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Nerve growth factor (NGF) pathway biomarkers in Down syndrome prior to and after the onset of clinical Alzheimer's disease: A paired CSF and plasma study

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Abstract

Background: The discovery that nerve growth factor (NGF) metabolism is altered in Down syndrome (DS) and Alzheimer's disease (AD) brains offered a framework for the identification of novel biomarkers signalling NGF deregulation in AD pathology.

Methods: We examined levels of NGF pathway proteins (proNGF, neuroserpin, tissue plasminogen activator [tPA], and metalloproteases [MMP]) in matched cerebrospinal fluid (CSF)/plasma samples from AD-symptomatic (DSAD) and AD-asymptomatic (aDS) individuals with DS, as well as controls (HC).

Results: ProNGF and MMP-3 were elevated while tPA was decreased in plasma from individuals with DS. CSF from individuals with DS showed elevated proNGF, neuroserpin, MMP-3, and MMP-9. ProNGF and MMP-9 in CSF differentiated DSAD from aDS (area under the curve = 0.86, 0.87). NGF pathway markers associated with CSF amyloid beta and tau and differed by sex.

Discussion: Brain NGF metabolism changes can be monitored in plasma and CSF, supporting relevance in AD pathology. These markers could assist staging, subtyping, or precision medicine for AD in DS.

1 | INTRODUCTION

Individuals with Down syndrome (DS) represent the largest genetically determined population at risk for Alzheimer's disease (AD).[1–6] A critical factor associated with cognitive decline in AD, both in the general population and in DS, is the degeneration of the cortical forebrain cholinergic system.[5,7–14] Cholinergic neurodegeneration in these disorders has been linked to dysregulation of the nerve growth factor (NGF) metabolic pathway.[15–19] This pathway controls the maturation and degradation of the neurotrophin NGF, on which forebrain cholinergic neurons depend.[20] In this pathway, the tissue plasminogen activator (tPA)/plasminogen system is responsible for the maturation of NGF from its precursor, proNGF, a process that is inhibited by neuroserpin.[20,21] The extracellular degradation of mature NGF is accomplished primarily by matrix metalloproteinase (MMP)-9,[20] which can be activated by MMP-3[22] and is inhibited by tissue inhibitor metalloproteinase (TIMP)-1.[23]

ProNGF levels have been found to be elevated in AD brains with advanced pathology.[24] The NGF metabolic pathway has likewise been found to be dysregulated in mild cognitive impairment (MCI), AD, and apparently healthy individuals with elevated brain amyloid[17,25,26] as well as in the brains of people with DS and AD dementia.[15] NGF dysregulation in the early phases of AD correlates to cholinergic dysfunction and cognitive decline,[26] and the ability to monitor this dysfunction through biomarkers would have high potential utility in research and clinical contexts.[27,28]

Correspondingly, an analysis of NGF metabolic pathway markers in plasma samples from people with DS has previously revealed that the levels of NGF metabolism-associated markers, including proNGF, MMP-1, MMP-3, and MMP-9 were elevated in individuals with DS, with and without the cognitive symptoms of AD.[16] Critically, it was demonstrated that longitudinal within-subjects increases in proNGF over 1 year correlated with cognitive decline over the subsequent 2 years.[16]

While plasma is the most accessible body fluid for the measurement of biomarkers, cerebrospinal fluid (CSF) can more faithfully reflect changes in the brain, as it is in direct contact with the extracellular central nervous system (CNS) milieu. Therefore, in this study, we analyzed a unique set of matched plasma and CSF samples from adults with DS with and without AD dementia, as well as from healthy, non-DS controls. The use of matched samples offers a unique opportunity to directly compare the performance of markers in the two most commonly analyzed biofluids as well as to assess their correspondence, which could support the ability of plasma markers to reflect changes in the CNS milieu. The present study confirms and elaborates upon our previous findings of dysregulation of NGF metabolism-associated proteins[16,29] in a new, independent clinical cohort of individuals with DS, and extends it by showing that such changes also exist in CSF and in fact better reflect the NGF metabolic changes observed in the brain. We also demonstrate that CSF proNGF and MMP-9 can effectively differentiate AD-symptomatic individuals with DS from those who were asymptomatic, and that proteins related to NGF metabolism correlate to core biomarkers of AD, especially when measured in CSF.

2 | MATERIALS AND METHODS

2.1 | Study cases

Adults with DS aged 18 years and older were recruited by the Alzheimer-Down Unit of the Fundació Catalana Síndrome de Down and the Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, totaling 37 cases who assented to blood and CSF extraction. Participants or their legal representative gave written informed consent, as approved by the ethics committee of Hospital Sant Pau following the standards for medical research in humans recommended by the Declaration of Helsinki and reported to the Ministry of Justice according to the Spanish law for research in people with intellectual disabilities. Subjects were required to understand and accept the study procedures to give informed consent. Genetic screening of trisomy 21 aneuploidy was done as previously described, confirming full trisomy of chromosome 21 in 95% of participants.[30]

Participants received a neurological and neuropsychological examination which included the Cambridge Cognitive Examination (CAMCOG) and Cambridge Examination for Mental Disorders of the Elderly-Down Syndrome (CAMDEX-DS) batteries, as previously described.[30] Behavioral measures were assessed with the Neuropsychiatric Inventory.[31] We stratified the level of intellectual disability according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition as mild, moderate, or severe or profound (which were grouped together), based on the individuals' best-ever level of functioning as determined from carers' reports. Participants were classified as asymptomatic, without AD-related cognitive impairment (aDS; n = 14), prodromal AD (n = 10), or AD dementia (n = 12) in a consensus meeting between the neurologist and the neuropsychologists who assessed them blind to the biomarker data, as previously described.[30] One participant was classified as 'uncertain' and excluded from analysis. Pilot studies with these samples indicated no significant differences in NGF biomarkers between the prodromal AD and AD

dementia groups, and these were therefore grouped together as a symptomatic AD group (referred to as DSAD) to conserve statistical power.

Control subjects were healthy volunteers of both sexes (age range 20 to 60 years, n = 16) recruited from the SPIN cohort (Sant Pau Initiative on Neurodegeneration).[32] Volunteers had no neurological deficits, a Clinical Dementia Rating (CDR) total score of zero, and with normal levels in core AD CSF biomarkers (amyloid beta [Aβ]42/Aβ40, total tau [t-tau] and phosphorylated tau [p-tau]) assessed by Lumipulse (LUMIPULSE G600II automated platform, Fujirebio).[33] For both cohorts (DS and controls), the period of recruitment was between November 2013 and November 2015. Apolipoprotein E (APOE) allele genotyping was done as previously described.[34] Table 1 illustrates the demographics of the populations included in this study.

2.2 | Plasma collection

Plasma samples were collected and processed following international protocols[35] and established procedures at the Sant Pau Memory Unit laboratory.[30] Briefly, fasting venous blood was collected in lavender EDTA-K2 BD vacutainer tubes and centrifuged at 2000 g for 10 minutes at 4°C for plasma separation. Plasma samples were separated in aliquots of 100, 250, and 500 μl with protease inhibitors (cOmplete, Mini Protease Inhibitors, Roche) and stored at –80°C. They were shipped on dry ice to McGill University and upon arrival were immediately stored at –80° until analysis. Samples were analyzed within 12-18 months after collection. Freeze-thaw cycles were minimized to three.

2.3 | CSF collection via lumbar puncture

A plasma-paired CSF sample of every subject was obtained at the time of blood collection. Lumbar puncture was performed by a spe-

RESEARCH IN CONTEXT

1. Systematic review: We used PubMed to review studies pertaining to the plasma/cerebrospinal fluid (CSF) biomarkers of central nervous system cholinergic deficit and nerve growth factor (NGF) metabolism in Down syndrome (DS). Basal forebrain cholinergic degeneration and NGF metabolic deficits have been demonstrated in DS brains. One study in 2016 showed an altered NGF metabolic signature in DS plasma. There were no reports in CSF.

2. Interpretation: Our findings indicate that NGF metabolic alterations can be monitored in plasma and CSF in DS, though CSF more closely reflects the changes observed in brain. Two CSF markers (proNGF 50 KDa and MMP-9) distinguished AD-symptomatic individuals from the overall DS population. These markers could have relevance for diagnosis, staging, subtyping, or precision medicine for AD in DS.

3. Future directions: NGF metabolic signatures should also be tested in biofluids from familial and sporadic AD, both pre- and post-symptomatically. In DS, associations with cognitive decline, basal forebrain imaging, and cholinergic positron emission tomography would be revealing.

cialized neurologist at the Sant Pau Hospital (Barcelona, Spain), following international consensus recommendations for CSF collection.[36–39] The procedure was performed either in sitting or lying position, using a pen-point atraumatic spinal needle (Whitacre-22G). CSF was collected by free-flow/dripping in 10 mL polypropylene tubes (Sarstedt, Ref# 62.610.018) and processed within 2 hours after acquisition and aliquoted as previously described.[33] The Memory Unit laboratory at

TABLE 1 Characteristics of the study cohort

	HC	aDS	DSAD	Test for difference
Number of cases	16	14	22	
Age (± SEM)	52.8 ± 1.1	39.9 ± 2.9	52.9 ± 2.9	1 way ANOVA, $P < .001$ (aDS lower than HC/DSAD)
Proportion female	0.44	0.50	0.50	Chi-square test of independence, $X^2 > 0.05$
APOE allele frequency (proportion)	ε4: 0.19 ε3: 0.78 ε2: 0.03	ε4: 0.11 ε3: 0.82 ε2: 0.07	ε4: 0.13 ε3: 0.85 ε2: 0.02	Chi-square test of independence, $X^2 > 0.05$
Intellectual disability (proportion)	N/A	Mild: 0.29 Moderate: 0.57 Severe: 0.14	Mild: 0.26 Moderate: 0.48 Severe: 0.26	Chi-square test of independence, $X^2 > 0.05$

Notes: Individuals with aDS were younger than those with DSAD as well as non-trisomic controls. Sex and APOE allele balance were equivalent between all groups. Underlying intellectual disability was equivalent between the aDS and DSAD groups.
Abbreviations: aDS, Alzheimer's disease asymptomatic; ANOVA, analysis of variance; APOE, apolipoprotein E; DSAD, Down syndrome Alzheimer's disease; HC, healthy control; SEM, standard error of the mean.

the Sant Pau Hospital participates in the Alzheimer's Association external quality control program for CSF biomarkers. CSF aliquots were stored at –80°C, shipped on dry ice to McGill University, and upon arrival were immediately stored at –80°C. Samples were analyzed within 12-18 months after collection. Freeze-thaw cycles were minimized to three.

2.4 | Analysis of proNGF and neuroserpin

Analysis of proNGF and neuroserpin in plasma and CSF was done by Western blotting, following established procedures at the McGill laboratory.[15–17,26,40] Briefly, plasma samples (diluted in phosphate buffered saline) and undiluted CSF were incubated in standard loading buffer under reducing conditions (5% β-mercaptoethanol), and loaded on 10% to 12% SDS-polyacrylamide gels and semi-dry transferred to nitrocellulose membranes (BioRad, USA) for 60 minutes at 220 mA. Membranes were blocked for 1 hour in 5% bovine serum albumin (Sigma, USA) in tris-buffered saline-polysorbate 20 0.1% (TBS-T) and incubated with anti-proNGF antibodies (Alomone Labs, Israel; at 1:2000) or anti-neuroserpin antibodies (kindly provided by Dr. Daniel Lawrence, University of Michigan, USA; at 1:5000), overnight at 4°C in blocking solution. Anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, USA) were applied for 1 hour at room temperature in blocking solution. Immunoblots were developed with the Western Lightning Plus-ECL substrate (PerkinElmer Inc, USA) on Hyblot CL films (Denville, Canada). Films were digitized and the relative levels of proNGF (27 kDa and 50 kDa bands) and neuroserpin (45 kDa band) were quantified with the Gel-Pro Analyzer (Media Cybernetics, Inc., USA) and the TotalLab CLIQS Software (TotalLab, UK). The densitometry of each sample was normalized to that of an internal control, a plasma or CSF sample that was loaded in all gels, and each value was expressed as relative fold-change with respect to the average of the control group. ProNGF and neuroserpin determinations were done in three independent experiments by independent investigators blinded to the clinical diagnosis of the cases. The values were averaged for analysis. Quantifiability across physiologically relevant concentrations/integrated optical density (IOD) values was established by reconstitution experiments in which plasma/CSF was spiked with 2, 5, 10, or 20 ng of human recombinant proNGF protein (Alomone Labs, Israel), and subsequently analyzed by Western blotting as described above. As shown in Figure S1 in the Supplementary Files, responses were linear across the range of 2 to 10 ng and showed a minimal (15%) increase in IOD response in the 20 ng sample in plasma (see Figure S1A and S1B). In CSF, all responses were linear (see Figure S1C and S1D). Spike-response patterns did not differ in plasma or CSF samples from controls (HC) versus individuals with DS (Figure S1A vs S1B; S1C vs S1D). All experimental values obtained by Western blotting fell within the linear range. The identity of each proNGF band was established and confirmed in previous reports by a variety of methods.[15–17,26,40]

2.5 | Analysis of metallo-proteases

The levels (pg/mL) of MMP-1, MMP-3, and MMP-9 in diluted plasma and CSF samples (1:10) were measured with a Multi-Spot MMP 3-Plex Ultra-Sensitive kit (MesoScale Discovery, USA), following manufacturer's instructions. Electrochemiluminiscent signals were read in a SECTOR Imager 2400 (MesoScale Discovery, USA) and the concentration of each metalloprotease was determined from a standard curve generated by the MSD Discovery Workbench software v. 4.0. (MesoScale Discovery, USA). Samples that fell below the detection threshold were not considered for analysis. Each sample was analyzed in three independent experiments from separate aliquots by independent investigators. The average values of all assays were used for analysis.

2.6 | Analysis of tPA

The plasma and CSF levels of tPA were measured with a Human Tissue Type Plasminogen Activator ELISA (enzyme-linked immunosorbent assay) kit (TPA; ab108914, Abcam, USA). tPA levels were measured in undiluted plasma and in diluted CSF samples (1:2), following the protocol detailed by the manufacturer. For the calibration curve used in the analysis of plasma samples, human tPA standards were diluted in tPA-depleted plasma, provided by the manufacturer. The assay range for tPA quantification was 0.02 to 10 ng/mL.

2.7 | Statistical analysis

Unless otherwise stated, data analyses were done with the software GraphPad Prism v7.00a (GraphPad, USA). Significance was set at $P < .05$. Normality of data distribution was tested with the D'Agostino and Pearson omnibus K2 test. If data normality was violated, analysis was done with non-parametric statistics. Outliers were removed at a threshold of 3x the standard error of the mean. Differential expression of NGF metabolism-related proteins across clinical classifications was analyzed with a one-way analysis of variance (ANOVA) and Bonferroni post hoc tests, if normally distributed, or Kruskal-Wallis tests and Dunn's post hoc correction if non-normal. The specificity of the biomarkers to DSAD was assessed by the area under a receiver operator characteristic (ROC) curve comparing the DSAD and aDS populations. Assessments of the effect of sex on biomarker expression was performed with a two-way ANOVA or Friedman non-parametric test, in the case of non-normally distributed data, looking for main effects of sex and sex-by-diagnosis interactions. Note that expression levels of each protein are not considered independent of one another and their relationships are described in Table S1 in the Supplementary Files. The relationships between the CSF and plasma levels of each NGF metabolism-related protein, as well as their associations with core CSF biomarkers of AD, displayed evidence of heteroskedasticity and were therefore assessed using the MM-estimation method of Crout

et al.,[41] which is robust to outliers and heterogeneity of variance. This analysis was performed using R (R Project for Statistical Computing, https://www.R-project.org).

3 | RESULTS

Table 1 summarizes the demographic information of the study participants. The aDS group was younger than DSAD and control individuals, although the age difference was not significant. There were no differences in the proportion of females or proportion of APOE ε4 carriers between clinical groups. The DSAD group had a higher proportion of individuals with severe intellectual disability than the aDS group.

3.1 | Increased proNGF levels in plasma and CSF from individuals with aDS and DSAD

As there are no existing ELISAs that can differentiate different forms of proNGF, we assessed them using Western blotting. This allowed the detection of a 27 kDa proNGF form corresponding to unprocessed proNGF,[42–44] and another one of higher molecular weight at 50 kDa, suggested to represent glycosylated proNGF[42–45] and/or complexes of proNGF with macromolecules such as α2-macroglobulin.[46]

Analysis of the 27 kDa form of proNGF revealed a significant 40% increase in plasma in aDS as well as in DSAD individuals compared to non-trisomic controls (Figure 1A, $P < .05$). An 80% upregulation of the 27 kDa proNGF band was observed in paired CSF samples (from the same individuals) in both the aDS and DSAD groups compared to non-trisomic controls (Figure 1B, $P < .01$ and $P < .001$). CSF and plasma levels of proNGF 27 kDa significantly correlated ($P < .01$, $r^2 = 0.12$, Table S1).

The 50 kDa form of proNGF was also upregulated in plasma from people with aDS and DSAD compared to controls (Figure 1C, $P < .05$), similarly to the 27 kDa form. CSF analysis further revealed higher levels of 50 kDa proNGF in the aDS and DSAD groups compared to non-trisomic controls, and further elevated levels in the DSAD group compared to the aDS group (Figure 1D, $P < .0001$ and $P < .01$). Plasma and CSF levels of proNGF 50 kDa did not correlate (Table S1).

3.2 | Neuroserpin and tPA levels in plasma and CSF from individuals with DS and DSAD

The plasma levels of neuroserpin were similar between all groups (Figure 2A, $P > .05$). In contrast, CSF neuroserpin levels were increased in subjects with DS (both aDS and DSAD), compared to non-trisomic controls (Figure 2B, $P < .001$, $P < .05$).

Plasma levels of the neuroserpin target, tPA, were reduced in aDS and DSAD compared to non-trisomic controls (Figure 2C, $P < .001$ and $P < .01$). However, no such alteration was detectable in CSF from the same individuals (Figure 2D, $P > .05$). We observed no correlation between plasma and CSF levels of neuroserpin or tPA (Table S1).
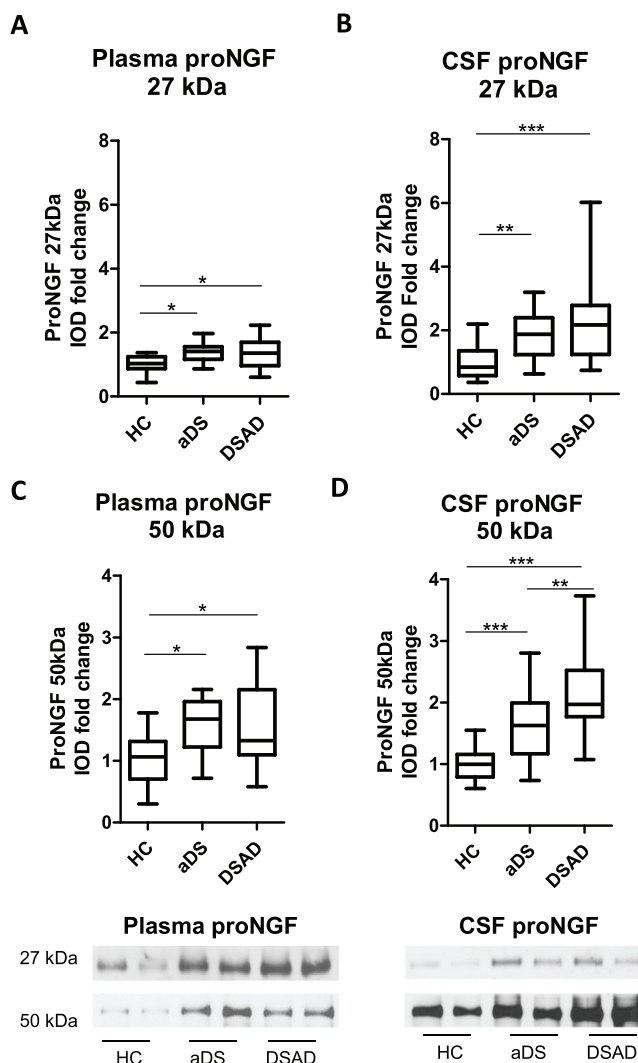

FIGURE 1 Upregulation of proNGF (nerve growth factor) in plasma and cerebrospinal fluid (CSF) from people with Down syndrome (DS). A, The 27 kDa form of proNGF, measured by Western blotting, is upregulated in plasma from Alzheimer's disease (AD)-asymptomatic people with Down syndrome (aDS) and in Down syndrome with symptomatic AD (DSAD) compared to non-trisomic controls, one-way analysis of variance (ANOVA), F = 5.03, $P = .01$ and Bonferroni post hoc correction; *$P < .05$. B, ProNGF (27 kDa) is also upregulated in CSF from people with aDS and DSAD compared to non-trisomic controls, Kruskal Wallis test $P = .0002$ H = 16.83 and Dunn's post hoc correction; **$P < .01$; ***$P < .001$. C, The 50 kDa form of proNGF is upregulated in plasma from people with aDS and DSAD compared to non-trisomic controls, one-way ANOVA, F = 5.62, $P = .006$ and Bonferroni post hoc correction; *$P < .05$. D, 50 kDa proNGF is upregulated in the CSF of people with aDS compared to non-trisomic controls, and further upregulated in people with DSAD, one-way ANOVA, F = 21.17, $P < .0001$ and Bonferroni post-hoc correction; ***$P < .001$. Data are displayed in box and whisker plots, in which the median is represented by the horizontal line and the whiskers go from each quartile to the minimum or maximum value. HC, Healthy controls: $n = 16$, aDS: $n = 14$, DSAD: $n = 22$; IOD, integrated optical density
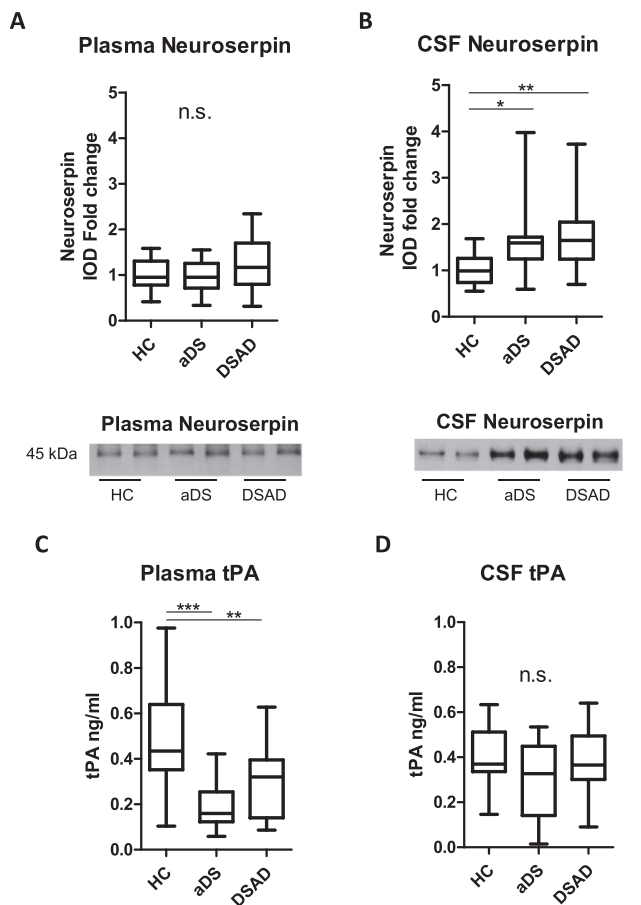
FIGURE 2 Dysregulation of neuroserpin and tissue plasminogen activator (tPA) in plasma and cerebrospinal fluid (CSF) from people with Down syndrome (DS). A, Neuroserpin levels were assessed by Western blotting. No significant change in plasma neuroserpin levels were observed between clinical groups, one-way analysis of variance (ANOVA), $P > .05$. B, Neuroserpin levels were found upregulated in CSF from AD-asymptomatic people with Down syndrome (aDS) and from people with Down syndrome and symptomatic Alzheimer's disease (DSAD) compared to non-trisomic controls, Kruskal Wallis test $P = .001$ H = 13.63 and Dunn's post hoc correction; *$P < .05$; **$P < .01$. C, tPA levels were reduced in plasma from people with aDS and DSAD, compared to non-trisomic controls, one-way ANOVA, F = 12.07, $P < .0001$ and Bonferroni post hoc correction; *$P < .05$. D, No change between clinical classifications in CSF levels of tPA, one-way ANOVA, $P > .05$. tPA levels were determined by enzyme-linked immunosorbent assay. Data are displayed in box and whisker plots, in which the median is represented by the horizontal line and the whiskers go from each quartile to the minimum or maximum value. HC, Healthy controls: $n = 16$, aDS: $n = 14$, DSAD: $n = 22$; IOD, integrated optical density

3.3 | Analysis of metalloproteases in plasma and CSF from individuals with aDS and DSAD

The NGF-degrading proteinase MMP-9 was found unaltered in plasma between the different groups (Figure 3A, $P > .05$). However, analysis of CSF samples revealed increased MMP-9 levels in the DSAD group compared to both the aDS group and the non-trisomic controls (Figure 3B,
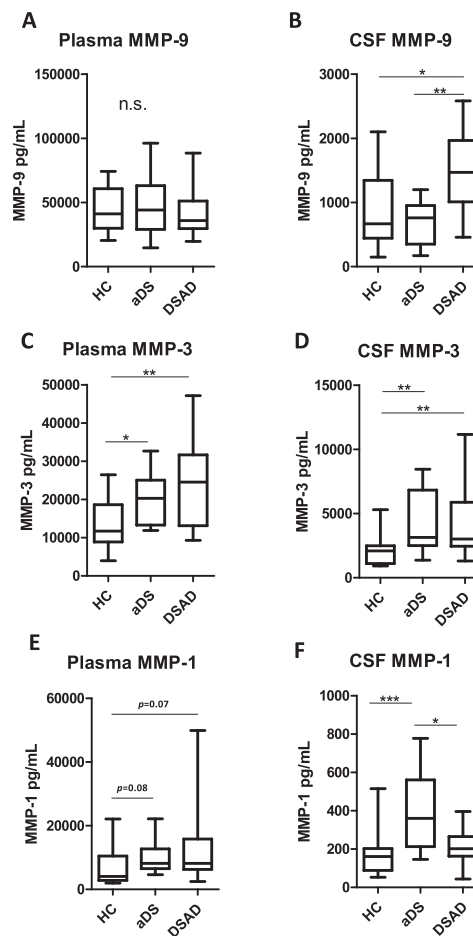

FIGURE 3 Dysregulation of matrix metalloproteinases (MMP) 9, 3, and 1 in plasma and cerebrospinal fluid (CSF) from people with Down syndrome (DS). A, No change between clinical classifications in plasma levels of MMP-9, Kruskal Wallis test, $P > .05$, Healthy controls (HC): $n = 16$, AD-asymptomatic Down syndrome (aDS): $n = 14$, Down syndrome with symptomatic Alzheimer's disease (DSAD): $n = 22$. B, CSF MMP-9 levels were elevated in people DSAD compared to aDS and non-trisomic controls, one-way analysis of variance (ANOVA), F = 5.90, $P = .007$ and Bonferroni post hoc correction; *$P < .05$, **$P < .01$ HC: $n = 13$, DS: $n = 10$, DSAD: $n = 12$. C, Both DSAD and aDS groups showed greater plasma levels of MMP-3 compared to non-trisomic controls, Kruskal Wallis test $P = .003$ H = 11.83 and Dunn's post hoc correction; *$P < .05$; **$P < .01$, HC: $n = 16$, DS: $n = 14$, DSAD: $n = 22$. D, CSF MMP-3 was likewise elevated in people with aDS and with DSAD compared to non-trisomic controls, Kruskal Wallis test $P = .001$ H = 13.47 and Dunn's post hoc correction; *$P < .05$; **$P < .01$, HC: $n = 16$, DS: $n = 13$, DSAD: $n = 21$. E, While no significant post hoc differences in plasma MMP-1 expression were observed between clinical classifications, trends of $P = .08$ and $P = .07$ toward upregulations in the aDS and DSAD groups compared to non-trisomic controls were observed $P = .03$, H = 6.85. F, MMP-1 was significantly upregulated in CSF from people with aDS compared to healthy controls and DSAD, Kruskal Wallis test $P = .0035$ H = 11.31 and Dunn's post hoc correction; *$P < .05$; ***$P < .001$, HC: $n = 15$, DS: $n = 14$, DSAD: $n = 20$. Metalloprotease levels were determined with a sensitive multiplex array from MesoScale Discovery. Data are displayed in box and whisker plots, in which the median is represented by the horizontal line and the whiskers go from each quartile to the minimum or maximum value

$P < .01$ and $P < .05$). CSF and plasma levels of MMP-9 did not correlate (Table S1).

MMP-3, which has been proposed to activate pro-MMP-9[47] and which we have also recently shown to degrade mature NGF but not its pro-form (Pentz et al.[88]), was equivalently increased in plasma from people with aDS and DSAD (Figure 3C, $P < .05$ and $P < .01$). MMP-3 was also elevated in both aDS and DSAD groups compared to controls in CSF samples (Figure 3D, $P < .01$). Plasma and CSF levels of MMP-3 significantly correlated ($P < .001$, $r^2 = 0.41$, Table S1).

MMP-1, which can be activated by MMP-3,[48] was unchanged across clinical classifications, though there was a trend toward upregulations in plasma in both the aDS and DSAD groups (Figure 3E, aDS vs HC: $P = .08$; DSAD vs HC: $P = .07$). In paired CSF samples, MMP-1 was elevated in the aDS group compared to both non-trisomic controls and to people with DSAD (Figure 3F, $P < .001$, $P < .05$). Plasma and CSF levels of MMP-1 did not correlate (Table S1).

3.4 | Specificity of NGF pathway markers to AD in DS

The ability of each marker of the NGF metabolic pathway to specifically signal changes associated with symptomatic AD in DS was assessed with the ROC curve analyses. Two candidates showed good and comparable performance: the 50 kDa form of proNGF measured in CSF (Figure 4A, AUC = 0.86) and CSF MMP-9 (Figure 4B, AUC = 0.87). These two markers showed better specificity for differentiating aDS and DSAD than two of three core biomarkers of AD that were compared for reference, including CSF 181-phospho-tau (p-tau, Figure 4C, AUC = 0.85) and total tau (t-tau, Figure 4C; AUC = 0.75). The CSF Aβ42/40 ratio performed slightly better than the proposed markers, with an AUC = 0.88 (Figure 4C). All other NGF metabolism-related markers had AUC values between 0.51 and 0.76 (Figure S2 in Supplementary Files).

3.5 | Associations between NGF metabolism-related proteins and core biomarkers of AD

Using robust regression (MM Estimation, following Croux et al.[41]), we next assessed the relationship between NGF metabolism-related proteins (which are not considered to be independent) and the established core AD biomarkers measured in CSF: the Aβ42/40 ratio, p-tau, and t-tau (see Tables 2 and 3). Adjusted r^2 values are given. In addition, we examined associations with each Aβ species individually and the p-tau/Aβ42 ratio (see Tables 2 and 3). The two composites have both been proposed to outperform their component individual measures as biomarkers of AD.[49-52]

The expression of NGF metabolism-related markers in plasma was typically uncorrelated to the three core AD CSF biomarkers, save neuroserpin, which correlated weakly to Aβ40 ($P = .02$, $r^2 = 0.07$, Table 2), Aβ42 ($P = .01$, $r^2 = 0.09$), and to the p-tau/Aβ42 ratio ($P = .01$,

A CSF ProNGF 50kDa ROC for AD in DS

AUC = 0.86

B CSF MMP9 ROC for AD in DS

AUC = 0.87

C Core Biomarkers ROC for AD in DS

pTau: AUC = 0.85
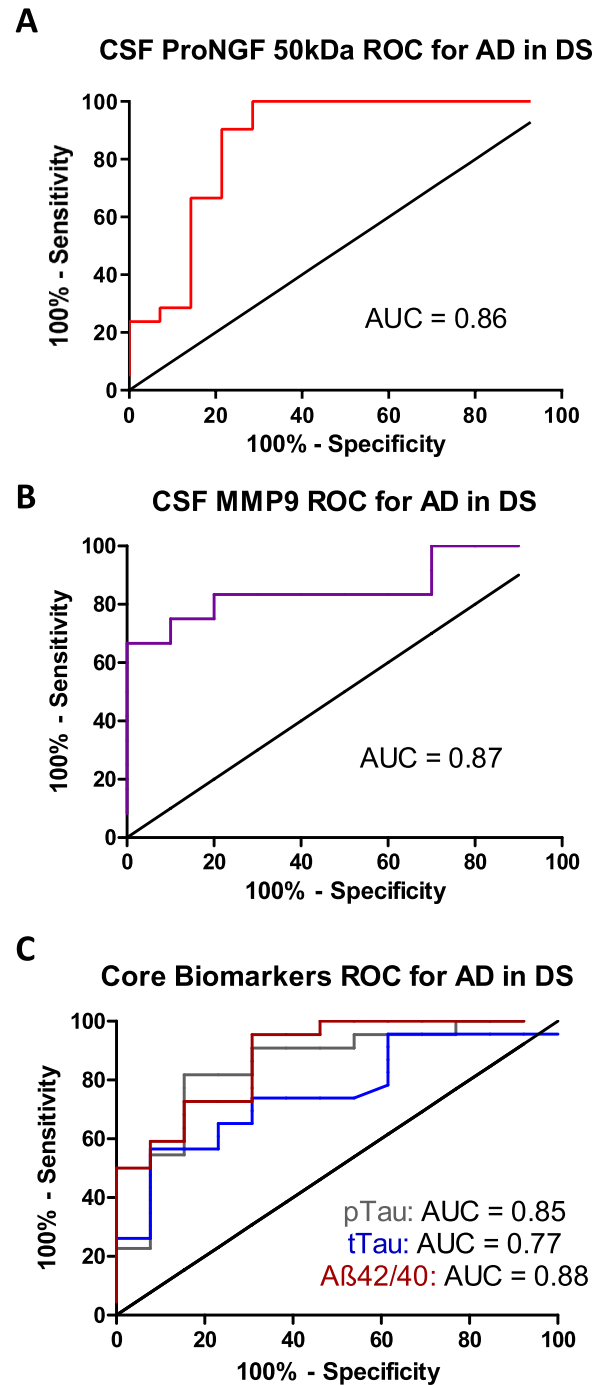tTau: AUC = 0.77
Aß42/40: AUC = 0.88

FIGURE 4 Cerebrospinal fluid (CSF) matrix metalloproteinase (MMP)-9 and proNGF (precursor of nerve growth factor) discriminate effectively between Alzheimer's disease (AD) symptomatic and asymptomatic people with Down syndrome (DS). A, CSF levels of the 50 kDa proNGF form effectively identify people with Down syndrome with symptomatic Alzheimer's disease (DSAD) from within the total DS sample group, with an area under the receiver operator characteristic (ROC) curve of 0.86. B, CSF MMP-9 levels effectively identify people with DSAD from within the total DS sample group, with an area under the ROC curve of 0.87. C, ROC curves for the core CSF biomarkers of AD demonstrate that MMP-9 and proNGF (50 kDa) measured in CSF outperformed CSF pTau-181 (AUC = 0.85) and total tau (AUC = 0.75), while being outperformed by the Aβ42/40 ratio (AUC = 0.88)

TABLE 2 Associations among plasma levels of NGF metabolism-related proteins, age, and core biomarkers of AD (individual components and composites) measured in cerebrospinal fluid, given by r^2 and P values as determined by the robust MM estimation method of Croux et al.[41] (all cases, n = 52)

Plasma marker	Age	Aβ40	Aβ42	p-tau-181	t-tau	Aβ42/40	p-tau/Aβ42
ProNGF 27 kDa	$P = .240$ $r^2 = 0.07$	$P = .66$ $r^2 = -0.02$	$P = .60$ $r^2 = -0.01$	$P = .63$ $r^2 = 0.02$	$P = .42$ $r^2 = 0.00$	$P = .31$ $r^2 = 0.01$	$P = .50$ $r^2 = -0.01$
ProNGF 50 kDa	$P = .348$ $r^2 = 0.04$	$P = .79$ $r^2 = -0.02$	$P = .67$ $r^2 = -0.02$	$P = .11$ $r^2 = 0.01$	$P = .08$ $r^2 = 0.03$	$P = 0.15$ $r^2 = 0.03$	$P = .09$ $r^2 = 0.02$
Neuroserpin	$P = .125$ $r^2 = 0.11$	$P = .02$ $r^2 = 0.07$	$P = .01$ $r^2 = 0.09$	$P = .14$ $r^2 = 0.02$	$P = .30$ $r^2 = 0.00$	$P = 0.15$ $r^2 = 0.03$	$P = .01$ $r^2 = 0.07$
tPA	$P = .945$ $r^2 = 0.00$	$P = .93$ $r^2 = -0.02$	$P = .72$ $r^2 = -0.02$	$P = .24$ $r^2 = 0.00$	$P = .34$ $r^2 = 0.00$	$P = .85$ $r^2 = -0.02$	$P = .51$ $r^2 = -0.01$
MMP-9	$P = .515$ $r^2 = 0.02$	$P = .46$ $r^2 = -0.01$	$P = .93$ $r^2 = -0.02$	$P = .09$ $r^2 = 0.02$	$P = .05$ $r^2 = 0.03$	$P = .46$ $r^2 = -0.01$	$P = .22$ $r^2 = 0.00$
MMP-3	$P = .491$ $r^2 = 0.02$	$P = .41$ $r^2 = -0.01$	$P = .40$ $r^2 = -0.01$	$P = .16$ $r^2 = 0.03$	$P = .08$ $r^2 = 0.05$	$P = .09$ $r^2 = 0.04$	$P = .22$ $r^2 = 0.02$
MMP-1	$P = .569$ $r^2 = 0.02$	$P = .76$ $r^2 = -0.02$	$P = .93$ $r^2 = -0.02$	$P = .66$ $r^2 = -0.02$	$P = .52$ $r^2 = -0.02$	$P = .88$ $r^2 = -0.02$	$P = .65$ $r^2 = -0.02$

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; MMP, matrix metalloproteinase; NGF, nerve growth factor; p-tau, phosphorylated tau; tPA, tissue plasminogen activator; t-tau, total tau.

$r^2 = 0.07$). The results of MM estimates for all associations between plasma NGF–related proteins and core AD biomarkers are given in Table 2.

In CSF, the 27 kDa form of proNGF correlated weakly to the Aβ42/Aβ40 ratio ($P = .04$, $r^2 = 0.07$, Table 3), as well as p-tau ($P = .008$, $r^2 = 0.10$) and t-tau levels ($P = .03$, $r^2 = 0.10$). A weak correlation to the p-tau/Aβ42 ratio was also observed ($P = .008$, $r^2 = 0.10$). In contrast, the 50 kDa proNGF form correlated well to the CSF Aβ42/Aβ40 ratio ($P < .0001$, $r^2 = 0.38$) as well as p-tau ($P = .01$, $r^2 = 0.26$), t-tau ($P < .005$, $r^2 = 0.31$), and the p-tau/Aβ42 ratio ($P = .02$, $r^2 = 0.28$). A weak correlation between 50 kDa proNGF in CSF and Aβ42 levels was also observed ($P = .04$, $r^2 = 0.07$). There were also significant correlations between CSF neuroserpin and the Aβ42/Aβ40 ratio ($P = .01$, $r^2 = 0.09$), as well as p-tau ($P = .04$, $r^2 = 0.05$) t-tau ($P = .04$, $r^2 = 0.07$), and the p-tau/Aβ42 ratio ($P = .01$, $r^2 = 0.06$). Conversely, no significant correlations were observed between tPA and core CSF biomarkers of AD. The results of MM estimates for all associations between CSF NGF–related proteins and core AD biomarkers are given in Table 3.

CSF MMP-9 only correlated to measures of tauopathy: p-tau ($P < .0001$, $r^2 = 0.22$) and t-tau ($P = .005$, $r^2 = 0.19$), and better still to the p-tau/ Aβ42 ratio ($P < .0001$, $r^2 = 0.26$). CSF MMP-3, in contrast, correlated well to the Aβ42/Aβ40 ratio ($P = .002$, $r^2 = 0.21$), though yet stronger was the association between MMP-3 levels and CSF Aβ40 alone ($P < .001$, $r^2 = 0.44$). CSF MMP-3 also correlation to t-tau ($P = .003$, $r^2 = 0.34$) and to the p-tau/ Aβ42 ratio ($P = .05$, $r^2 = 0.19$), though a strong positive association between CSF MMP-3 and p-tau did not survive correction ($P = .17$, $r^2 = 0.50$). Last, CSF MMP-1 correlated to individual measures of CSF Aβ42 ($P = .02$, $r^2 = 0.33$), but not to Aβ42, to their ratio, or to any measures of tauopathy. The results of MM estimates for all associations between CSF NGF–related proteins and core AD biomarkers are given in Table 3.

3.6 | Effects of sex on the plasma and CSF expression of NGF metabolism-related proteins

Given that some of our markers showed specificity to symptomatic AD in DS (ie, proNGF 50 kDa and MMP-9) or efficacy as measurements of pathological processes associated with AD, we investigated whether sex influenced the expression of NGF pathway proteins, as this could have implications for setting biomarker thresholds. We observed an interaction between sex and clinical diagnosis in the expression of CSF neuroserpin, with males displaying higher levels of neuroserpin in the non-trisomic controls and DSAD groups, but with females displaying higher levels in the aDS group (Figure 5A, two-way ANOVA, $P_{interaction} < .05$). MMP-9 was differentially expressed by sex in plasma, with males expressing higher levels across all clinical conditions (Figure 5B, two-way ANOVA, $P_{main\ effect\ sex} < .01$). Likewise, males had consistently greater levels of MMP-3, both in plasma (Figure 5C, two-way ANOVA, $P_{main\ effect\ sex} < .001$) and in CSF (Figure 5D, two-way ANOVA, $P_{main\ effect\ sex} < .05$). No other main effects of sex or interactions between sex and clinical diagnosis were observed for other NGF pathway markers (Figure S3 in Supplementary Files).

4 | DISCUSSION

People born with DS inexorably develop AD neuropathology through their lifetimes,[1–3,53–57] with lifetime risk estimates now more than 90%.[58] The primary cause of this vulnerability is attributed to the triplication of chromosome 21, which encodes the amyloid precursor protein (APP) gene, from which Aβ peptides are produced,[6,59–61] though other triplicated genes in chromosome 21 are also known to contribute to cognitive impairment and to influence the development of

TABLE 3 Associations among CSF levels of NGF metabolism-related proteins, age, and core biomarkers of AD (individual components and composites) measured in CSF, given by r^2 and p values for each association as determined by the robust MM estimation method of Croux et al.[41] (all cases, n = 52)

CSF marker	Age	Aβ40	Aβ42	p-tau-181	t-tau	Aβ42/40	p-tau/Aβ42
ProNGF 27 kDa	P = .705 r^2 = 0.00	P = .28 r^2 = 0.00	P = .60 r^2 = -0.01	P = .008 r^2 = 0.10	P = .03 r^2 = 0.10	P = .04 r^2 = 0.07	P = .008 r^2 = 0.10
ProNGF 50 kDa	P = .461 r^2 = 0.01	P = .33 r^2 = -0.01	P = .04 r^2 = 0.07	P = .01 r^2 = 0.26	P = .005 r^2 = 0.31	P < .001 r^2 = 0.38	P = .02 r^2 = 0.28
Neuroserpin	P = .249 r^2 = 0.03	P = .22 r^2 = 0.00	P = .42 r^2 = -0.01	P = .04 r^2 = 0.05	P = .04 r^2 = 0.07	P = .01 r^2 = 0.09	P = .01 r^2 = 0.06
tPA	P = .462 r^2 = 0.01	P = .94 r^2 = -0.02	P = .44 r^2 = -0.01	P = .37 r^2 = -0.01	P = .25 r^2 = -0.01	P = .36 r^2 = 0.00	P = .38 r^2 = -0.01
MMP-9	P = .051 r^2 = 0.10	P = .89 r^2 = -0.03	P = .10 r^2 = 0.04	P < .001 r^2 = 0.22	P = .005 r^2 = 0.19	P = .06 r^2 = 0.09	P < .001 r^2 = 0.26
MMP-3	P = .108 r^2 = 0.05	P < .001 r^2 = 0.44	P = .35 r^2 = 0.04	P = .17 r^2 = 0.50	P = .003 r^2 = 0.34	P = .002 r^2 = 0.21	P = .05 r^2 = 0.19
MMP-1	P < .001 r^2 = 0.38	P = .08 r^2 = 0.19	P = .02 r^2 = 0.33	P = .44 r^2 = -0.01	P = .45 r^2 = -0.02	P = .62 r^2 = -0.01	P = .69 r^2 = -0.02

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; CSF, cerebrospinal fluid; MMP, matrix metalloproteinase; NGF, nerve growth factor; p-tau, phosphorylated tau; tPA, tissue plasminogen activator; t-tau, total tau.

AD pathology in DS.[62–65] The inexorable progression of AD pathology in this population, generally unmixed with other age-related diseases, makes it ideal for assessing the ability of biofluid proteins to reflect pathological changes occurring in the brain and thereby to support these alterations. Biomarkers of preclinical AD-associated processes could be especially valuable, as this is the stage at which therapeutic intervention is most likely to be viable.[66]

AD, both in DS and in the sporadic population, is associated with the degeneration of basal forebrain cholinergic neurons.[5,7–14] This phenomenon contributes to cognitive decline in the early phases of the disease[67,68] and is associated with disruptions of the NGF metabolic pathway,[15–18] as basal forebrain cholinergic neurons depend on a continuous target-derived supply of NGF for the maintenance of a healthy cholinergic phenotype.[18,19,69–71]

Initial studies on the expression of NGF metabolism-related proteins in frontal, temporal, and parietal cortex had shown that adults with DS and AD-dementia express higher levels of proNGF and neuroserpin protein, lower levels of tPA mRNA, and higher levels of MMP-9 activity and mRNA (likely reflecting higher MMP-9 protein expression),[15] and indicating reduced maturation of proNGF to mNGF and increased degradation of mNGF. Furthermore, these changes also occur in sporadic AD, both at symptomatic stages and prior to the onset of overt dementia, where they correlate to cognitive decline and brain cholinergic dysfunction.[25,26]

The present results in matched body fluids from people with DS indicate that brain NGF pathway alterations are reflected in plasma and, more faithfully, in CSF, as plasma did not reveal the upregulations in MMP-9 or neuroserpin seen in DS brains.[15] The finding of NGF metabolic changes in individuals asymptomatic for AD supports an early dysregulation of NGF metabolism alongside AD pathology in DS and the utility of the methods described here for monitoring these changes in research and potentially clinical settings.

Both the 27 and 50 kDa forms of proNGF were increased in DS plasma and CSF (irrespective of AD symptomatology) compared to non-trisomic controls; however, the 50 kDa form was further elevated in the DSAD group compared to aDS. While the 27 kDa proNGF band represents the unmodified neuronal form of the NGF precursor,[72–74] higher molecular weight forms have also been frequently reported, including a 41 kDa form secreted by cortical cells[20] and a 50 kDa glycosylated form.[42,75,76]

It is unsurprising that CSF markers outperformed plasma markers as signals of neuropathology. First, the CSF compartment is in direct contact with the CNS. Second, brain-derived pools of molecules unaltered in plasma, such as MMP-9 and neuroserpin, may simply be too small compared to those derived from other sources, such as macrophages and neutrophils.[77,78] Importantly, the plasma results confirm the dysregulation of NGF metabolism-related proteins that had been reported previously in a different and independent DS population from Sicily, Italy, which similarly demonstrated the upregulation of proNGF, MMP-9, and MMP-3 in plasma samples, with no changes to neuroserpin.[16] Novel biomarkers are needed in DS, in both research and clinical contexts, to track specific pathological events and thereby enable AD precision medicine.[79,80] In this respect, we investigated whether NGF pathway biomarkers reflected processes specific to symptomatic individuals with DS that distinguished them from the rest of the DS population. ROC analysis revealed that MMP-9 (AUC = 0.87) and the 50 kDa form of proNGF measured in CSF (AUC = 0.86) outperformed classical CSF biomarkers of AD, including p-tau (AUC = 0.85) and t-tau (0.75), but not the Aβ42/40 ratio (0.88). Thus, we propose that our markers are reflecting changes that are associated with the onset of symptomatic AD in DS. Furthermore, proNGF and MMP-9 measured in CSF could assist in the identification of AD-related NGF dysmetabolism and cholinergic dysfunction in the DS population. This claim could be further investigated in future studies using markers
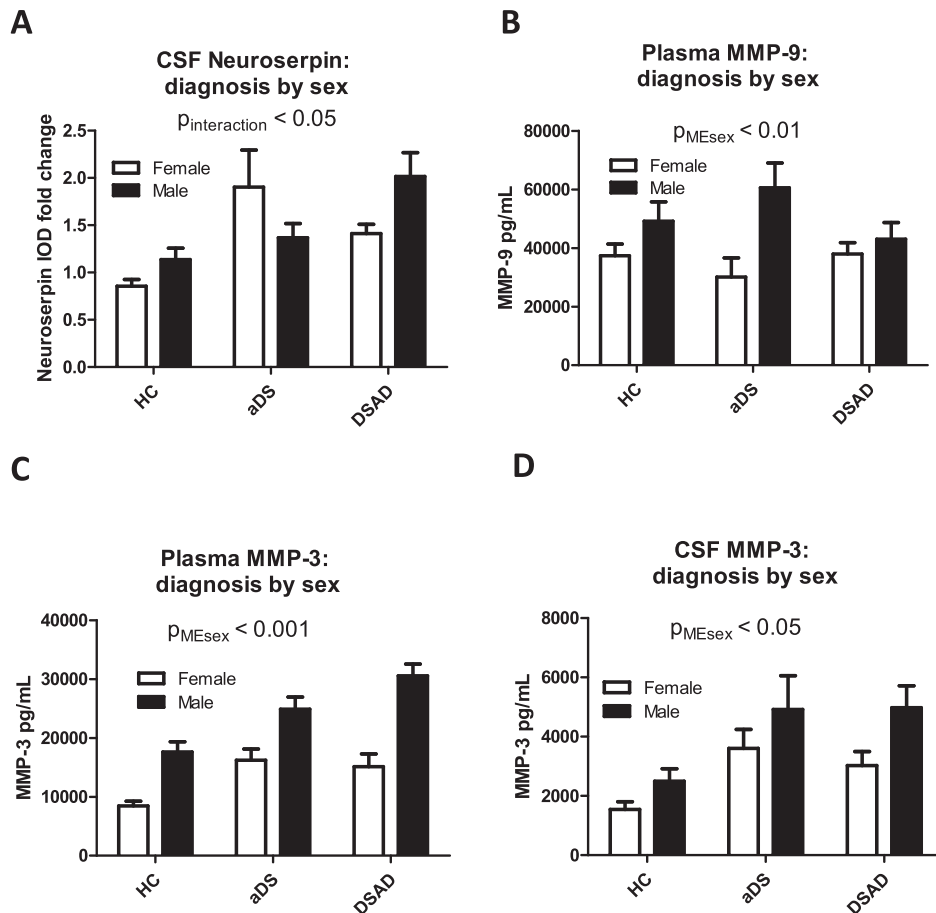
A

**CSF Neuroserpin:
diagnosis by sex**

$p_{interaction} < 0.05$

B

**Plasma MMP-9:
diagnosis by sex**

$p_{MEsex} < 0.01$

C

**Plasma MMP-3:
diagnosis by sex**

$p_{MEsex} < 0.001$

D

**CSF MMP-3:
diagnosis by sex**

$p_{MEsex} < 0.05$

FIGURE 5 Sex influences the expression of certain nerve growth factor (NGF) metabolism-related proteins in plasma and cerebrospinal fluid (CSF). A, Sex/group interaction in the expression of CSF neuroserpin (two-way analysis of variance, interaction $P < .05$). B, Main effect of sex in the expression of plasma matrix metalloproteinase (MMP)-9 with consistently higher levels in males. C, Main effect of sex in the expression of plasma MMP-3 ($P < .01$) with consistently higher levels in males. D, Main effect of sex ($P < .05$) in the expression of CSF MMP-3 with consistently higher levels in males

of cholinergic dysfunction accessible in plasma or CSF, or preferably, by basal forebrain volumetry with structural magnetic resonance imaging[79,80] or the positron emission tomography imaging of cholinergic synapses.[81,82] It is nonetheless worth noting that the direct impact of NGF dysregulation on the cholinergic/cognitive phenotype has been demonstrated experimentally in rodents[18,19] and that NGF dysmetabolism correlates to early cognitive decline and cholinergic dysfunction in the human AD brain.[26]

While current biomarkers are able to effectively diagnose DSAD from the wider DS population, proNGF could also play a role in subtyping and staging AD given that AD pathology and cholinergic degeneration differ in extent among neuroanatomical subtypes of AD.[83] The ability of proNGF to predict responses to cholinesterase inhibitor therapy should also be investigated in this regard. Importantly, it is likely that no single marker will prove to be an adequate diagnostic tool for AD in isolation; proNGF and other related markers may therefore have utility as part of a larger diagnostic panel.

Significant sex-related dimorphisms have been observed in the incidence and nature of AD, with important implications for diagnosis

and treatment.[84] The present study further suggests that sex-specific thresholds may also aid the clinical implementation of biomarkers. We observed sex-related differences in MMP-9 (most significantly in plasma but also in CSF), which was one of the markers best able to identify individuals with AD dementia from DS at large, as well as MMP-3, which has been shown to inform longitudinal risk of AD-associated cognitive decline and has been reported to be increased in males in other studies.[16,85] Therefore, these results would indicate that any clinical thresholds established for these markers should take the differential expression of males and females into account. These differences may also be relevant to the sex-related dimorphisms observed in the cholinergic system and its dysfunction in AD.[86]

Whether the NGF biofluid signature reported in DS plasma and CSF is unique to AD in DS remains to be thoroughly examined. Neuropathological studies have shown that proNGF (both forms), neuroserpin, MMP-3, and MMP-9 (activity) are all increased, while tPA is unchanged in the frontal cortex from people with sporadic AD or MCI, as well as in cognitively normal people with elevated levels of brain amyloid.[26] These results accord well with the biomarker signature reported in the

present study, and support its generalizability. These results are also in accord with other accounts of increased proNGF in CSF from people with AD,[87] and with the altered expression of MMP-3, neuroserpin, and plasminogen reported in CSF from AD and MCI participants.[40]

Overall, the results of the present work arrive in a timely manner, considering the new landscape of AD clinical trial initiatives being launched for the DS population, in which biomarkers will play a crucial role in AD staging and subtyping during recruitment as well as for monitoring treatment response. Based on our results in this and other studies we propose that proNGF and MMP-9, could play a significant role in monitoring NGF metabolism, cholinergic dysfunction, and early cognitive decline in this population– a proposition awaiting further confirmatory studies.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

REFERENCES

1. Lott IT. Down's syndrome, aging, and Alzheimer's disease: a clinical review. *Ann N Y Acad Sci.* 1982;396:15-27.
2. Lott IT, Head E. Dementia in Down syndrome: unique insights for Alzheimer disease research. *Nat Rev Neurol.* 2019;15:135-147.
3. Wisniewski KE, Wisniewski HM, Wen GY. Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann Neurol.* 1985;17:278-282.
4. Zigman WB, Devenny DA, Krinsky-McHale SJ, et al. Alzheimer's disease in adults with Down syndrome. *Int Rev Res Ment Retard.* 2008;36:103-145.
5. Mann DM, Yates PO, Marcyniuk B. Alzheimer's presenile dementia, senile dementia of Alzheimer type and Down's syndrome in middle age form an age related continuum of pathological changes. *Neuropathol Appl Neurobiol.* 1984;10:185-207.
6. Glenner GG, Wong CW. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun.* 1984;122:1131-1135.
7. Bowen DM, Smith CB, White P, Davison AN. Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain.* 1976;99:459-496.
8. Davies P, Maloney AJ. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet.* 1976;2:1403.
9. Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science.* 1982;215:1237-1239.
10. Pearson RC, Sofroniew MV, Cuello AC, et al. Persistence of cholinergic neurons in the basal nucleus in a brain with senile dementia of the Alzheimer's type demonstrated by immunohistochemical staining for choline acetyltransferase. *Brain Res.* 1983;289:375-379.
11. Yates CM, Simpson J, Gordon A, et al. Catecholamines and cholinergic enzymes in pre-senile and senile Alzheimer-type dementia and Down's syndrome. *Brain Res.* 1983;280:119-126.
12. Casanova MF, Walker LC, Whitehouse PJ, Price DL. Abnormalities of the nucleus basalis in Down's syndrome. *Ann Neurol.* 1985;18:310-313.
13. Mufson EJ, Bothwell M, Kordower JH. Loss of nerve growth factor receptor-containing neurons in Alzheimer's disease: a quantitative analysis across subregions of the basal forebrain. *Exp Neurol.* 1989;105:221-232.
14. Coyle JT, Price DL, DeLong MR. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science.* 1983;219:1184-1190.
15. Iulita MF, Do Carmo S, Ower AK, et al. Nerve growth factor metabolic dysfunction in Down's syndrome brains. *Brain.* 2014;137:860-872.
16. Iulita MF, Ower A, Barone C, et al. An inflammatory and trophic disconnect biomarker profile revealed in Down syndrome plasma: relation to cognitive decline and longitudinal evaluation. *Alzheimers Dement.* 2016;12:1132-1148.
17. Bruno MA, Leon WC, Fragoso G, Mushynski WE, Almazan G, Cuello AC. Amyloid beta-induced nerve growth factor dysmetabolism in Alzheimer disease. *J Neuropathol Exp Neurol.* 2009;68:857-869.
18. Allard S, Leon WC, Pakavathkumar P, Bruno MA, Ribeiro-da-Silva A, Cuello AC. Impact of the NGF maturation and degradation pathway on the cortical cholinergic system phenotype. *J Neurosci.* 2012;32:2002-2012.
19. Allard S, Jacobs ML, Do Carmo S, Cuello AC. Compromise of cortical proNGF maturation causes selective retrograde atrophy in cholinergic nucleus basalis neurons. *Neurobiol Aging.* 2018;67:10-20.
20. Bruno MA, Cuello AC. Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. *PNAS.* 2006;103:6735-6740.
21. Krueger SR, Ghisu G-P, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. *J Neurosci.* 1997;17:8984-8996.

22. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem*. 1992;267:3581-3584.

23. Gomis-R F-X, Maskos K, Betz M, et al. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. *Nature*. 1997;389:77.

24. Peng S, Wuu J, Mufson EJ, Fahnestock M. Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer disease. *J Neuropathol Exp Neurol*. 2004;63:641-649.

25. Bruno MA, Mufson EJ, Wuu J, Cuello AC. Increased matrix metalloproteinase-9 activity in mild cognitive impairment. *J Neuropathol Exp Neurol*. 2009;68:1309.

26. Pentz R, Iulita MF, Ducatenzeiler A, Bennett DA, Cuello AC. The human brain NGF metabolic pathway is impaired in the pre-clinical and clinical continuum of Alzheimers disease. *Mol Psychiatry*. 2020:1-15.

27. Cuello AC, Pentz R, Hall H. The brain NGF metabolic pathway in health and in Alzheimer's pathology. *Front Neurosci*. 2019;13:62.

28. Hampel H, Mesulam M-M, Cuello AC, et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain*. 2018;141:1917-1933.

29. Florencia Iulita M, Claudio Cuello A. The NGF metabolic pathway in the CNS and its dysregulation in Down syndrome and Alzheimer's disease. *Curr Alzheimer Res*. 2016;13:53-67.

30. Fortea J, Carmona-Iragui M, Benejam B, et al. Plasma and CSF biomarkers for the diagnosis of Alzheimer's disease in adults with Down syndrome: a cross-sectional study. *Lancet Neurol*. 2018;17:860-869.

31. Cummings JL, Mega M, Gray K, Rosenberg-Thompson S, Carusi DA, Gornbein J. The neuropsychiatric inventory: comprehensive assessment of psychopathology in dementia. *Neurology*. 1994;44:2308-2314.

32. Alcolea D, Clarimón J, Carmona-Iragui M, et al. The Sant Pau Initiative on Neurodegeneration (SPIN) cohort: a data set for biomarker discovery and validation in neurodegenerative disorders. *Alzheimers Dement (N Y)*. 2019;5:597-609.

33. Alcolea D, Pegueroles J, Munoz L, et al. Agreement of amyloid PET and CSF biomarkers for Alzheimer's disease on Lumipulse. *Ann Clin Transl Neurol*. 2019;6:1815-1824.

34. Carmona-Iragui M, Balasa M, Benejam B, et al. Cerebral amyloid angiopathy in Down syndrome and sporadic and autosomal-dominant Alzheimer's disease. *Alzheimers Dement*. 2017;13:1251-1260.

35. O'Bryant SE, Gupta V, Henriksen K, et al. Guidelines for the standardization of preanalytic variables for blood-based biomarker studies in Alzheimer's disease research. *Alzheimers Dement*. 2015;11:549-560.

36. Alcolea D, Martinez-Lage P, Izagirre A, et al. Feasibility of lumbar puncture in the study of cerebrospinal fluid biomarkers for Alzheimer's disease: a multicenter study in Spain. *J Alzheimers Dis*. 2014;39:719-726.

37. del Campo M, Mollenhauer B, Bertolotto A, et al. Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. *Biomark Med*. 2012;6:419-430.

38. Alcolea D, Carmona-Iragui M, Suarez-Calvet M, et al. Relationship between beta-Secretase, inflammation and core cerebrospinal fluid biomarkers for Alzheimer's disease. *J Alzheimers Dis*. 2014;42:157-167.

39. Mattsson N, Andreasson U, Persson S, et al. CSF biomarker variability in the Alzheimer's association quality control program. *Alzheimers Dement*. 2013;9:251-261.

40. Hanzel CE, Iulita MF, Eyjolfsdottir H, et al. Analysis of matrix metalloproteases and the plasminogen system in mild cognitive impairment and Alzheimer's disease cerebrospinal fluid. *J Alzheimers Dis*. 2014;40:667-678.

41. Croux C, Dhaene G, Hoorelbeke D. Robust standard errors for robust estimators. *CES-Discussion paper series (DPS) 0316*. 2004:1-20.

42. Edwards R, Selby M, Garcia PD, Rutter W. Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. *J Biol Chem*. 1988;263:6810-6815.

43. Seidah NG, Benjannet S, Pareek S, et al. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochem J*. 1996;314:951-960.

44. Trabjerg E, Kartberg F, Christensen S, Rand KD. Conformational characterization of nerve growth factor-β reveals that its regulatory propart domain stabilizes three loop regions in its mature part. *J Biol Chem*. 2017;292:16665-16676.

45. Soligo M, Protto V, Florenzano F, et al. The mature/pro nerve growth factor ratio is decreased in the brain of diabetic rats: analysis by ELISA methods. *Brain Res*. 2015;1624:455-468.

46. Barcelona PF, Saragovi HU. A pro-nerve growth factor (proNGF) and NGF binding protein, α2-macroglobulin, differentially regulates p75 and TrkA receptors and is relevant to neurodegeneration ex vivo and in vivo. *Mol Cell Biol*. 2015;35:3396-3408.

47. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J Biol Chem*. 1999;274:13066-13076.

48. Nagase H, Suzuki K, Morodomi T, Enghild J, Salvesen G. Activation mechanisms of the precursors of matrix metalloproteinases 1, 2 and 3. *Matrix (Stuttgart, Germany) Supplement*. 1992;1:237-244.

49. Lewczuk P, Esselmann H, Otto M, et al. Neurochemical diagnosis of Alzheimer's dementia by CSF Aβ42, Aβ42/Aβ40 ratio and total tau. *Neurobiol Aging*. 2004;25:273-281.

50. Maddalena A, Papassotiropoulos A, Müller-Tillmanns B, et al. Biochemical diagnosis of Alzheimer disease by measuring the cerebrospinal fluid ratio of phosphorylated tau protein to β-amyloid peptide42. *Arch Neurol*. 2003;60:1202-1206.

51. Dumurgier J, Schraen S, Gabelle A, et al. Cerebrospinal fluid amyloid-β 42/40 ratio in clinical setting of memory centers: a multicentric study. *Alzheimers Res Ther*. 2015;7:30.

52. Hansson O, Lehmann S, Otto M, Zetterberg H, Lewczuk P. Advantages and disadvantages of the use of the CSF Amyloid β (Aβ) 42/40 ratio in the diagnosis of Alzheimer's Disease. *Alzheimers Res Ther*. 2019;11:34.

53. Teller JK, Russo C, DeBusk LM, et al. Presence of soluble amyloid beta-peptide precedes amyloid plaque formation in Down's syndrome. *Nat Med*. 1996;2:93-95.

54. Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: implications for initial events in amyloid plaque formation. *Neurobiol Dis*. 1996;3:16-32.

55. Mori C, Spooner ET, Wisniewsk KE, et al. Intraneuronal Abeta42 accumulation in Down syndrome brain. *Amyloid*. 2002;9:88-102.

56. Leverenz JB, Raskind MA. Early amyloid deposition in the medial temporal lobe of young Down syndrome patients: a regional quantitative analysis. *Exp Neurol*. 1998;150:296-304.

57. Handen BL, Cohen AD, Channamalappa U, et al. Imaging brain amyloid in nondemented young adults with Down syndrome using Pittsburgh compound B. *Alzheimers Dement*. 2012;8:496-501.

58. McCarron M, McCallion P, Reilly E, Dunne P, Carroll R, Mulryan N. A prospective 20-year longitudinal follow-up of dementia in persons with Down syndrome. *J Intellect Disabil Res*. 2017;61:843-852.

59. Glenner G, Wong C, Quaranta V, Eanes E. The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. *Appl Pathol*. 1984;2:357-369.

60. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci*. 1985;82:4245-4259.

61. Rumble B, Retallack R, Hilbich C, et al. Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N Engl J Med*. 1989;320:1446-1452.

62. Gardiner K, Davisson M. The sequence of human chromosome 21 and implications for research into Down syndrome. *Genome Biol.* 2000;1:Reviews0002.

63. Gardiner K, Slavov D, Bechtel L, Davisson M. Annotation of human chromosome 21 for relevance to Down syndrome: gene structure and expression analysis. *Genomics.* 2002;79:833-843.

64. Gardiner K, Fortna A, Bechtel L, Davisson MT. Mouse models of Down syndrome: how useful can they be? Comparison of the gene content of human chromosome 21 with orthologous mouse genomic regions. *Gene.* 2003;318:137-147.

65. Gardiner K, Herault Y, Lott IT, Antonarakis SE, Reeves RH, Dierssen M. Down syndrome: from understanding the neurobiology to therapy. *J Neurosci.* 2010;30:14943-14945.

66. McDade E, Bateman RJ. Stop Alzheimer's before it starts. *Nature.* 2017;547:153.

67. Mesulam M. The cholinergic lesion of Alzheimer's disease: pivotal factor or side show?. *Learn Mem.* 2004;11:43-49.

68. Grothe M, Zaborszky L, Atienza M, et al. Reduction of basal forebrain cholinergic system parallels cognitive impairment in patients at high risk of developing Alzheimer's disease. *Cereb Cortex.* 2010;20:1685-1695.

69. Hefti F. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J Neurosci.* 1986;6:2155-2162.

70. Debeir T, Saragovi HU, Cuello AC. TrkA antagonists decrease NGF-induced ChAT activity in vitro and modulate cholinergic synaptic number in vivo. *J Physiol Paris.* 1998;92:205-208.

71. Cuello AC. Trophic responses of forebrain cholinergic neurons. *Prog Brain Res.* 1993;98:265-273.

72. Darling T, Petrides P, Beguin P, et al. The biosynthesis and processing of proteins in the mouse 7S nerve growth factor complex. *Cold Spring Harbor Symposia on Quantitative Biology*: Cold Spring Harbor Laboratory Press; 1983:427-434.

73. Ullrich A, Gray A, Berman C, Dull TJ. Human β-nerve growth factor gene sequence highly homologous to that of mouse. *Nature.* 1983;303:821.

74. Selby M, Edwards R, Sharp F, Rutter W. Mouse nerve growth factor gene: structure and expression. *Mol Cell Biol.* 1987;7:3057-3064.

75. Lakshmanan J, Burns C, Smith R. Molecular forms of nerve growth factor in mouse submaxillary glands. *Biochem Biophys Res Commun.* 1988;152:1008-1014.

76. Reinshagen M, Geerling I, Eysselein VE, et al. Commercial recombinant human β-Nerve growth factor and adult rat dorsal root ganglia contain an identical molecular species of nerve growth factor prohormone. *J Neurochem.* 2000;74:2127-2133.

77. Ardi VC, Kupriyanova TA, Deryugina EI, Quigley JP. Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc Natl Acad Sci.* 2007;104:20262-20267.

78. Kennedy SA, van Diepen AC, van den Hurk CM, et al. Expression of the serine protease inhibitor neuroserpin in cells of the human myeloid lineage. *Thromb Haemost.* 2007;97:394-399.

79. Schmitz TW, Spreng RN, Weiner MW, et al. Basal forebrain degeneration precedes and predicts the cortical spread of Alzheimer's pathology. *Nat Commun.* 2016;7:13249.

80. Grothe MJ, Heinsen H, Amaro Jr E, Grinberg LT, Teipel SJ. Cognitive correlates of basal forebrain atrophy and associated cortical hypometabolism in mild cognitive impairment. *Cereb Cortex.* 2016;26:2411-2426.

81. Aghourian M, Legault-Denis C, Soucy J, et al. Quantification of brain cholinergic denervation in Alzheimer's disease using PET imaging with [18 F]-FEOBV. *Mol Psychiatry.* 2017;22:1531-1538.

82. Nabulsi NB, Holden D, Zheng M-Q, et al. Evaluation of 11C-LSN3172176 as a novel PET tracer for imaging M1 muscarinic acetylcholine receptors in nonhuman primates. *J Nucl Med.* 2019;60:1147-1153.

83. Al-Shaikh FSH, Duara R, Crook JE, et al. Selective vulnerability of the nucleus basalis of meynert among neuropathologic subtypes of Alzheimer disease. *JAMA Neurol.* 2019;77:225-233.

84. Ferretti MT, Iulita MF, Cavedo E, et al. Sex differences in Alzheimer disease—the gateway to precision medicine. *Nat Rev Neurol.* 2018;14:457-469.

85. Iulita MF, Ganesh A, Pentz R, et al. Identification and preliminary validation of a plasma profile associated with cognitive decline in dementia and at-risk individuals: a retrospective cohort analysis. *J Alzheimers Dis.* 2019;67:327-341.

86. Giacobini E, Pepeu G. Sex and gender differences in the brain cholinergic system and in the response to therapy of Alzheimer disease with cholinesterase inhibitors. *Curr Alzheimer Res.* 2018;15:1077-1084.

87. E Counts S, He B, G Prout J, et al. Cerebrospinal fluid proNGF: a putative biomarker for early Alzheimer's disease. *Curr Alzheimer Res.* 2016;13:800-808.

88. Pentz R, Iulita MF, Mikutra-Cencora M, Ducatenzeiler A, Bennett DA, & Cuello AC. A new role for matrix metalloproteinase-3 in the NGF metabolic pathway: Proteolysis of mature NGF and sex-specific differences in the continuum of Alzheimer's pathology. *Neurobiology of Disease.* 2020:105150.

SUPPORTING INFORMATION

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