Received: 15 October 2024 | Revised: 25 February 2025 | Accepted: 4 March 2025

DOI: 10.1002/alz.70137

Alzheimer's & Dementia®
THE JOURNAL OF THE ALZHEIMER'S ASSOCIATION

RESEARCH ARTICLE

Nerve growth factor precursor alterations in neuron-derived extracellular vesicles from individuals with Down syndrome along the Alzheimer's disease continuum

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Abstract

BACKGROUND: In Down syndrome (DS) and Alzheimer's disease (AD), nerve growth factor precursor protein (proNGF) accumulates in the brain. However, its non-invasive detection using neuron-derived extracellular vesicles (NDEVs) from plasma remains unexplored.

METHODS: We included 139 adults with DS (45 asymptomatic [aDS], 94 symptomatic for AD [sDS]) and 37 healthy controls. NDEVs were isolated from plasma. ProNGF and tetraspanin (CD81) were quantified by enzyme-linked immunosorbent assay. We assessed proNGF/CD81 changes with age, along the AD continuum (aDS and sDS), and

Funding information
Jerome Lejeune Foundation (France), Grant/Award Numbers: PDC-2023-51, 1913, 1941; Instituto de Salud Carlos III (Ministerio de Asuntos Económicos y Transformación Digital, Gobierno de España), Grant/Award Numbers: PI21/01395, PI24/01087, FI22/00077, PI20/01473, PI23/01786, CP20/00038, PI22/00307, PI18/00435, PI20/01330, INT21/00073; National Institutes of Health, Grant/Award Numbers: R01AG056850, R21AG056974, R01AG061566, R01AG081394, R61AG066543, P30AG066512, R01AG087002, P01AG060882; Department de Salut de la Generalitat de Catalunya, Pla Estratègic de Recerca I Innovació en Salut, Grant/Award Numbers: SLT006/17/00119, SLT002/16/00408; Fundación Tatiana Pérez de Guzmán el Bueno, Grant/Award Number: IIBSP-DOW-2020-151; Horizon Europe - Research and Innovation Funding Programme, Grant/Award Number: H2020-SC1-BHC-2018-2020; Alzheimer's Association

associations with cerebrospinal fluid (CSF), plasma biomarkers, episodic memory, and basal forebrain volume.

RESULTS: In DS, proNGF/CD81 levels increased with age and were higher in NDEVs from asymptomatic and symptomatic individuals compared to controls, with the highest levels in the symptomatic group. ProNGF correlated with CSF phosphorylated tau (p-tau)181, plasma p-tau217, neurofilament light chain, and episodic memory.

DISCUSSION: ProNGF/CD81 levels in NDEVs increase along the AD continuum in DS and parallel tau pathology, indicating the potential as a promising biomarker for monitoring disease progression in plasma.

KEYWORDS
Alzheimer's disease, Down syndrome, nerve growth factor precursor protein, neuronal injury, neuron-derived extracellular vesicle, tau pathology

Highlights

- Nerve growth factor precursor protein (ProNGF)/tetraspanin (CD81) ratio increased in the third decade of life, 20 years before Alzheimer's disease (AD) symptom onset in Down syndrome (DS).
- proNGF/CD81 concentrations were significantly higher in individuals with DS compared to controls and were notably elevated in individuals with DS and symptomatic AD compared to asymptomatic AD.
- proNGF/CD81 concentrations were associated with tau pathology and neuronal injury.

1 | BACKGROUND

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease and the leading cause of dementia worldwide, responsible for 50% to 75% of dementia cases.[1] Down syndrome (DS) is a genetically determined AD form of the disease, due to the triplication of chromosome 21.[2–4] Trisomy 21 includes the triplication of the amyloid precursor protein (*APP*) gene, leading to an increased APP expression and the accumulation of amyloid beta (Aβ) peptides.[5] Consequently, individuals with DS exhibit progressive AD neuropathology from an early age, making them a crucial population for studying the early changes associated with AD-related progression.

Previous neuropathological studies have shown that the nerve growth factor (NGF) precursor protein (proNGF) is elevated in the brains of individuals with AD dementia,[6] mild cognitive impairment,[7,8] as well as in the brains of adults with DS.[9] This increase in proNGF results from a failure in the enzymatic mechanism responsible for its maturation to the active form (NGF), leading to a reduction in mature NGF levels. These alterations ultimately result in an increased susceptibility of basal forebrain cholinergic neurons to neurodegeneration,[6] a process linked to the dysregulation of retrograde NGF availability, on which these neurons depend for survival.[10]

Accordingly, earlier research from our group in both plasma and cerebrospinal fluid (CSF) revealed increased levels of proNGF in individuals with DS. However, only proNGF measured in CSF was able to distinguish symptomatic participants (prodromal AD and AD dementia) from those without cognitive impairment.[11] These findings support the hypothesis that proNGF measured in CSF may serve as a biomarker of brain degeneration linked to AD pathology. However, despite its safety,[12] lumbar puncture is a minimally invasive procedure, limiting its widespread use, particularly in longitudinal studies. As a result, there have been persistent efforts to identify blood biomarkers as an alternative approach.[11]

Extracellular vesicles (EVs) are lipid bilayer–delimited nanoparticles released by all cells into the extracellular space, carrying compounds from their cell of origin.[13] Additionally, they can be selected and enriched by immunocapture based on cell surface markers related to their cellular origin, constituting a representative sample of the environment of a specific tissue.[14–22] In neurodegenerative diseases, EVs have garnered significant attention for their ability to reflect the pathological state of their cells of origin and to promote the intercellular spread of toxic proteins, such as Aβ and tau, across the brain.[15,23,24] Therefore, EVs isolated from blood are emerging as a non-invasive tool for biomarker research across a range of diseases.

The present study aimed to expand our previous work on the NGF pathway[9,25] and investigate whether changes in proNGF can be assessed in plasma and plasma neuron-derived extracellular vesicles (NDEVs) in adults with DS.

2 | METHODS

2.1 | Study population

This was a single-center cross-sectional study conducted with human plasma samples from individuals with DS and euploid healthy controls (HCs). Adults with DS were recruited from the population-based research cohort Down Alzheimer Barcelona Neuroimaging Initiative (DABNI)[26] between 2014 and 2023. Our inclusion criteria comprised individuals with DS of both sexes (age > 18 years) with mild or moderate levels of intellectual disability and an available plasma sample. HCs of both sexes were part of the SPIN (Sant Pau Initiative on Neurodegeneration) cohort.[27] The study was approved by the ethics committee of Hospital Sant Pau and adhered to the standards for medical research involving humans as recommended by the Declaration of Helsinki. All participants and/or their legal representatives signed the written informed consent.

Participants with DS received a clinical and cognitive evaluation, and all cases were discussed in a consensus meeting between the neurologists and neuropsychologists who assessed them independently, blinded to biomarker data. Participants were classified into asymptomatic (aDS), when there was no clinical or neuropsychological suspicion of AD-related cognitive decline (i.e., absence of cognitive impairment beyond the intellectual and developmental disabilities, or functional decline compared to the previous functioning) or symptomatic (sDS), when there was a cognitive decline beyond the intellectual and developmental disabilities suggestive of an AD-related cognitive decline. The sDS group included both participants in the prodromal stage of AD (pDS)—those with clinical symptoms suggestive of AD but not fulfilling criteria of dementia (i.e., cognitive impairment without associated functional deterioration)—and with AD dementia (dDS)—those with evidence of cognitive impairment and that interfered with everyday activities (i.e., functional decline compared to prior levels). Functional status to differentiate prodromal and dementia stages was assessed based on anamnesis, the Dementia Questionnaire for Persons with Mental Disorders of the Elderly, modified for people with AD (CAMDEX-DS). Our diagnostic procedures followed the recommendations of the Working Group for the Establishment of the Criteria for the Diagnosis of Dementia in Individuals with Developmental Disability.[28]

In individuals with DS, global cognition was assessed with the Cambridge Cognitive Examination adapted for individuals with Down syndrome and other intellectual disabilities, which evaluates orientation, language, memory, attention, praxis, abstract thinking, and perception (CAMCOG-DS, Spanish version).[29,30] Episodic memory was evaluated with the modified Cued Recall Test (mCRT), adapted for people with intellectual disabilities.[31] The level of intellectual disability was stratified according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, categorizing it as mild, moderate, or profound. This stratification was based on the highest level of functioning observed in the individuals, as reported by their caregivers. To account for the effect of intellectual disability on cognitive performance, we excluded severe or profound cases to prevent floor effects.[31]

RESEARCH IN CONTEXT

1. **Systematic review**: Previous research has established elevated nerve growth factor precursor protein (proNGF) levels in the brains and cerebrospinal fluid of individuals with Alzheimer's disease (AD) and Down syndrome (DS). However, the utility of plasma neuron-derived extracellular vesicles (NDEVs) as non-invasive tools for assessing proNGF levels as a biomarker in DS, its evolution with age and AD progression, as well as its association with AD pathophysiology had not been comprehensively examined.
2. **Interpretation**: proNGF levels in NDEVs progressively increased with age and along the AD continuum in DS, distinguishing between asymptomatic and symptomatic individuals, and correlated with tau, neurodegeneration biomarkers, and cognitive performance. Our findings suggest that NDEVs are a feasible non-invasive approach for monitoring proNGF-related changes, which are linked to neuronal injury and tau pathology, demonstrating its relevance in tracking disease progression in DS.
3. **Future directions**: Future research should focus on longitudinal studies to validate the utility of proNGF in NDEVs as an early marker for the onset of AD dementia in individuals with DS.

All HC participants were cognitively unimpaired and evaluated by neurologists. Briefly, they had scores between 27 and 30 on the Mini-Mental State Examination (MMSE) test, absence of subjective memory complaints or objective memory deficits (measured with the Free and Cued Selective Reminding Test—FCSRT), and a score on the Clinical Dementia Rating (CDR) scale of 0. In addition, levels of core AD biomarkers in CSF were within the normal range.[27]

For the optimization and validation of the NDEV enrichment protocol, we used plasma from HCs of both sexes. For the analysis of proNGF, we selected plasma samples from participants of both sexes meeting the study entry criteria including the following groups: HC, aDS, pDS, and dDS. For the analysis of biomarkers, plasma and CSF were obtained in a subset of participants with DS and processed as previously described and stored at −80°C prior to analysis.[32] We analyzed paired plasma samples to obtain NDEVs.

2.2 | Apolipoprotein E genotyping

DNA was extracted from peripheral blood and genotyping was determined by Sanger sequencing, as previously described.[32] Although the most frequent genetic form of AD is apolipoprotein E (*APOE*) ε4 homozygosity,[33] given that only six participants were homozygotes for *APOE* ε4, we combined *APOE* ε3/ε4 and *APOE* ε4/ε4 to dichotomize individuals in two groups: *APOE* ε4 carriers or non-carriers (Table 1).

TABLE 1 Study participants.

	Controls	aDS	sDS	*p* value
Sample size (*N*)	37	45	94	NA
Demographics				
Age, median (IQR), years	60.12 (55.23–64.39)	41.78 (33.63–46.61)	51.42 (48.58–56.48)	<0.001[a]
Sex (female), *N* (%)	21 (57%)	19 (42%)	45 (48%)	0.400[b]
*APOE ε*4 genotype	8 (22%)	5 (11%)	22 (23%)	0.200[b]
Mild ID, *N* (%)	NA	13 (29%)	18 (19%)	
CSF biomarkers; median (IQR)				
CSF A*β*42/A*β*40 ratio (*n* = 97)	0.10 (0.10–0.11)	0.07 (0.06–0.08)	0.04 (0.04–0.05)	<0.001[a]
CSF p-tau181 (*n* = 97)	34.35 (25.63–46.68)	52.10 (26.60–84.86)	164.05 (89.63–245.13)	<0.001[a]
CSF NfL (*n* = 93)	505.21 (393.16– 687.48)	412.30 (239.63–565.18)	832.85 (692.35–,481.38)	<0.001[a]
Plasma biomarkers; median (IQR)				
Plasma p-tau217 (*n* = 127)	0.31 (0.23–0.43)	0.38 (0.31–0.55)	2.08 (1.39–3.22)	<0.001[a]
Plasma NfL (*n* = 139)	9.08 (7.23–13.17)	8.35 (6.15–12.39)	21.21 (16.12–29.10)	<0.001[a]
Cognitive test score; median (IQR)				
mCRT	NA	25.00 (17.50–36.00)	13.00 (6.00–23.75)	<0.001[a]
Neuroimaging biomarkers; median (IQR)				
amBF volume (cm^3)	1.35 (1.30–1.40)	1.30 (1.25–1.40)	1.16 (1.09–1.22)	<0.001[a]
pBF volume (cm^3)	1.02 (0.97–1.06)	1.12 (1.07–1.16)	0.94 (0.88–1.03)	<0.001[a]

Abbreviations: A*β*, amyloid beta; aDS, asymptomatic Down syndrome; aDS, Down syndrome with symptomatic Alzheimer's disease; amBF, anteromedial basal forebrain; *APOE*, apolipoprotein E genotype; mCRT, modified cued recall test; CSF, cerebrospinal fluid; dDS, symptomatic Down syndrome with Alzheimer's disease dementia; ID, intellectual disability; IQR, interquartile range; NA, not applicable; NfL, neurofilament light chain; pBF, posterior basal forebrain; p-tau, phosphorylated tau; sDS, symptomatic Down syndrome.
[a]Kruskal–Wallis test.
[b]Chi-squared test.

2.3 | Isolation of NDEVs

The isolation of EVs from ethylenediaminetetraacetic acid plasma (500 µL aliquots) was performed using polymer-based precipitation with ExoQuick (System Biosciences), based on previously reported methods.[15,34,35] Briefly, plasma was defibrinated with thrombin and ExoQuick was incubated for 1 hour at 4°C. The enrichment of EVs of neuronal origin was performed by immunoprecipitation with the biotinylated CD171 (L1CAM) antibody (Life Sciences, Invitrogen) for 16 hours at 4°C and subsequent streptavidin beads incubation (ThermoFisher) for 4 hours at 4°C. After centrifugation, NDEVs were precipitated, while the resulting supernatant contained the non-NDEV fraction, representing EVs of non-neuronal origin. To dissociate the streptavidin beads from the EVs, an acidic glycine-HCl solution was applied, followed by centrifugation. The final supernatant containing the NDEVs was subsequently treated with a basic tris-HCl solution to restore pH balance. Vesicles were lysed with a soft buffer (M-PER, ThermoFisher) and stored at −80°C until analysis (Figure 1).

2.4 | Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to measure the particle concentration and size distribution of fresh non-lysed EV preparations (Institute of Materials Science of Barcelona). NDEV suspensions (10 µL) were diluted with phosphate-buffered saline according to the detection range of the instrument (20–100 particles/frame). The Nanosight software (NTA 3.4 Build 3.4.003) recorded three 60 second videos with settings as follows: syringe flow rate at 30 a.u, camera level at 13, and detection threshold at 5. The particle concentrations were corrected for the input sample volume, volume of EV resuspension, and dilution necessary for NTA reading.

2.5 | Cryo-electron microscopy

Cryo-electron microscopy (Cryo-EM) was used to confirm the presence and morphology of EVs in non-lysed NDEV suspensions. Samples were vitrified using a Leica EM GP plunge freezer (Leica Microsystems, Germany) on Holey carbon grids with the following settings: 3.9 µL sample, wait time 10 seconds, blot time 2 seconds. EVs were examined with a transmission electron microscope (JEOL LTD) equipped with a CCD Gatan 895 USC 4000 camera (Gatan 626, GAtan) at 200 kV (Microscopy Unit, Universitat Autònoma de Barcelona). Micrographs were acquired using Digital Micrograph 1.8 software at defocus varying between 3 and 5 µm at 29,000 × or 80,000 × magnification (dose of 20 e− Å−2).

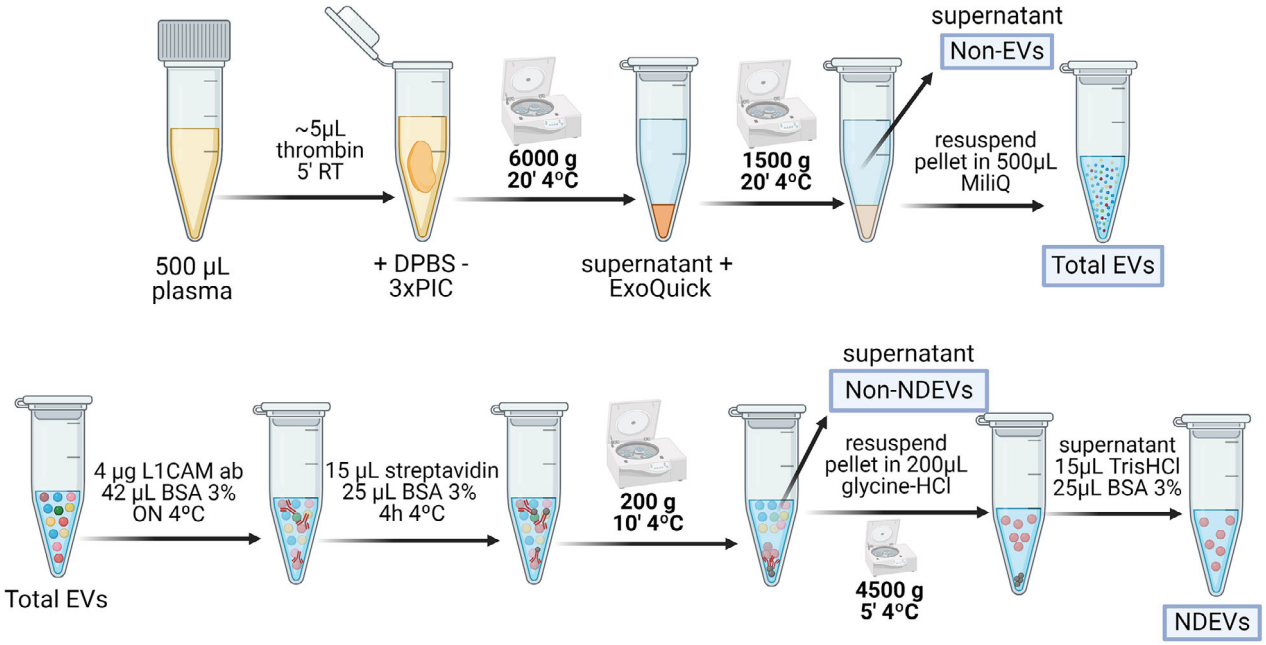

FIGURE 1 Step-by-step outline of the NDEVs enrichment protocol. This methodology involves an initial defibrination of plasma, followed by polymer-based precipitation using ExoQuick to precipitate the total EV fraction. The resultant supernatant constitutes the non-EV fraction, which is collected as EV-depleted plasma. The total EV fraction is subsequently subjected to immunoprecipitation with a biotinylated L1CAM antibody and streptavidin beads, which after centrifugation, allow to precipitate the NDEVs. The resultant supernatant constitutes the non-NDEV fraction, which are the EVs with non-neuronal origin. The bonds between the streptavidin beads and the EVs are disrupted using an acidic glycine-HCl solution, followed by centrifugation. The resulting supernatant, which constitutes the NDEVs, is then treated with a basic tris-HCl solution to equilibrate the pH. Ab, antibody; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate-buffered saline; EV, extracellular vesicle; L1CAM, L1 cell-adhesion molecule; MiliQ, ultrapure water; NDEVs, neuron-derived extracellular vesicles; non-EVs, EV-depleted plasma; non-NDEVs, extracellular vesicles from non-neuronal origin; ON, overnight; PIC, protease inhibitor cocktail; RT, room temperature.

2.6 | Analysis of L1CAM, neurofilament light chain, and phosphorylated tau181 in NDEVs

The obtained NDEV and non-NDEV fractions were used to corroborate the enrichment of neuronal biomarkers in the NDEV fraction compared to the non-NDEV one. L1CAM was quantified by enzyme-linked immunosorbent assay (ELISA; CloudCorp) following the manufacturer's recommendations in non-lysed NDEVs and non-NDEVs fractions. Neurofilament light chain (NfL) and phosphorylated tau (p-tau)181 were quantified by single molecule array (Simoa) technology in diluted lysed samples (1:4) with commercial kits (Quanterix), using the SR-X instrument. Samples were tested in duplicate.

2.7 | Analysis of vesicular and contamination markers

Lysed EV fractions and diluted plasma samples were used for western blot analysis to confirm the presence of vesicular markers. The samples were supplemented with 4X Laemmli sample buffer, boiled for 10 minutes at 90°C and loaded onto 10% stain-free acrylamide gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done at 80 V for 30 minutes, followed by 200 V for 40 minutes. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes through a semi-dry system at 25 V, 2.5 A for 10 minutes. Post-transfer the membranes were submerged in Ponceau staining for 5 to 10 minutes with gentle rotation, and the excess of stain was removed by gentle rinsing with distilled H_2O before imaging the blot. Ponceau was completely removed with four washes in Tris-buffered saline/0.1% Tween 20. Subsequently, the membrane was blocked and incubated overnight with antibodies to Alix (1:1000; Millipore), CD9 (1:1000; Cell Signaling), CD63 (1:1000, Santa Cruz Biotechnology), or calnexin (1:1000; Genetex). Protein bands were visualized with a chemiluminescence imaging system after the incubation of the blots for 5 minutes with a chemiluminescent substrate (ThermoFisher).

To verify the absence of the co-precipitating contaminant apolipoprotein B (ApoB), their levels were analyzed in plasma (dilution 1:1000), EVs (1:1000), and NDEVs (undiluted) using a commercial ELISA (R&D Systems) following manufacturer's recommendations. Samples were analyzed at least in duplicate. Plates were read at 450 nm using a microplate reader.

2.8 | Analysis of proNGF and CD81

ProNGF levels were measured in lysed NDEVs (dilution 1:4), with a commercial ELISA assay (Biosensis), following the manufacturer's recommendations. Samples were analyzed at least

in duplicate. Plates were read at 450 nm using a microplate reader.

Given the previously reported endosomal abnormalities in DS,[36] the levels of tetraspanin (CD81), a recognized EV hallmark, were subsequently quantified. To measure the levels of proNGF per NDEV based on EV quantity, CD81 levels in lysed NDEVs (diluted 1:4) were measured using a commercial ELISA assay (CUSABIO) according to the manufacturer's instructions. Each sample was analyzed at least in duplicate, and the plates were read at 450 and 570 nm with a microplate reader. These values were then used to normalize proNGF levels to compensate for differences in EV secretion, and those proNGF/CD81 ratio values, representing the amount of proNGF per NDEV, were used for downstream analyses (Table S1 in supporting information).

2.9 | Analysis of CSF and plasma biomarkers

CSF Aβ40, Aβ42, and p-tau181 proteins were measured with the Lumipulse G600II fully automated platform (Lumipulse, Fujirebio-Europe).[27] CSF NfL concentration was quantified by ELISA (Uman-Diagnostics), following the manufacturer's recommendations. Plasma concentrations of p-tau217 and NfL were measured with Simoa (Quanterix). NfL was analyzed at Hospital Sant Pau, whereas p-tau isoforms were analyzed at the University of Gothenburg, using standardized protocols.[26,32,37]

2.10 | Image acquisition and processing of basal forebrain volume

Magnetic resonance imaging data were collected using a 3T Philips Achieva scanner (Philips Healthcare) at Hospital del Mar (Barcelona) or a 3T Siemens Prisma scanner (Siemens Healthcare) at Hospital Clínic (Barcelona) between 2011 and 2022. T1-weighted images underwent processing using the voxel-based morphometry pipeline implemented in the Computational Anatomy Toolbox (CAT12; Christian Gaser and Robert Dahnke; http://dbm.neuro.uni-jena.de/cat/) for Statistical Parametric Mapping software (SPM12; http://www.fil.ion.ucl.ac.uk/spm/software/spm12/), as previously described.[38] We derived volumes of the anteromedial and posterior regions of the basal forebrain (BF; amBF and pBF, respectively). The amBF encompasses histopathologically defined cholinergic nuclei 1 through 3 (Ch1–3), projecting to the hippocampal complex and olfactory bulbs. The pBF corresponds to cholinergic nucleus 4 (Ch4), projecting to the cerebral cortex, particularly the limbic and paralimbic regions, and amygdala. We used the same quality assessment as previously described.[38]

2.11 | Statistical analyses

Statistical analyses were performed using R software, version 3.6.3. The association between proNGF and age was assessed in DS and HCs with locally estimated scatterplot smoothing (LOESS) curves, using a first-order LOESS model with a tricubic weighting function and a span parameter of 0.75 to capture the non-linear patterns in the data across both groups. Group comparisons among HC, aDS, and sDS were tested with Kruskal–Wallis and Dunn post hoc tests. The influence of biological sex and *APOE* ε4 genotype on proNGF in NDEVs across age in DS was assessed with a Mann–Whitney test. Spearman correlation analysis was used to assess the association between proNGF and amyloid/tau/neurodegeneration (AT[N]) biomarkers and cognitive performance in participants with DS, as our data did not meet the normality assumption by the Shapiro–Wilk test. The threshold for statistical significance was established at P value < 0.05. For the correlation with cognitive performance, we used the CAMCOG-DS total scores and the mCRT immediate free recall scores in participants with mild and moderate intellectual disability.

3 | RESULTS

3.1 | Demographics

We included a total of 176 individuals: 37 HC, 45 aDS, and 94 sDS (41 pDS and 53 dDS). Asymptomatic individuals with DS were younger (median age [interquartile range]: 41.78 [33.63–46.61] years) than those with symptomatic AD (51.42 [48.58–56.48] years), and HCs (60.12 [55.23 –64.39] years). The HC group was significantly older than the aDS and sDS groups. There were no significant differences between groups in the overall proportion of males and females or in the proportion of *APOE* ε4 carriers (Table 1).

3.2 | Validation of neuronal EV enrichment protocol

The size profile of vesicles analyzed in the NDEV fractions was homogeneous, with a mean size of 105 ± 1.2 nm, consistent with that of small EVs, including exosomes and ectosomes (Figure 2A). Their vesicular morphology was corroborated by cryo-EM (Figure 2A, inset). We then tested all fractions throughout the isolation procedure for the presence of vesicular and non-vesicular markers. We found that the luminal marker Alix and the transmembrane proteins CD9 and CD63, frequently identified in EVs, were present in NDEVs (Figure 2B). These markers were also found in the non-NDEV fraction (supernatant of the IP), corresponding to other EV populations in blood, which are not L1CAM positive and therefore may have different cell origins. The endoplasmic reticulum marker Calnexin, which is typically used as a cellular contamination marker, was not detected in the NDEV fraction (Figure 2B).

We verified that the immunocapture step with L1CAM and the subsequent wash steps in our protocol effectively removed contamination of lipoproteins like ApoB, which can co-precipitate with EVs. As depicted in Figure 2C, ApoB levels were lower and minimal in the NDEV fraction compared to plasma. Finally, we ran additional tests to verify

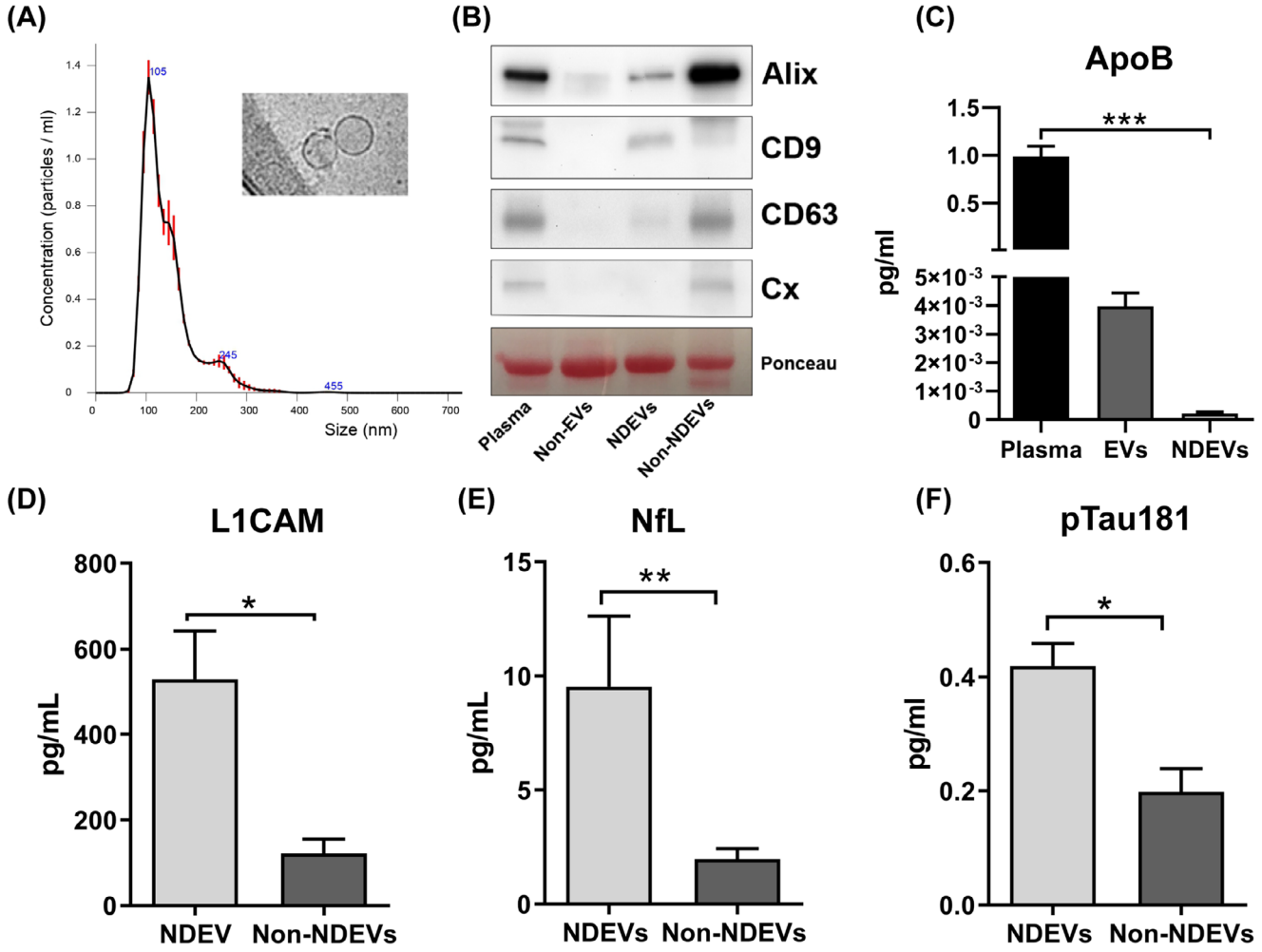

FIGURE 2 Validation of neuronal EV enrichment protocol from human plasma. NDEVs were obtained from healthy donors with no cognitive impairment. A, NTA showed a mean size of 105 ± 1.2 nm. The inset corroborates the EV morphology by cryo-EM. B, The presence of the vesicular markers Alix, CD63, and CD81 in the NDEV fraction was confirmed by western blotting, as well as the lack of the cellular contamination marker calnexin in this same fraction. Ponceau staining was used for total protein load estimation. C, The NDEV fraction showed minimal levels of the co-precipitant contaminant apolipoprotein B ($p < 0.001$). D, NDEV fraction showed higher levels of L1CAM ($p = 0.03$), NfL ($p = 0.004$), and p-tau181 ($p = 0.03$) compared to the non-NDEV fraction. Significance values are expressed as $^{*}p = 0.05$, $^{**}p = 0.01$, $^{***}p = 0.001$. Two-group comparisons were tested with a Mann–Whitney test; three-group comparisons were tested with Kruskal–Wallis and Dunn post hoc tests. Alix, CD9, CD81, vesicular markers; ApoB, apolipoprotein B; Cx, calnexin; L1CAM, L1 cell-adhesion molecule; NDEVs, neuron-derived extracellular vesicles; NfL, neurofilament light chain; non-EVs, EV-depleted plasma; non-NDEVs, extracellular vesicles from non-neuronal origin; p-tau181, phosphorylated tau 181.

the enrichment of neuronal markers in our NDEV preparations, including the analysis of L1CAM (Figure 2D), NfL (Figure 2E), and p-tau181 (Figure 2F). As seen in Figure 2D-F, the levels of these markers were significantly higher in the NDEV fraction compared to the non-NDEV fraction.

3.3 | proNGF levels in NDEVs increase with age and along the AD continuum in DS

We evaluated the detectability of proNGF in free plasma, CSF, and plasma-derived NDEVs from HCs and individuals with DS. In plasma, proNGF was detectable only in undiluted samples from HCs and in CSF only in undiluted samples from individuals with DS. Nevertheless, proNGF showed good detectability in NDEVs across all groups (Figure S1 in supporting information). These findings highlight the utility of NDEVs as the preferred biological sample for studying alterations in proNGF, thus prompting us to use NDEVs in this study.

The levels of NDEV proNGF were low throughout the lifespan in the HC group but increased with age in the DS group from the third decade of life (Figure 3). Overall, the aDS group exhibited higher NDEV proNGF levels although they were comparable to those of the HC group, whereas the sDS group displayed greater levels (Figure S2 in supporting information).

We did not observe differences in NDEV proNGF between men and women across the different AD clinical stages or in *APOE* ε4

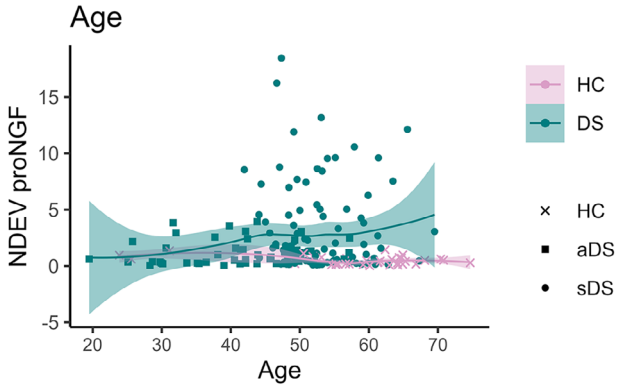

FIGURE 3 NDEV proNGF across age. NDEV proNGF increases with age in Down syndrome. NDEV proNGF stands for the ratio proNGF/CD81. LOESS smoothing line with shaded confidence interval for each etiology group to highlight the trend. aDS, asymptomatic Down syndrome; CD81, tetraspanin; DS, Down syndrome; HCs, healthy controls; LOESS, locally estimated scatterplot smoothing; NDEV, extracellular vesicle from non-neuronal origin; proNGF, nerve growth factor precursor protein; sDS, symptomatic Down syndrome.

carriers versus non-carriers between the asymptomatic and symptomatic groups in DS (Figure S3 in supporting information).

ProNGF was significantly elevated in NDEVs obtained from individuals with DS compared to HCs, both in the aDS ($p < 0.001$) and sDS ($p < 0.001$) groups. There was a trend toward significance ($p = 0.045$) indicating increased levels of proNGF in aDS compared to sDS (Figure 4A).

As expected, CD81 levels were higher in individuals with DS compared to controls in both aDS ($p = 0.01$) and sDS ($p < 0.01$; Figure 4B).

The NDEV proNGF levels were significantly higher in individuals with DS compared to those from HCs. The ratio also increased along the AD continuum in DS (Figure 4C), and was higher in the symptomatic stage compared to the asymptomatic ($p = 0.01$).

3.4 | Association between proNGF and AT(N) biomarkers in both CSF and plasma

NDEV proNGF showed significant correlations with tau and neurodegeneration biomarkers in both CSF and plasma in the DS population. More specifically, we observed a statistically significant positive correlation with p-tau181 in CSF ($r = 0.351$; $p < 0.001$), and with plasma p-tau217 ($r = 0.321$; $p < 0.001$) and plasma NfL ($r = 0.265$; $p < 0.01$). Correlations with CSF Aβ42/Aβ40 ratio ($r = -0.152$; $p = 0.163$) and CSF NfL ($r = 0.168$; $p = 0.108$) did not reach significance (Figure 5). Interestingly, when we stratified according to the presence of AD symptoms, NDEV proNGF significantly and positively correlated with CSF p-tau181 in the aDS ($r = 0.356$; $p = 0.036$) and sDS ($r = 402$; $p = 0.001$) groups (Table S2 in supporting information).

Given the variability of NDEV proNGF levels within the sDS group, we aimed to identify potential contributors. Because NDEV proNGF concentrations were similar between individuals with pDS and dDS (mean pDS = 3.16 vs. mean dDS = 3.27; $p = 0.73$), we decided to compare whether individuals at the extremes of the distribution of NDEV proNGF levels differed in any of the diverse biomarkers determined in our study. Our results demonstrated that participants with sDS and the highest concentrations of NDEV proNGF (high quartile [sDS_HQ]) had higher p-tau181 levels than those with the lowest NDEV proNGF levels (low quartile [sDS_LQ]; mean sDS_HQ = 648.57 vs. mean sDS_LQ = 133.01; $p = 0.0108$). No statistically significant differences were found when comparing the levels of the other biomarkers.

3.5 | Association between proNGF and cognitive decline

The levels of NDEV proNGF showed a significant positive correlation with episodic memory, measured with the mCRT immediate free recall test ($r = 0.375$; $p < 0.001$). When stratified by intellectual disability, this correlation remained significant in the moderate intellectual disability group ($r = 0.394$; $p < 0.001$), but not in the mild intellectual disability group ($r = 0.321$; $p = 0.09$; Figure 6). When we further categorized participants with moderate intellectual disability by the presence or absence of AD symptoms, the correlations remained significant in the asymptomatic ($r = -0.535$; $p = 0.002$), but not in the symptomatic group ($r = -0.244$; $p = 0.095$; Figure S4 in supporting information).

3.6 | Association between proNGF and basal forebrain volume

In adults with DS, we found no significant correlation between NDEV proNGF and the adjusted anteriomedial or posteriomedial basal forebrain volumes ($r = -0.166$; $p = 0.093$ and $r = -0.153$; $p = 0.122$, respectively). Additionally, after stratifying participants by the presence or absence of AD symptoms, the correlations remained non-significant in both asymptomatic (amBF: $r = -0.154$, $p = 0.375$; pBF: $r = 0.089$, $p = 0.610$) and symptomatic groups (amBF: $r = 0.089$; $p = 0.610$; pBF: $r = -0.123$; $p = 0.319$; Figure S5 in supporting information).

4 | DISCUSSION

To the best of our knowledge, this is the first study to analyze proNGF levels in plasma NDEVs in individuals with DS. We demonstrate an increase along the AD continuum in DS, which distinguishes asymptomatic from symptomatic participants. Notably, we found that proNGF levels start to rise as early as the third decade of life (Figure 3), > 20 years before symptom onset. These findings underscore the potential of NDEVs as a non-invasive tool for monitoring disease progression in populations at high risk for developing AD, opening new avenues for research into early detection, when timely intervention could significantly impact the disease trajectory in patients.

Our results emphasize the challenges in identifying and validating novel plasma biomarkers due to the lower concentration of

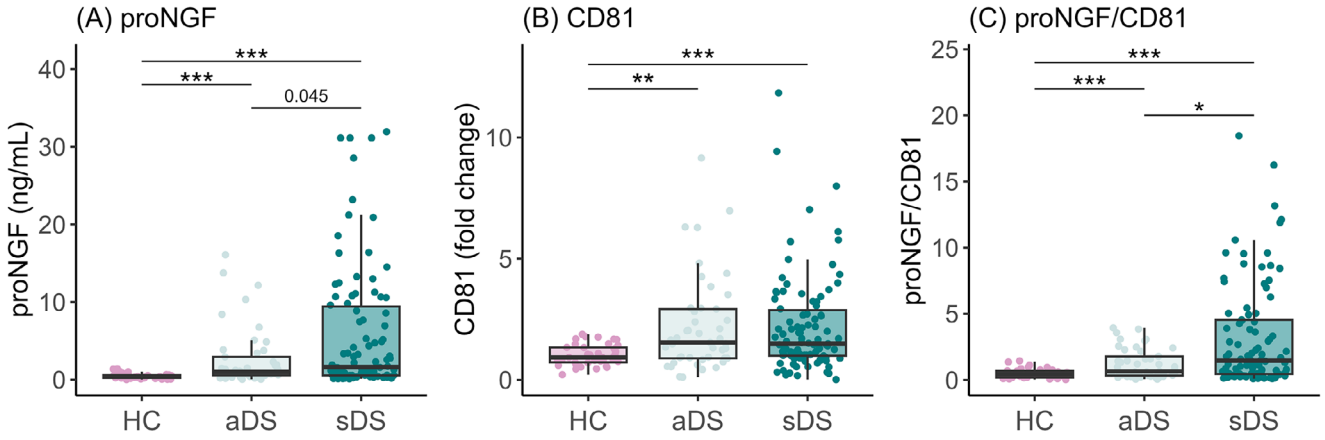

FIGURE 4 Changes in NDEV proNGF in adults with Down syndrome. A, proNGF showed significantly increased levels in NDEVs from the DS groups compared to the healthy control group ($p < 0.001$). B, CD81 was higher in individuals with DS compared to healthy controls (HC vs. aDS: $p = 0.01$; HC vs. sDS: $p < 0.01$). C, The ratio proNGF/CD81 significantly increased in individuals with DS in the symptomatic AD stage compared to the asymptomatic ($p = 0.01$) and was also higher in both aDS and sDS compared to the HC group ($p < 0.01$). ProNGF levels in (A) are expressed as pg/mL. CD81 in (B) is expressed as the fold difference with respect to the HC group. Significance values are expressed as * $P = 0.05$, ** $p = 0.01$, *** $p = 0.001$. Analysis performed using Kruskal–Wallis and Dunn post hoc test. aDS, asymptomatic Down syndrome; CD81, tetraspanin, vesicular marker; DS, Down syndrome; HCs, healthy controls; NDEV, extracellular vesicle from non-neuronal origin; proNGF, nerve growth factor precursor protein; sDS, symptomatic Down syndrome.

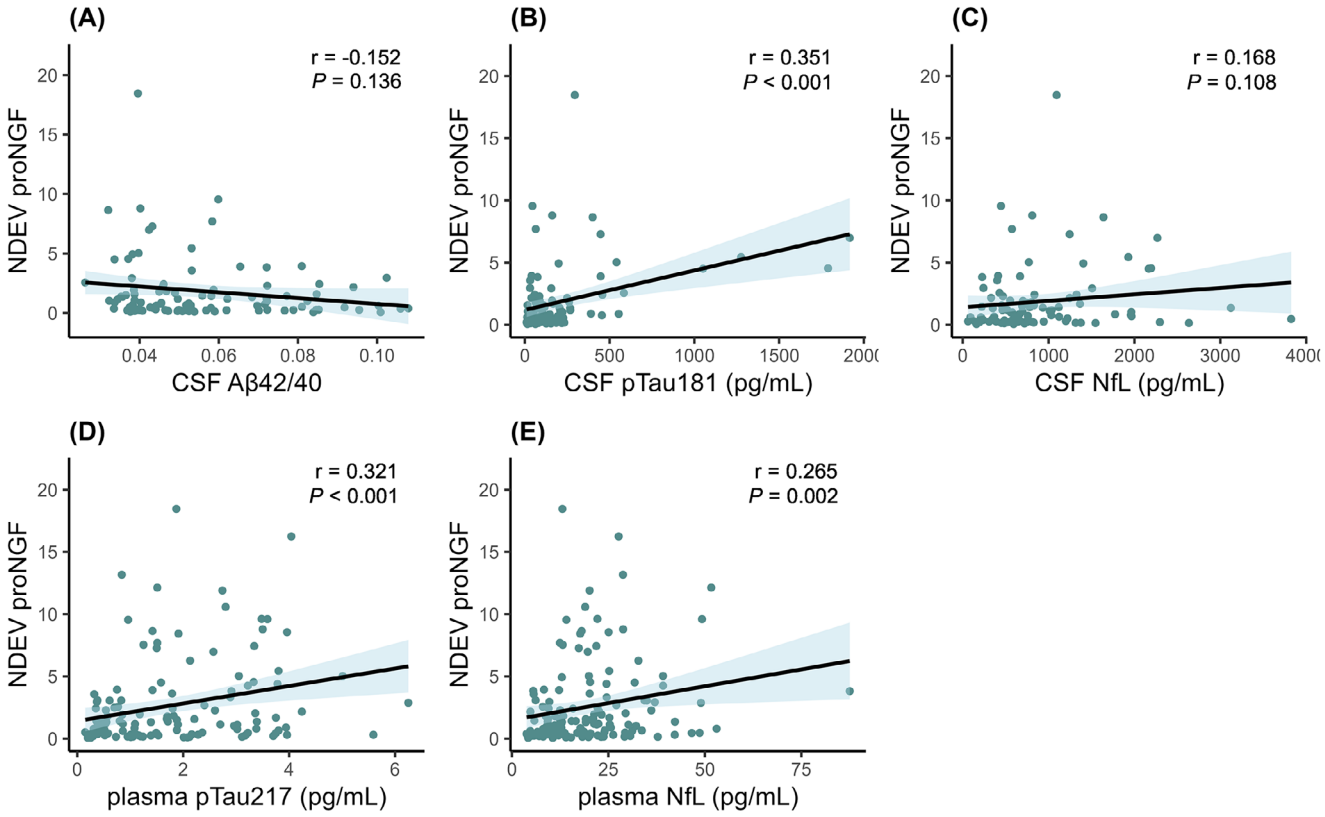

FIGURE 5 Correlations between NDEV proNGF and AT(N) biomarkers in CSF and plasma of DS individuals. NDEV proNGF showed significant correlation with (B) CSF p-tau181 ($r = 0.351$; $p < 0.001$) and with (D) plasma p-tau217 ($r = 0.321$; $p < 0.001$), and (E) plasma NfL ($r = 0.265$; $p < 0.01$) and no significant correlations with (A) CSF Aβ42/Aβ40 ratio ($r = -0.152$; $p = 0.136$) and (C) NfL ($r = 0.168$; $p = 0.108$). NDEV proNGF stands for the ratio proNGF/CD81. Light blue shaded areas represent 95% confidence intervals. *R* and *p* values obtained with Spearman correlation ($p < 0.05$). Aβ, amyloid beta; AD, Alzheimer's disease; CD81, tetraspanin; CSF, cerebrospinal fluid; DS, Down syndrome; NDEV, extracellular vesicle from non-neuronal origin; NfL, neurofilament light chain; proNGF, nerve growth factor precursor protein; p-tau, phosphorylated tau.

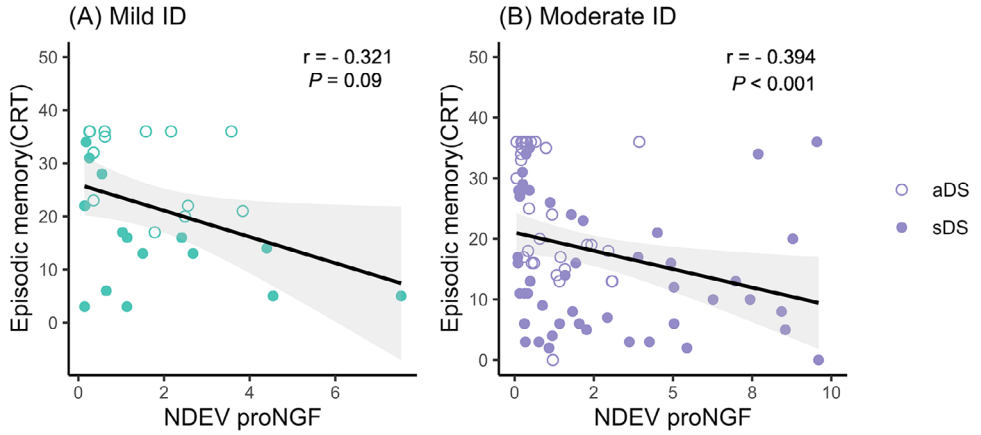

FIGURE 6 Correlations between NDEV proNGF and cognitive decline. NDEV proNGF showed significant correlation with (B) CRT individuals with moderate intellectual disability ($r = 0.394$; $p < 0.001$) and no significant correlation in the case of individuals with (A) mild intellectual disability ($r = -0.321$; $p = 0.09$). NDEV proNGF stands for the ratio proNGF/CD81. Shaded areas represent 95% confidence intervals. *R* and *P* values obtained with Spearman correlation ($p < 0.05$). CD81, tetraspanin, vesicular marker; CRT, cued recall test; ID, intellectual disability; NDEV, extracellular vesicle from non-neuronal origin; proNGF, nerve growth factor precursor protein.

brain-derived analytes and the complexity of the blood matrix, which can interfere with detection. These findings highlight EVs as a promising solution. Immunoaffinity methods targeting cell surface markers inherited from the cell population of origin enable selective isolation of EVs. Therefore, the use of NDEVs in this study represents a liquid biopsy–based approach for the non-invasive analysis of the intracellular neuronal environment from peripheral blood. Our findings in NDEVs consistently align with previously observed results in CSF from individuals with DS, importantly demonstrating the capability to detect alterations in proNGF in asymptomatic individuals with DS for AD in a non-invasive manner.

We identified significant associations between NDEV proNGF and plasma NfL concentrations. Interestingly, NfL levels increase at ≈ 30 years of age in DS,[26] and the elevation coincides with the rising of NDEV proNGF levels. We also found positive correlations between NDEV proNGF and p-tau181 in CSF, a marker that starts to increase in the mid 30s.[26] We did not find significant correlations with the Aβ42/Aβ40 ratio in CSF, possibly because of the floor effects in late preclinical and symptomatic stages of the disease. Previous studies performed in vitro[9] and in animal models[39] have demonstrated that proNGF levels can be altered in the presence of amyloid and the absence of tau. Our findings suggest that in DS, proNGF levels start to rise early in preclinical AD but show a stronger correlation with neurodegeneration and tau biomarkers throughout the AD continuum. Moreover, although a significant increase in proNGF levels was observed in NDEVs from sDS compared to aDS participants, we acknowledge the variability within the sDS group data. Therefore, we explored the potential contribution of other biomarkers. Our analyses revealed that p-tau181 contributes to the variability observed in the levels of NDEV proNGF in sDS patients. Furthermore, previous research reported that in aging and AD, mitochondrial dysfunction and excessive microglial activation, combined with a simultaneous decline in antioxidant defenses, drive the overproduction of reactive oxygen and nitrogen species (ROS/RNS) observed in the AD brain.[40–43] The presence of these species induce the nitration of proNGF and reduce the expression of its receptor (tropomyosin-related kinase A [TrkA]),[44] leading to the loss of neurotrophic function of proNGF and, consequently, leading to the activation of apoptotic pathways and decreased cell viability.[45–47] This modulatory effect of ROS and RNS may explain the lower proNGF levels in certain sDS patients, who are also older than the aDS individuals. A significant negative correlation was also observed between NDEV proNGF and episodic memory performance in individuals with moderate intellectual disability. When the groups were stratified based on symptom presence, the correlation remained significant in the asymptomatic group but not in the symptomatic group. This finding is consistent with the early floor effects observed in the mCRT evaluated in symptomatic individuals.[48]

Previous research from our group has demonstrated a decrease in BF volume in individuals with DS with age and along the AD continuum.[38] We did not find a correlation between NDEV proNGF and the amBF or pBF volume, despite its association with NfL levels. NGF is expected to drive BF atrophy, as it is the key neurotrophin for the survival of this brain region. However, quantifying mature NGF presents a challenge due to the lack of high-throughput assays capable of specifically detecting this protein without cross-reactivity with its precursor, proNGF. Additionally, NGF exhibits a rapid turnover and a brief extracellular presence, owing to its degradation by the metalloproteases MMP9 and MMP3.[10,49] Moreover, it is important to note that NGF metabolic deregulation is not an acute phenomenon, but rather a prolonged and persistent process that takes years to induce the progressive atrophy of the BF cholinergic neurons.[7,8] Consequently, although we did not identify any single-point correlation between the degree of BF atrophy and proNGF levels, it is plausible that a correlation exists between the duration of NGF metabolic deregulation and the subsequent atrophy of the BF or earlier cholinergic degeneration processes like cholinergic pre-synaptic button atrophy.

Indeed, correlations between proNGF and the vesicular acetylcholine transporter (VAChT) have been documented in AD animal models and human neuropathological tissue.[11,39] Therefore, these findings focused on the NGF precursor represent a significant advancement in understanding cholinergic impairment and in the discovery of AD-related biomarkers.

We found that proNGF was not detectable in undiluted free plasma and CSF across all clinical groups when using the ELISA kit used in this study, despite previous reports demonstrating its detectability in these biofluids through western blot with a different primary antibody.[11] While ELISA offers quantitative advantages over western blot, including higher sensitivity and high-throughputness, western blot allows for the detection of different isoforms by molecular weight, which immunoassays do not provide. These different proNGF isoforms are altered differently in HCs and people with DS as AD progresses.[50] In this study, NDEVs offered a clear advantage for detecting proNGF from plasma sources.

There are certain limitations in the current study. Regarding the methodology used for the isolation of EVs, it has been proposed that the polymer-based precipitation approach is subject to co-precipitation of lipoproteins together with the EVs. To address this, we mitigated this limitation with the additional immunocapture step using L1CAM antibodies and subsequent washes to selectively bind to NDEVs, thus restricting the lipoprotein impurities, and confirming minimal ApoB levels in the NDEV fraction compared to plasma. We also chose this method because of its simplicity, lack of use of specific devices, and because it has been the method of choice in nearly all neurology-related studies on the use of EVs for biomarker discovery,[51] thus permitting cross-study comparisons. Furthermore, given the known endosomal dysregulation in DS[52] and the previously reported increase in EV release,[36] we anticipated that EV levels would vary across our experimental groups. To accurately measure proNGF levels per NDEV, we used CD81, a canonical tetraspanin found in EVs, as a normalization marker. Although CD81 levels differed between HC and DS participants, consistent with an elevated number of EVs in individuals with DS, they remained similar when comparing aDS and sDS groups. CD81 levels were quantified using a commercially available ELISA, following the methodology used in previous studies involving DS and AD cohorts.[15,17,34,53–55] This approach enhanced the rigor of our results and compensated for the higher EV yield observed in the DS group. A limitation of our study is that only CD81 was used for normalization. The inclusion of additional EV markers, such as CD9 or CD63, could provide further support for our findings with CD81 and should be considered for future research. Finally, longitudinal studies in additional cohorts[56] are needed to confirm the age-related increase in proNGF that we report in this cross-sectional study. That said, the predictable nature of AD pathology in the DS population makes such comparisons quasi-longitudinal.[26]

In summary, our findings reveal that proNGF levels measured in NDEVs progressively increase along the AD continuum in individuals with DS, and directly correlate with biomarkers of neuronal injury in CSF and plasma, as well as with episodic memory performance. These results suggest that EVs offer promising avenues for examining brain-specific targets in peripheral fluids, enabling the monitoring of disease progression.

AUTHOR CONTRIBUTIONS

Natalia Valle-Tamayo, M. Florencia Iulita, Juan Fortea, and Oriol Dols-Icardo conceived and designed the study. Natalia Valle-Tamayo, Mateus Rozalem Aranha, Rocío Pérez-González, Sara Serrano-Requena, Isabel Barroeta, Laura Videla, Bessy Benejam, Gemma Chiva-Blanch, Amanda Jimenez, Jorge Busciglio, Thomas Wisniewski, Alexandre Bejanin, Olivia Belbin, Daniel Alcolea, María Carmona-Iragui, Alberto Lleó, A. Claudio Cuello, Juan Fortea, Oriol Dols-Icardo, and M. Florencia Iulita acquired and interpreted the data. Natalia Valle-Tamayo and Mateus Rozalem Aranha performed the statistical analysis. Natalia Valle-Tamayo, M. Florencia Iulita, Juan Fortea, and Oriol Dols-Icardo drafted the manuscript, which all authors critically reviewed for important intellectual content.

ACKNOWLEDGMENTS

The authors thank all study participants and caregivers for their support and dedication. The authors acknowledge the help of Soraya Torres, Shaimaa El Bounasri El Bennadi, and Oriol Sanchez Lopez for sample handling and preparation. The authors also recognize the collaboration of José Amable Bernabé from the ICTS "NANBIOSIS," unit 6 (CIBER in Bioengineering, Biomaterials & Nanomedicine) at the Barcelona Materials Science Institute, and Marti de Cabo Jaume from the Electron Microscopy Unit at Universitat Autonoma de Barcelona. The authors also extend our appreciation to the Fundació Catalana Síndrome de Down (https://fcsd.org/) for their overall support, and to Tania Martínez, Cristina Pastor, and Ana Sebastián for administrative assistance, as well as Aida Sanjuan and Mireia Carreras for nursing support. This study was funded by Jerome Lejeune Foundation (France), through projects Project #1941 to MFI and #1913 to MCI and the Instituto de Salud Carlos III (Ministerio de Asuntos Económicos y Transformación Digital, Gobierno de España) through the projects PI21/01395 (and associated FI22/00077 to N.V.T. and O.D.I.) and PI24/01087 to O.D.I.; INT21/00073, PI20/01473 and PI23/01786 to J.F., CP20/00038 and PI22/00307 to A.B., PI18/00435 to D.A., PI20/01330 to A.L.) and the Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas Program 1, partially jointly funded by Fondo Europeo de Desarrollo Regional, Unión Europea, Una Manera de Hacer Europa, and cofunded by the European Regional Development Fund/European Social Fund (ERDF/ESF), 'A way to make Europe'/'Investing in your future'. This study was also supported by the Jerome Lejeune Foundation #51 (PDC-2023-51) to O.D.I. This work was also supported by the National Institutes of Health grants (R01 AG056850; R21 AG056974, R01 AG061566, R01 AG081394 and R61AG066543 to J.F. and P30AG066512, R01AG087002 and P01AG060882 to T.W.), the Department de Salut de la Generalitat de Catalunya, Pla Estratègic de Recerca I Innovació en Salut (SLT006/17/00119 to J.F.; SLT002/16/00408 to A.L. It was also supported by Fundación Tatiana Pérez de Guzmán el Bueno

(IIBSP-DOW-2020-151 to J.F.) and Horizon 2020–Research and Innovation Framework Programme from the European Union (H2020-SC1-BHC-2018-2020 to J.F.) also supported this work. The Alzheimer Association also participated in the funding of this work (AARG-22-923680 to A.B. and AARF-22-924456 to ODI).

CONFLICT OF INTEREST STATEMENT

O.B. reported receiving personal fees from Adx NeuroSciences outside the submitted work. D.A. reported receiving personal fees for advisory board services and/or speaker honoraria from Fujirebio-Europe, Roche, Nutricia, Krka Farmacéutica, and Esteve, outside the submitted work. A.L. has served as a consultant or on advisory boards for Almirall, Fujirebio-Europe, Grifols, Eisai, Lilly, Novartis, Roche, Biogen, and Nutricia, outside the submitted work. J.F. reported receiving personal fees for service on the advisory boards, adjudication committees or speaker honoraria from AC Immune, Adamed, Alzheon, Biogen, Eisai, Esteve, Fujirebio, Ionis, Laboratorios Carnot, Life Molecular Imaging, Lilly, Lundbeck, Perha, Roche, and outside the submitted work. O.B., D.A., A.L., and J.F. report holding a patent for markers of synaptopathy in neurodegenerative disease (licensed to ADx, EPI8382175.0). The remaining authors declare no competing interests. M.F.I. is currently an employee of Altoida Inc. Author disclosures are available in the supporting information.

CONSENT STATEMENT

All participants and/or their legally authorized representatives gave written informed consent.

DATA AVAILABILITY STATEMENT

The authors may share de-identified data that underlie the results reported in this article upon reasonable request to the corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Valle-Tamayo N, Aranha MR, Pérez-González R, et al. Nerve growth factor precursor alterations in neuron-derived extracellular vesicles from individuals with Down syndrome along the Alzheimer's disease continuum. *Alzheimer's Dement*. 2025;21:e70137. https://doi.org/10.1002/alz.70137