with passive, nonspecific tau release consequent to neuron death, since only a small proportion of the brain is involved in neurofibrillary degeneration at Braak stage III when limited cognitive dysfunction is initially observed (Braak and Braak, 1991). Meanwhile, AD EVs p-tau181 levels were similar to those of non-AD control patients in later Braak stages V to VI of AD (Saman et al., 2012b). Additionally, the mouse model (Guo et al., 2016) demonstrated efficient tau propagation potency in EV-tau in comparison with vesicle-free tau, suggesting that EVs show higher transmissibility of tau than free tau. Meanwhile, tau phosphorylation itself facilitates tau transmission and propagation (Takeda et al., 2015), EVs secretion mechanisms offer clues to new avenues of research into how tau misprocessing and producing pathological changes may be connected to AD pathology (Takeda et al., 2015).

Furthermore, it has been shown that aggregation-prone proteins such as Aβ, α-synuclein, and prion protein are also secreted via EVs (Fevrier et al., 2004; Rajendran et al., 2006; Emmanouilidou et al., 2010). For Aβ, it has been shown that neurotoxic, oligomeric forms of this protein are associated with EVs isolated from brain tissue and that these vesicles can facilitate intraneuronal propagation of Aβ. EVs that contain Aβ were also shown to be neurotoxic to primary cultured neurons indicating, at least in vitro (Sardar Sinha et al., 2018). This makes EVs-tau secretion in early AD particularly interesting, as those proteins interact with tau and intensify the oligomerization and neurotoxicity of protein (Cushman et al., 2010). In conclusion, these observations suggest that neuronal EVs may act as vehicles for the intercellular transport of APP and its catabolites, Aβ, and exert neurotoxicity in AD processes.

Aims and Hypothesis

Previous studies have primarily focused on plasma-derived EVs to identify AD biomarkers. However, CSF, being in direct contact with the brain, is a more promising source for studying these markers. Therefore, general aim of this thesis was to seek and provide deeper insights into the molecular changes associated with AD biomarkers and EVs and evaluate the efficacy of EVs as diagnostic and prognostic tools.

Aim 1: Explore the Acoustic Trapping for CSF EVs.

The first aim of this study was to investigate the feasibility and effectiveness of acoustic trapping as a novel method for isolating EVs from CSF. Acoustic trapping is a fast, gentle technique that utilizes sound waves to concentrate and capture particles, such as EVs, and in parallel remove any impurities. This method offers several advantages over traditional isolation techniques, including preserving the integrity of EVs and allowing them to work with as small volumes of CSF that are more relevant for clinical settings.

Hypothesis: Acoustic trapping can effectively isolate EVs from CSF, providing a viable alternative to conventional methods. We hypothesized that this technique would not only allow for the isolation of a broad range of EV sizes but also preserve their structure and the bioactive molecules contained within these vesicles, enabling a more accurate analysis of their potential as biomarkers for AD.

Aim 2: Characterize EVs in AD in Terms of Numbers, Size, Detailed Phenotypes, and P-tau Content.

The second aim of this thesis was to characterize the EVs isolated from CSF in patients with AD and cognitively unimpaired (CU) individuals. This characterization involved a detailed analysis of EV numbers, size distribution, phenotypic markers, and the content of phosphorylated tau proteins (P-tau181 and P-tau217), which are key biomarkers of AD pathology.

Hypothesis: We hypothesized that EVs from AD patients will exhibit distinct characteristics compared to those from CU individuals, and display alterations in their numbers, size, and phenotypic markers, reflecting the pathological processes of AD. Additionally, we expect that the content of P-tau within these EVs will be elevated in AD patients, correlating with the disease's progression and severity.

Materials and Methods

Here are brief descriptions of the methods used in the thesis. For more detailed protocols, see the attached manuscript.

CSF samples selection

The study was carried out in two phases. For preliminary studies to optimize the acoustic trapping for CSF, we used de-identified CSF samples from 10 AD patients and 10 controls. AD patients were selected based on having a positive CSF Aβ42/Aβ40 ratio and a Mini-Mental State Examination (MMSE) score below 25.

For the main experiments, we included CU subjects (n = 20) and AD patients (n = 20). CSF concentrations of the established AD markers Aβ42/Aβ40 and P-tau181 were measured with Meso Scale Discovery (MSD). All AD patients had positive levels of Aβ42/Aβ40 and P-tau181, while all CU individuals had negative levels of these biomarkers. The cut-off points for AD were, for P-tau181 >50 ng/l and for Aβ42/Aβ40 ratio <0.89.

CSF samples were collected by lumbar puncture using a sterile needle and transferred into sterile polypropylene tubes. Care was taken to avoid any contamination during the collection process. All procedures were conducted in accordance with relevant ethical guidelines and approved by the ethical review authority. Informed consent was obtained from all study participants. Samples were de-identified before analysis and provided by obtained from the Memory Clinic at Skåne University Hospital, Malmö.

EVs Isolation from CSF using acoustic trapping

EVs were isolated from CSF samples using an acoustic trapping method, employing the AcouTrap 2 device from AcouSort AB. This method involved trapping EVs within a glass capillary (2 mm by 200 μm) that was coupled with a piezoelectric microtransducer, which created an acoustic standing wave to capture EVs. Unlike prior methods, this study utilized silica beads as seed particles instead of

polystyrene, offering higher retention force and throughput. Before trapping, CSF samples were centrifuged at 100 g for 10 minutes to eliminate large particles. The silica beads formed clusters in the microfluidic channel, where 75 µl of CSF was aspirated and subjected to acoustic trapping at a frequency of 4 MHz and 10 V peak-to-peak voltage. The EVs were subsequently washed with phosphate-buffered saline (PBS) to remove soluble compounds, and then collected by deactivating the ultrasound. The trapped EVs were stored at -80°C for further analysis.

Nanoparticle tracking analysis

The size distribution and concentration of EVs were assessed using Nanoparticle Tracking Analysis (NTA) on a NanoSight LM10 instrument (Malvern Analytical). The trapped EV samples were diluted with particle-free PBS to achieve a 1 ml measurement volume. NTA tracked the Brownian motion of the nanoparticles, allowing for the calculation of size distribution and concentration. Each sample was measured through five 90-second video recordings and analysed with NTA 3.4 software to obtain statistical data.

CSF aspiration volumes and EVs lysis methods

We performed preliminary experiments to optimize the CSF aspiration volume for the EV P-tau217 assay, by lysis EVs and using P-tau217 concentration as the outcome measure. We investigated CSF samples that were aspirated at volumes set at 200 µl, 150µl, 100 µl, 75 µl, 50 µl and 25 µl for acoustic trapping. We have concluded that an aspirated CSF volume of 75 µl is optimum for P-tau assay, which allows for receiving a signal above a lower detection limit and achieving a low CSF consumption.

Multiple experiments have also concluded that EVs were best lysed using 0.5% Tween when compared to Tween, Triton x at a variety of concentrations, as well as physical EVs lysis using bio rapture. NTA has shown a rapid and drastic drop-down of particles counted after mixing isolated EVs with lyse buffer. P-tau assay revealed as well that we see a significant boost in signal in samples with 0.5% Tween. Meanwhile, it did not show a negative effect on raw CSF samples, with a non-significant increase in the signal.

Transmission electron microscopy

CSF and trapped EVs samples derived from one AD and one control patient (selected based on a high concentration of EVs according to NTA) were analysed by Transmission Electron Microscopy **(**TEM). Copper TEM grids (400 mesh) were pre-treated with piliform, carbon coated, glow discharged. Trapped samples were vortexed and diluted with 4 % PFA in a 1:1 ratio and incubated for 10 mins at room temperature (RT) to fix the EVs. Then 10 µl of sample was deposited on each grid and incubated at RT for 20 minutes without drying out. The samples were then labelled, fixed, and stained by floating on top of droplets sequentially as described. Grids were then incubated in PBS for 5 mins. Followed by blocking with bovine serum albumin (1% BSA in PBS) and washing in PBS. Samples were subsequently incubated with chosen primary antibodies, diluted 1:30 in PBS-1%, for 1 hour at RT. We studied the tetraspanins CD9, CD63, and CD81. Primary antibodies used include CD81 Monoclonal Antibody (M38) (Catalogue # 10630D, Thermofisher), CD9 Recombinant Rabbit Monoclonal Antibody (SA35-08) (Catalog # MA5-31980, Thermofisher), CD63 Monoclonal Antibody (Ts63) (Catalog # 10628D Thermofisher), ATP1A3 Monoclonal Antibody (XVIF9-G10) (Catalog # MA3-915 Thermofisher) and GLT-1 Recombinant Rabbit Monoclonal Antibody (9H9L17) (Catalog# 701988 Thermofisher). Grids were then washed three times in PBS for 5 minutes each time. Followed by 40 minutes of incubation using appropriate secondary antibodies diluted in PBS-1% (15 nm gold colloidal goat anti-mouse and/or 10 nm gold colloidal goat anti-rabbit). The grids were washed again with PBS three times for 5 minutes. Samples were then placed in glutaraldehyde (1% in PBS) for 5 minutes at RT and then washed in distilled water for 5 minutes. Finally, the grids were incubated for 5 minutes in 1% uranyl acetate. Samples were then left to dry before sample grids were observed under a transmission electron microscope (FEI Tecnai Biotwin 120 kV).

Meso scale discovery assay

Levels of P-tau181 and P-tau217 in the CSF and EVs samples were measured using immunoassays on the MSD platform (Meso Scale Discovery, Rockville, MD, USA) developed by Lilly Research laboratories. Biotinylated (Thermo Scientific) IBA493 (Lilly) and IBA406 (Lilly) were used as capture antibodies in the P-tau217 and P-tau181 assay, respectively. SULFO-TAG (Meso Scale Discovery) conjugated 4G10-E2 antibody (Lilly) was used as the detector. The assays were calibrated with synthetic p-tau217 and ptau181 peptides. The samples were thawed on wet ice. To perform the assays, MSD small-spot streptavidin-coated plates (Meso Scale Discovery) were blocked for 1 hour at RT with 200 µl of 3% BSA in DPBS with 650 rpm mixing on a plate shaker. The plates were then washed three times with

200 µl of wash buffer (PBS + 0.05% Tween 20) and 25 µl of biotinylated capture antibody (either IBA 406for P-tau181 at 1 µg/ml or IBA493 for P-tau217 at 0.1 µg/ml) were added to the wells and incubated for 1 hour at RT with 650 rpm shaking on a plate shaker. The plates were again washed three times with 200 µl of wash buffer. 50 µl of diluted calibrator or sample, either non-lysed control or treated sample with tween 0.5%, was added to each well and incubated for 2 hours at RT with 650 rpm shaking on a plate shaker. The plates were then washed three times with 200 µl of wash buffer and 25 µl of SULFO-TAG-4G10-E2 detection antibody was added at 0.02 µg/ml to the plates and incubated for 1 hour at RT with 650 rpm shaking on a plate shaker. The plates were washed a final time with 200 µl of wash buffer. Finally,150 µl of 2x MSD Read Buffer T with Surfactant (Meso Scale Discovery) was added to each plate and read on the MSD SQ120 within 10 minutes of read buffer addition. Samples were measured with two technical replicates and the mean of duplicates was used in statistical analysis. Levels of CSF P-tau181 were measured out before that (Cat. # 231654, Fujirebio Diagnostics, US, Malvern, PA) using the Lumipulse G1200 automated immunoassay platform according to the method described (Gobom et al., 2022).

Results

This section summarizes key results from the manuscript. For more detailed results, see the attached manuscript.

Initial Experiments

NTA of EVs and CSF

In a series of initial experiments, we established a fundamental protocol for the study. First, we performed NTA on unprocessed CSF samples (raw-CSF) from AD (n=1) and control samples (n=1). We quantified and compared particle size distribution and concentration (**Fig. 4a**). Data shows the variation in particle size distribution and concentration between raw CSF samples and isolated EVs. Trapped EVs had a mean size of 62.5 nm, compared to 125.2 nm in raw CSF samples, indicating our isolation technique preferentially traps smaller particles. Raw NTA dada for (**Fig. 4a**).

Additionally, to improve the reliability of our results, we planned to expand the sample size. In order to process a large volume of samples we analysed the effect of flow rate on the number EVs trapped, (**Fig. 4b**). Results showed that varying the acoustic trapping flow rates did not affect the number of EVs trapped. During the isolation process, silica seed particles were utilized to capture EVs from CSF. The efficiency of EV trapping was assessed under different washing flow rates. Our results indicated that the trapping efficiency was consistent when the washing flow rate was increased from 30 µl/min to 200 µl/min, with no significant differences observed in the yield of EVs under these conditions.

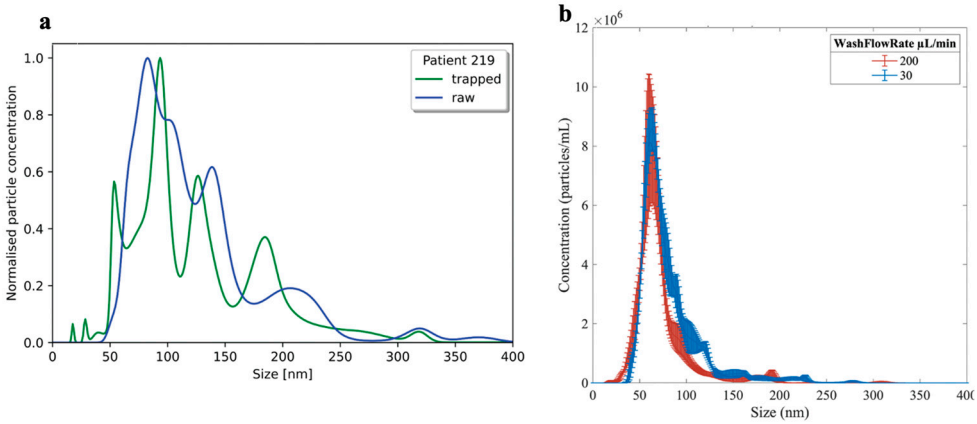

Figure 4. Comparative nanoparticle tracking analysis (NTA) of CSF-derived particles, extracellular vesicles (EVs). Panel (a) Results of NTA performed on raw-CSF (blue) and trapped (green)of a cognitively unimpaired (CU) patient. The CSF was subsequently diluted to a total volume of 1 ml in preparation for NTA. The mean particle size 127 nm and the mode 83 nm. Size distribution of trapped EVs from CSF. The results, aggregated from five separate captures, show that the trapped samples have a mean particle size of 145 nm mode 93 nm. The graphical profiles display an NTA analysis of EVs based on three 90-second videos capturing the trapped EVs. (b) Effects of different wash flow rates on EV yield using NTA. The figure shows the NTA of EVs isolated from the CSF of a healthy control subject. The EVs were trapped using two distinct washing flow rates, set to 200 µl/min or 30 µl/min, to evaluate the impact of flow rate on EV capture efficiency. In each condition, the trapping procedure was performed in triplicate, and the resultant EVs from these three runs were combined. The pooled samples were then diluted to a final volume of 1 ml to facilitate NTA measurement of the concentration of particles and different wash flow rates.

EVs Lysis Methods

We also investigated lysis methods to make sure that we rapture all EVs, thus, releasing enough cargo for MSD to get reading. We compared multiple lysis methods, Bioruptor Plus (an acoustic lysis of EVs), Triton-X, and Tween. The lysis efficiency of different EV rupture methods was validated using NTA. (**Fig. 5**) shows the particle distribution profiles resulting from Triton-X treatment, Tween-20x treatment, and Bioruptor. Each treatment condition was compared to an untreated reference, which represents the intact EV distribution. Tween-20X treatment at 0.5%, demonstrated a significantly higher reduction of EVs after 5 minutes and was superior to Triton-X 0.1% (even after 25 minutes) and Bioruptor. Therefore, we established that Tween-20X treatment at 0.5%, is optimum and thus we used it throughout the study.

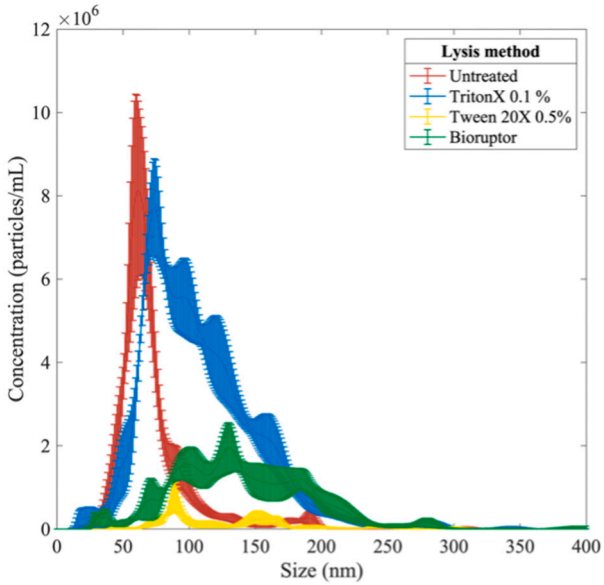

Figure 5. Evaluation of EVs Rupture Methods from Trapped CSF. This analysis was conducted on EVs isolated from the CSF of a healthy control subject, following washing at a flow rate of 200 µl/min. Illustrates particle distribution profiles derived from NTA after applying various EV rupture techniques. The methodologies tested included Triton X at 1% 25 min incubation (represented in Blue), Tween 20X at 0.5% (Yellow), and a mechanical rupture method, Biorupture, performed for 20 cycles (alternating 30 seconds off and on) (Green). A control sample is also presented (Red) for comparison. Each rupture method was assessed for its impact on particle distribution, with results indicating distinctive effects from each treatment compared to the CU.

Aspiration Volumes

We were next interested in the maximum capacity of acoustics. In this initial study, we performed experiments to optimise the CSF aspiration volume for the EV P-tau217 assay, by lysis EVs and using P-tau217 concentration as the outcome measure (**Fig. 6**). The highest P-tau217 concentration was observed at 100 µl, as no significant increase in P-tau217 concentration was noted at 200 µl (data not shown). This indicates that 75 µl is the optimal volume of CSF to aspirate for effective EV trapping, in terms of P-tau217 levels inside EVs. Therefore, for the rest of the experiments, we used a 75 µl aspiration volume. Although we did not quantify the EVs in this experiment, these findings suggest this volume could be close to the maximum capacity for EV trapping. Additionally, the increase in P-tau217 following lysis supports the notion that P-tau217 is transported within EVs. The non-lysed EVs from the 75 µl trapped sample had only 0.15 ng/l of P-tau217, whereas lysis increased the P-Tau217 concentration to 0.33 ng/l.

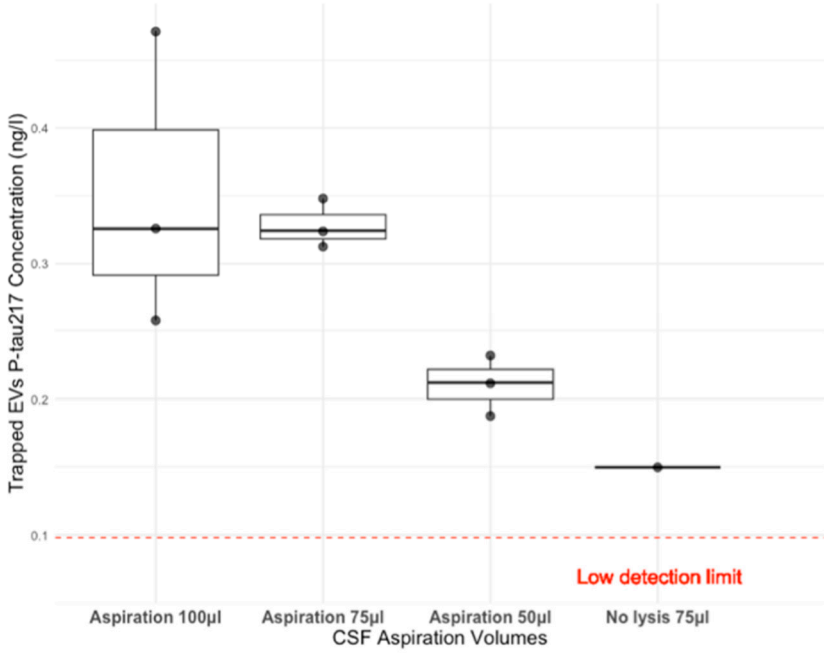

Figure 6. Assessment of P-tau217 levels from lysed EVs from trapped CSF samples using meso scale discovery (MSD). This assay quantifies P-tau217 in EVs isolated from the CSF of an AD patient (n=1) from the preliminary group, from three different MSD plates. Each sample underwent two trapping cycles at specified aspiration volumes to ensure consistency. CSF samples were aspirated at volumes set at 100 µl, 75 µl, and 50 µl for acoustic trapping and then EVs were lysed using 0.5% Tween. In the final column is the signal from a sample trapped from 75 µl but without lysis. The assay's sensitivity was calibrated to detect P-tau217 at a minimum of twice the concentration of the lowest standard, ensuring reliable low-level detection. The low-level detection limit is average n=3.

EVs characterisation with TEM

To support NTA results we carried out a series of TEM experiments alongside immunogold labelling (**Fig. 7**). Targeting the EV-specific marker CD9 and brain-derived marker ATP1A3, we utilized primary antibodies against CD9 and ATP1A3, which were then visualized using secondary antibodies conjugated with gold particles measuring 15 and 10 nm, respectively (**Fig. 7a**). This allowed for specific visualization of the EVs. The analysis included samples from the trapped CSF specimens, which underwent this comprehensive visualization protocol. CD81 positive EVs are depicted in (**Fig. 7b**), using secondary antibodies conjugated with gold particles measuring 15 nanometres. CD63 positive EVs are depicted in (**Fig. 7c**), using secondary antibodies conjugated with gold particles measuring 15 nanometres. An astrocyte-specific EVs maker GLT-1 positive EVs are depicted in (**Fig. 7d**), using secondary antibodies conjugated with gold particles measuring 15

nanometres. Importantly, corresponding negative control images with secondary antibodies revealed no specific binding, data is not presented. Furthermore, we did not observe any "cup-shape" EVs, indicating that EVs were not dehydrated, or damaged during or damaged during (Zhao et al., 2021).

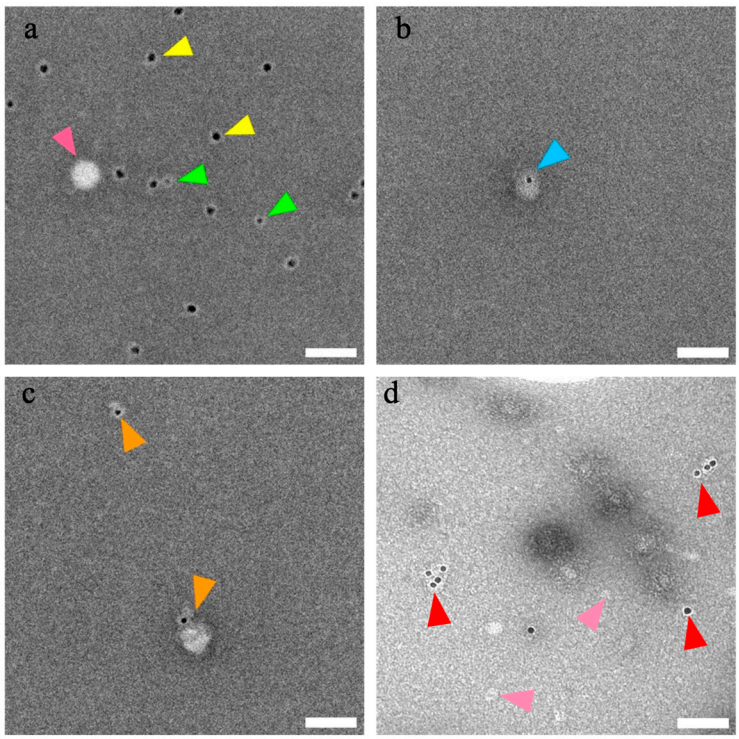

Figure 7. TEM analysis of extracellular vesicles in trapped CSF. This figure shows TEM images resulting from the immunogold labelling technique to visualize EVs isolated from CSF. (a) EVs have been immunolabeled with primary antibodies against CD9 and ATP1A3, followed by 15 nm and 10 gold-conjugated secondary antibodies, with yellow arrows highlighting CD9 positive, green ATP1A3 positive stained EVs. The pink arrow indicates non-labelled EVs. The scale bar represents 100 nm. (b) EVs have been immunolabeled with primary antibodies against CD81 followed by 10nm gold-conjugated secondary antibodies, with a blue arrow highlighting CD81 positive. The scale bar represents 100 nm. (c) EVs have been immunolabeled with primary antibodies against CD63 followed by 10nm gold-conjugated secondary antibodies, with orange arrows highlighting CD63 positive. The scale bar represents 100 nm. (d) EVs have been immunolabeled with primary antibodies against GLT-1 followed by 10nm gold-conjugated secondary antibodies, with red arrows highlighting CD81 positive. The pink arrow indicates non-labelled EVs. The scale bar represents 100 nm.

Main results

In our experiments, first of all, we demonstrated for the first time that acoustic trapping enables the isolation of EVs from CSF and demonstrates that EVs from AD have differential content of P-tau variants when compared to CU. AD patients have higher levels of P-tau181 and notably lower levels of P-tau217 in CSF-derived EVs when compared to CU individuals. Meanwhile, when examining the levels of P-tau217 in raw CSF, AD patients exhibited higher concentrations than CU subjects. Furthermore, in the EVs, the P-tau181/P-tau217 ratio was higher in the CU group. The observed trends of biomarker levels in EVs and CSF provide complementary insights into the pathophysiology of AD, potentially this could indicate a difference in the expression or clearance rates of specific variants of P-tau in EVs in AD.

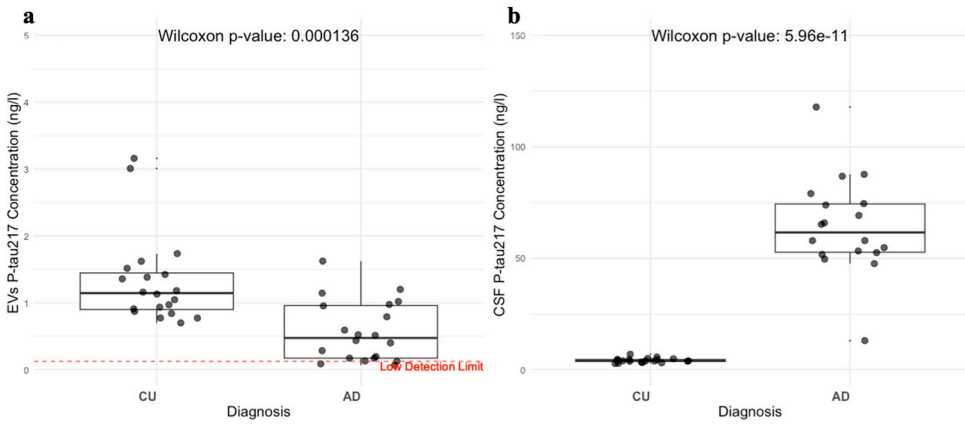

Figure 8. P-tau217 in CSF and EVs. (a) Meso Scale Discovery assay of P-tau217 from trapped EVs from the same CSF samples. Aspiration of 75µl at 200µl 1/m. EVs were lysed with tween 0.5%. The red dashed line is a low detection limit (twice the lowest value on the calibration standard). A Wilcoxon rank-sum test indicates a statistically significant difference between the groups for both CSF and EVs but with opposite directions. (b) Meso Scale Discovery assay of P-tau217 from CSF of AD (n=20) and CU (n=20) samples.

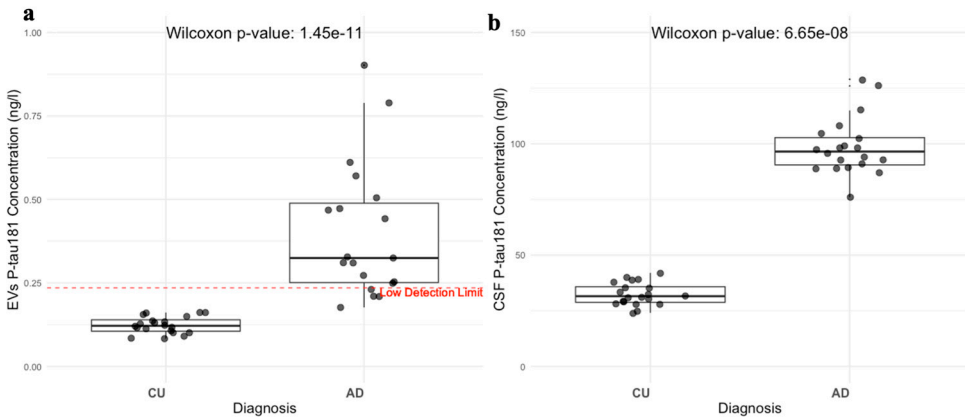

Figure 9. P-tau181 in CSF and EVs. Meso Scale Discovery assays of P-tau181 from CSF of AD (n=20) and CU (n=20) samples. (a) Meso Scale Discovery assay of P-tau181 from trapped EVs isolated from the 40 patient CSF samples. Aspiration of 75 µl at 200 µl l/m. EVs were lysed with tween 0.5%. The red dash line is a low detection limit (twice the lowest value of a standard). The Wilcoxon rank-sum test indicates a statistically significant difference between the groups for both CSF and EVs, with a similar correlation trend (b) Meso Scale Discovery assay of P-tau181 from unprocessed CSF of the same patient samples.

Meanwhile, NTA analysis revealed no statistically significant difference in the size distribution of EVs between the two groups. The mean particle size in AD samples was 144.5 nm, closely matching the CU group's mean of 144.0 nm. Additionally, our analysis showed that neither gender nor age significantly influenced the size distribution of EVs. This could suggest that, within the scope of this study, the EV size distribution alone may not serve as a distinguishing factor between AD and CU individuals. This is a potentially underlying need to switch focus for further investigations into the content and potential biomarkers within EVs, instead of EVs themselves.

Discussion

This thesis is the first demonstration of how a novel approach, acoustic trapping, can be implemented for isolating EVs from human CSF and subsequently quantifying EV cargo in the form of phosphorylated tau proteins in AD patient samples. Along with that, we established a protocol for EV lysis with (Tween-X), where we determined the time (> 5min), and concentration (0.5%), to ensure a release of all the cargo from EVs. It is essential to find optimum lysis methods which can have different effects depending on vesicle subpopulations (Osteikoetxea et al., 2015). Using non-ionic detergents, at the suggested concentrations, such as Tween-X, allows to break lipid–lipid interactions and lipid-protein interactions rather than protein-protein interactions. That enables it to differentiate EVs from protein aggregates inside of EVs. Thus, enclosed proteins, RNAs, and metabolites may be released for detection.

Using MSD assays, we identified significant differences in EV cargo levels of phosphorylated forms of tau between AD patients and CU individuals. Our findings reinforce the importance of EVs' critical role in the pathophysiology of AD, particularly in the transport and propagation of specific tau protein variants. The role of tau in AD, as highlighted in the Introduction, involves its hyperphosphorylation and subsequent aggregation into neurofibrillary tangles, a key pathological hallmark of the disease. Furthermore, it supports the notion that tau proteins, particularly in their phosphorylated forms, are actively secreted via EVs rather than merely being released as a result of neuronal death. Meanwhile, the majority of tau is secreted in a free form, approximately 90% (Dujardin et al., 2014). Our observations are consistent with previous research indicating that tau is involved in an active secretory process, with a significant portion being associated with EVs, including exosomes(Dujardin et al., 2014; Mudher et al., 2017).

Understanding these mechanisms is crucial for improving disease monitoring and developing targeted therapies, as pathological tau can transfer between cells, promoting the spread of neurodegeneration across brain regions(Frost, Jacks and Diamond, 2009; Yamada et al., 2011; Mudher et al., 2017). Interestingly, while we observed higher levels of P-tau181 in the AD group relative to the CU group, the levels of P-tau217 were paradoxically higher in EVs from CU individuals. This contrasts with the elevated P-tau217 levels found in AD CSF from the same cohort (as well as in other cohorts, where CSF P-tau217 has consistently been found to be markedly increased in AD) (Janelidze et al., 2020; Ashton et al., 2023). The

difference in the ratio of P-tau181/P-tau217 between EVs and CSF could imply a different mechanism of tau variant expression or clearance within EVs. Although it is unknown why the metabolism should differ so between these two P-tau variants, the finding aligns with studies suggesting that EV-mediated tau secretion plays a significant role in the abnormal processing of tau and the origin of elevated CSF tau in early AD (Saman et al., 2012c; Stancu et al., 2015; Yuyama et al., 2015).

Our analysis also revealed that demographic factors such as age and gender did not significantly influence the levels of P-tau in EVs or CSF, reinforcing the idea that these biochemical differences are more directly related to the underlying disease processes rather than demographic variations. Furthermore, our analysis showed no significant correlation between the CSF Aβ42/Aβ40 ratio and trapped P-tau levels.

NTA analysis showed variability in EV concentrations, with a mean size of 144 nm, indicating that while EVs are carriers of disease-relevant biomarkers, their absolute counts or size distributions may not directly reflect the pathological state, in the format of our research, emphasizing the importance of focusing on the content of EVs rather than their quantity or size alone for disease characterization. The mean size of isolated EVs was consistent with previous reports (Guha et al., 2023; Sandau et al., 2024), although our findings did not show significant differences in EV concentration or size between AD and CU groups, contrasting with other studies that reported higher EV concentrations and smaller sizes in AD CSF.

Further, analyses of EVs with TEM provided additional insights, revealing a predominance of small EVs, particularly those less than 50 nm in diameter, which NTA may miss due to its detection limits. TEM provided qualitative analysis, thus contributing to the variance of average size. In summary, TEM analysis also confirmed the presence of classical EV markers, such as CD9, CD63, and CD81. Studies indicate that these markers are not equally expressed in all EVs but show heterogeneity that reflects the expression levels of their cell of origin, such as neuronal or glial cells (Kugeratski et al., 2021). Our study observed all CD9, CD63, and CD81 positive EVs, generally considered classical EV markers (Andreu, Zoraida, Yanez-Mo, 2014). All markers indicate that EV populations in various subpopulations are present. This heterogeneity introduces an extra level of complexity in the study of EV function in AD.TEM also revealed a notably higher presence of EVS that are just CD9-positive. Studies indicate, that small-sized EVs are CD9 positive, medium-sized EVs are CD81 positive, and larger EVs are positive for both CD81 and CD9 (Zhang et al., 2023b).

Furthermore, the presence of ATP1A3 suggested the presence of neuronal EVs (You et al., 2023) and GLT-1 expression, and glia-related EVs (Ahmad et al., 2022). This heterogeneity underscores the complex role of EVs in AD, particularly in glial-to-neuronal communication, where microglia and astrocytes play pivotal roles in supporting neuronal functions and potentially propagating tau pathology(van de Wakker et al., 2023).

That opens the opportunity to investigate roles in EVs related to their cell origin. The pivotal role of EVs in glial-to-neuronal communication has gained attention in the field of molecular neuroscience. (Frühbeis et al., 2013; Ahmad et al., 2022). Concerning AD propagation, microglia are directly associated with Aβ and tau density(Smith et al., 2022). EVs secreted by astrocytes are key factors in supporting neuronal functions(Li et al., 2021). Both microglia depletion and inhibition of EV production decreased tau propagation in experimental models (Agosta et al., 2014; Clayton et al., 2021). Investigating if CSF EVs are released by activated microglia and astrocytes in response to AD pathology is crucial, as these EVs carry pro-inflammatory molecules, such as cytokines and chemokines, which can induce inflammatory responses in recipient cells, leading to neuronal damage and exacerbating AD pathology (Van Hezel et al., 2017; Gabrielli et al., 2022).

Additionally, EVs hold significant promise for clinical and research applications. The genetic and lipid content of EVs presents viable targets for biomarkers associated with neurological diseases. Multiple studies have shown that using CSF and plasma has identified panels of EV-related mRNA panels that show expression differences between control and diseased individuals (Cheng et al., 2015; Saugstad et al., 2017). Furthermore, these mRNA are distinct from the mRNA profile of astrocytes. (Men et al., 2019). Furthermore, mRNA (MiR-124a, inflammatory-associated) loaded in neuronal EVs can regulate astrocyte functions by being directly uptaken into astrocytes, regulate extracellular glutamate levels, and modulate synaptic activation. Furthermore, as mentioned previously, neuroinflammation is an important AD factor. EVs may also induce neuroinflammation, and impair neuronal functions, which can lead to neuronal death, after brain injury (Yin et al., 2020). Overall, it has been well-documented that EVs facilitate the secretion of pro-inflammatory proteins (Ridder et al., 2014).

Conclusion and perspective on future development

During the optimization of our methodology, we developed a protocol for accurately quantifying P-tau levels within EVs from CSF. Our findings demonstrated that EVs indeed carry P-tau, with the detergent lysis method playing a crucial role in releasing these proteins for detection. The presence of P-tau in EVs, confirmed through increased levels post-lysis, highlights the potential of EVs as carriers of tau pathology in AD. The differential levels of P-tau181 and P-tau217 in EVs and their distinct difference when compared to raw CSF underline the potential importance of EVs as a source of biomarkers for neurodegenerative diseases, particularly AD. Therefore, this research establishes the groundwork for using CSF-derived EV cargo to study disease propagation using AcouTrap 2 (AcouSort AB).

In conclusion, this study provides compelling evidence that smaller EVs, with confirmed cerebral origins via ATP1A3 and GLT-1 expression, can be effectively isolated using acoustic trapping from the CSF of AD and CU patients. The observed differences in P-tau variants within these EVs open new avenues for biomarker discovery and enhance our understanding of tau propagation in AD. Future studies should focus on characterizing specific EV subpopulations, particularly those derived from microglia, to further elucidate their role in tau spread and AD pathology. However, understanding which neuronal subtypes release disease-associated EVs would be important, for understanding AD pathology. Furthermore, it would be important to establish if there is a presence of Aβ in EVs, and how it correlates to Tau in EVs, and how the EV content correlates to the deposition of Aβ and tau in the brain, as well as downstream events such as atrophy and cognitive decline. Future studies may also validate these findings in larger cohorts and explore these EV's toxicity and use in AD cell models. Finally, research is needed to clarify the relationship between these EV changes and other aspects of AD, such as inflammation markers.

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