

RESEARCH ARTICLE

CSF proteomic profiles of neurodegeneration biomarkers in Alzheimer's disease

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Abstract

INTRODUCTION: We aimed to unravel the underlying pathophysiology of the neurodegeneration (N) markers neurogranin (Ng), neurofilament light (NfL), and hippocampal volume (HCV), in Alzheimer's disease (AD) using cerebrospinal fluid (CSF) proteomics.

METHODS: Individuals without dementia were classified as A+ (CSF amyloid beta [A β]42), T+ (CSF phosphorylated tau181), and N+ or N– based on Ng, NfL, or HCV separately. CSF proteomics were generated and compared between groups using analysis of covariance.

RESULTS: Only a few individuals were A+T+Ng–. A+T+Ng+ and A+T+NfL+ showed different proteomic profiles compared to A+T+Ng– and A+T+NfL–, respectively. Both Ng+ and NfL+ were associated with neuroplasticity, though in opposite directions. Compared to A+T+HCV–, A+T+HCV+ showed few proteomic changes, associated with oxidative stress.

DISCUSSION: Different N markers are associated with distinct neurodegenerative processes and should not be equated. N markers may differentially complement disease staging beyond amyloid and tau. Our findings suggest that Ng may not be an optimal N marker, given its low incongruency with tau pathophysiology.

KEYWORDS

Alzheimer's disease, biomarkers, cerebrospinal fluid, hippocampal volume, neurodegeneration markers, neurofilament light, neurogranin, pathophysiology, proteomics

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Highlights

- In Alzheimer's disease, neurogranin (Ng)+, neurofilament light (NfL)+, and hippocampal volume (HCV)+ showed differential protein expression in cerebrospinal fluid.
- Ng+ and NfL+ were associated with neuroplasticity, although in opposite directions.
- HCV+ showed few proteomic changes, related to oxidative stress.
- Neurodegeneration (N) markers may differentially refine disease staging beyond amyloid and tau.
- Ng might not be an optimal N marker, as it relates more closely to tau.

1 | BACKGROUND

The biological ATN staging scheme for Alzheimer's disease (AD) categorizes individuals based on amyloid (A), tau (T), and neurodegeneration (N) biomarkers in cerebrospinal fluid (CSF) or on imaging.¹ Proposed N biomarkers include, among others, neurogranin (Ng), neurofilament light (NfL), and hippocampal volume (HCV).^{1,2} To date, the pathophysiological processes underlying these candidate N biomarkers within the ATN staging scheme in AD are not fully understood.

The initially proposed N markers were CSF total tau (t-tau), glucose metabolism on fluorodeoxyglucose positron emission tomography, and atrophy on structural magnetic resonance imaging (MRI) in AD-related brain regions, including the hippocampus.¹ More recently, other N markers in CSF have been proposed, including Ng and NfL proteins.² In the present study, we focus on the candidate N markers CSF Ng, CSF NfL, and HCV. Ng is a neuron-specific postsynaptic protein, which plays a role in synaptic plasticity. Increased CSF Ng levels have been reported specifically in AD compared to other neurodegenerative diseases, and are presumably associated with synaptic dysregulation.²⁻⁵ NfL is an intermediate filament, which plays a role in the assembly and maintenance of the neuronal cytoskeleton. High CSF NfL levels are associated with large-caliber axonal degeneration in AD, but also in other neurodegenerative diseases.^{2,6,7} The hippocampus is part of the limbic system and plays an important role in memory and learning. It is among the earliest regions showing atrophy in AD. Reduced HCV is an important early marker of brain atrophy in AD, as well as in other neurodegenerative diseases.^{8,9} To date, it remains unclear to what extent different N markers reflect similar or distinct neurodegenerative processes in AD. Previous studies showed that CSF Ng reflects amyloid beta (A β)-dependent neurodegeneration, while CSF NfL is associated with neurodegeneration independently of A β pathology.¹⁰ CSF and neuroimaging biomarkers of neurodegeneration often show low correlation, which suggests that these markers may reflect different neurodegenerative aspects.^{11,12}

To our knowledge, no study has yet compared the proteomic signature underlying distinct N markers, that is, Ng, NfL, and HCV. Therefore, the aim of this study is to use large-scale proteomics to assess commonalities and differences across Ng, NfL, and HCV, and to understand whether these N markers reflect similar or distinct under-

lying pathophysiological processes in AD. CSF protein level alterations reflect ongoing biochemical and metabolic changes in the brain and studying a large number of proteins can provide a robust characterization of the underlying pathophysiological mechanisms in AD.^{13,14} In the present study, we included individuals without dementia and with abnormal amyloid and tau markers (A+T+) and compared CSF proteomic profiles of those with an abnormal N marker to those with a normal N marker. Secondary analyses were conducted in A+T- individuals without dementia using the same methodology. More knowledge about the underlying biological processes of the different N markers in AD will be important for improving AD staging, which may be relevant for the design of clinical trials.¹⁵⁻¹⁸

2 | METHODS

2.1 | Participants

Four hundred seven participants were enrolled from the Maastricht BioBank Alzheimer Center Limburg cohort (BB-ACL, $n = 52$) memory clinic study,¹⁹ the Washington University (WashU) Knight Alzheimer Disease Research Center (ADRC, $n = 90$), study²⁰ and the European Medical Information Framework for Alzheimer's Disease Multimodal Biomarker Discovery study (EMIF-AD MBD, $n = 265$).²¹ All patients provided informed consent for research. All centers approved participation in this study after local medical ethics committee approval. Participants were included in the current project if they had normal cognition/subjective cognitive decline (NC) or mild cognitive impairment (MCI), baseline CSF samples available, and baseline data of CSF A β 42 and CSF phosphorylated tau (p-tau)181 measures, and at least one of the following baseline measures: CSF NfL, CSF Ng, or HCV.

2.2 | Neuropsychological assessment

All participants were administered a neuropsychological assessment, including the Mini-Mental State Examination (MMSE) and tests assessing several cognitive domains, including memory. Memory tests differed between centers but most common tests were the Rey Auditory

Verbal Learning Test (BB-ACL and EMIF-AD MBD studies) and the Free and Cued Selective Reminding Test (WashU Knight ADRC study). Detailed information about the neuropsychological tests and calculation of Z scores can be found elsewhere.^{19,21,22} NC was defined as neuropsychological test performance ranged within 1.5 standard deviation (SD) of the average corrected for age, sex, and years of education. MCI was defined according to the criteria of Petersen, and based on <1.5 SD in at least one of the neuropsychological tests assessing several cognitive domains.^{21,23}

2.3 | CSF protein analysis

CSF was obtained by lumbar puncture, centrifuged, and stored at -80°C in polypropylene tubes. CSF samples were shipped on dry ice to the neurochemistry lab of the University of Gothenburg in Mölndal, Sweden, where central proteomic and peptidomic analyses were performed using an untargeted tandem mass tag (TMT) technique with 10+1 plexing, using high-pH reverse phase high-performance liquid chromatography for peptide prefractionation^{14,24,25} to quantify ≈ 500 proteins as well as endogenous peptides in the same CSF sample aliquot. More information is described elsewhere.^{14,24,25} In total, 3102 proteins were quantified using TMT spectrometry. We selected proteins that had at least one third of observations per participant group. For related proteins that had identical values due to fragment non-specificity, we randomly selected one protein for analysis.¹⁴ All analyses were performed according to the manufacturer's instructions and using two batches (batch 1 $n = 285$, batch 2 $n = 122$) of reagents by board-certified laboratory technicians who were blinded to clinical information.

Targeted analyses were performed for well-established CSF markers, that is, $\text{A}\beta 40$, $\text{A}\beta 42$, $\text{A}\beta 42/40$ ratio, p-tau181, t-tau, NfL, and Ng. For the BB-ACL and the EMIF-AD MBD cohorts, the neurochemistry lab of University of Gothenburg in Sweden analyzed centrally the levels of $\text{A}\beta 40$, $\text{A}\beta 42$ (using V-PLEX Plus $\text{A}\beta$ Peptide Panel 1 [6E10] Kit from Meso Scale Discovery [MSD]), NfL (using NF-light enzyme-linked immunosorbent assay [ELISA], UmanDiagnostics), and Ng (using an in-house immunoassay). Moreover, $\text{A}\beta 42$, t-tau and p-tau levels were measured locally with INNOTEST ELISAs (Fujirebio) and for a subset with AlzBio3 xMAP Luminex ($n = 29$). For the WashU Knight ADRC cohort, levels of $\text{A}\beta 40$, $\text{A}\beta 42$, t-tau, and p-tau were measured by chemiluminescent enzyme immunoassay with a LUMIPULSE G1200 (Fujirebio), NfL was measured with the NF-light ELISA (UmanDiagnostics), and Ng was measured by quantitative fluorescent two-site immunoassays using single-molecule counting (SMC) technology on the Singulex Erenna platform.^{26,27}

2.4 | Genetic analysis

Protocols for apolipoprotein E (APOE) genotyping are described elsewhere.^{21,28,29} In brief, APOE genotype was assessed using polymerase chain reaction (PCR) techniques for two single nucleotide

RESEARCH IN CONTEXT

1. **Systematic review:** The authors reviewed the literature using PubMed. In the ATN framework of Alzheimer's disease (AD), various neurodegeneration (N) markers have been suggested, such as neurogranin (Ng), neurofilament light (NfL), and hippocampal volume (HCV). It is unclear whether these N markers reflect similar or distinct underlying mechanisms.
2. **Interpretation:** Ng, NfL, and HCV were associated with distinct cerebrospinal fluid proteomic profiles, reflecting different neurodegenerative processes in AD. While Ng and NfL were both associated with neuroplasticity, they acted in opposite directions. Only a few individuals were $\text{A}+\text{T}+\text{Ng}-$, indicating that Ng may not be an optimal N marker. Different N markers should not be equated and may differentially complement disease staging beyond amyloid and tau.
3. **Future directions:** Future studies should characterize further the dysregulated processes associated with each neurodegeneration biomarker, as well as the associated clinical outcomes, to improve AD diagnosis and prognosis, which may be relevant for the design of clinical trials.

polymorphisms (SNPs; rs429358 for the " $\epsilon 4$ allele" and rs7412 the " $\epsilon 2$ allele"). Participants were classified as APOE $\epsilon 4$ carriers or non-carriers, determined by the presence of at least one APOE $\epsilon 4$ allele.

In a subset of participants of the EMIF-AD MBD study ($n = 234$), polygenic risk score (PGRS) analyses were performed on imputed genome-wide SNP genotyping data generated with the Global Screening Array (Illumina, Inc.) using PRSice (v2.3).³⁰ PGRSs were calculated by adding the sum of each allele weighted by the strength of its association with AD risk as calculated previously by a genome-wide association study (GWAS) on AD.³¹ Prior to calculating PGRS, clumping was performed to remove SNPs that are in linkage disequilibrium ($r^2 > 0.1$) within a slicing 1 M bp window. After clumping, PGRS were computed using various SNP inclusion thresholds.¹⁴ PGRS results were validated in the WashU Knight ADRC cohort ($n = 91$).^{32,33}

2.5 | Image analysis

At each site, MRI scans were acquired using local protocols. For all studies, a thorough quality check was performed. Images were segmented using FreeSurfer (version 5.3.0 for EMIF-AD MB and BB-ACL studies and version 5.0 for the WashU Knight ADRC study, <https://surfer.nmr.mgh.harvard.edu>).³⁴ Subcortical volumes (including HCV) were normalized by total intracranial volume (TIV).^{35,36}

2.6 | Participant classification

Persons were classified using the ATN scheme. Local CSF A β 42 was used as a measure of amyloid (A) and local CSF p-tau as a measure of tau (T). We used cohort-specific cut-offs to define abnormal biomarker levels. The A β 42 cut-offs were redefined for each cohort using unbiased Gaussian mixture modeling, as different methodologies had been used by the centers to define A β 42 cut-offs (Table S1 in supporting information). CSF NfL, CSF Ng, or HCV were used as a measure of neurodegeneration (N). As those parameters were measured centrally for EMIF-AD MBD and BB-ACL studies, common cut-offs were calculated for both studies and separately for the WashU Knight ADRC cohort, using unbiased Gaussian mixture modeling.^{37,38} If the Gaussian mixture modeling showed only one distribution, the Youden index was used.³⁹ The cut-offs for N markers are presented in Figure S1 in supporting information. For primary analysis, we included individuals with A+T+N+ and A+T+N-. For secondary analysis, we included individuals with A+T-N+ and A+T-N-. Individuals with NC A-T- were included as control group.

2.7 | Pathway enrichment analysis

Gene Ontology (GO) enrichment analysis was performed using Protein ANalysis THrough Evolutionary Relationships (PANTHER, version 15.0)⁴⁰ to identify the biological processes, cellular components, and molecular functions related to the increased or decreased proteins of each group comparison. This tool used the Fisher exact test with false discovery rate (FDR; using the Benjamini-Hochberg procedure⁴¹) and we only reported pathways with an FDR-corrected *P* value < 0.05.^{42,43} Associated GO terms were clustered in broader categories to reduce redundancy and facilitate interpretation. We validated these pathways and categories using the online database STRING version 11.0⁴⁴ and ClueGO, a Cytoscape plug-in.⁴⁵ We further annotated proteins as indicative of increased blood-brain barrier (BBB) permeability from Neumeier et al.,⁴⁶ Dayon et al.,⁴⁷ and Rapoport and Pettigrew⁴⁸ or as highly expressed by the choroid plexus (ChP) of the lateral ventricles according to the Allen Brain Map⁴⁹ through Harmonizome⁵⁰ and ABAEnrichment analysis.⁵¹

2.8 | Statistical analysis

To investigate the associations among the different ATN biomarkers, we performed Spearman rank correlation tests. For parametric analyses, we transformed the biomarker values to Z scores as absolute values for biomarkers varied across assays. To characterize the N groups, clinical, CSF, and imaging measures were compared between groups using analyses of covariance (ANCOVA) corrected for age and sex for continuous variables and chi-square for categorical variables. CSF protein levels were normalized according to the mean and SD of the control group. PGRS were compared between groups using linear models.

Individuals with NC and MCI were combined for analyses. For the main analysis, we compared proteomic profiles of individuals without dementia and with A+T+N+ to those with A+T+N-. In secondary analysis, we compared proteomic profiles of individuals without dementia and with A+T-N+ to those with A+T-N-. Groups were also compared to controls (NC A-T-). In post hoc analyses, proteomic profiles were studied separately in individuals with NC and MCI. In post hoc analyses, our main analyses were corrected for potential batch effects, and as there is an age-dependent increase of CSF NfL, we also determined post hoc an age-adjusted cut-off of ≥ 2 SD, using previously reported age-adjusted Z score formulas.⁵² For EMIF-AD MBD and Maastricht BB-ACL, the formula for age-adjusted Z scores was: $Z \text{ score} = (\log^2[\text{NfL value}] - [5.957 + (\text{age} \times 0.053)]) / 396.549$. For WashU Knight ADRC, the formula for age-adjusted Z scores was: $Z \text{ score} = (\log^2[\text{NfL value}] - [6.911 + (\text{age} \times 0.038)]) / 507.565$.

Statistical analyses were performed using R 4.1.3. and IBM SPSS Statistics version 26. Two-sided statistical significance was used and set at *P* < 0.05.

3 | RESULTS

3.1 | CSF proteomic profiles for neurodegeneration biomarkers

We compared CSF protein levels in persons with AD (A+T+) with an abnormal N marker to those with a normal N marker. Comparisons to controls are presented in Tables S2-S4 in supporting information. A similar secondary analysis in A+T- individuals is presented in [Supplementary Results](#) and in Tables S2-S5 in supporting information.

3.1.1 | CSF neurogranin as a neurodegeneration marker

Using Ng as N marker, 12 participants were classified as A+T+N- and 143 as A+T+N+ (Figure 1A). A+T+N+ individuals showed higher levels of A β 40, A β 42, p-tau, and t-tau compared to A+T+N-. No significant differences in the A β 42/40 ratio were found between A+T+N+ and A+T+N-. There were no A+T+N- individuals with NC (Table 1). No significant AD PGRS differences were found between N- and N+ (Figure S2A in supporting information).

CSF proteomic profiling showed that 177 proteins were increased and 14 were decreased in A+T+N+ compared to A+T+N- (Figure 2A, Table S2A). Increased proteins were associated with nervous system development, cell adhesion and migration, protein secretion and modification, and blood vessel development. No pathways were related to the decreased proteins (Figure 2B,C, Table S2B). Twenty-nine percent (four proteins) of those decreased proteins were associated with increased BBB permeability.

In A+T- individuals, similar proteomic results were found when comparing N+ to N- (Figure 1B, [Supplementary Results](#), Figure S3 in supporting information, Table S2C,D).

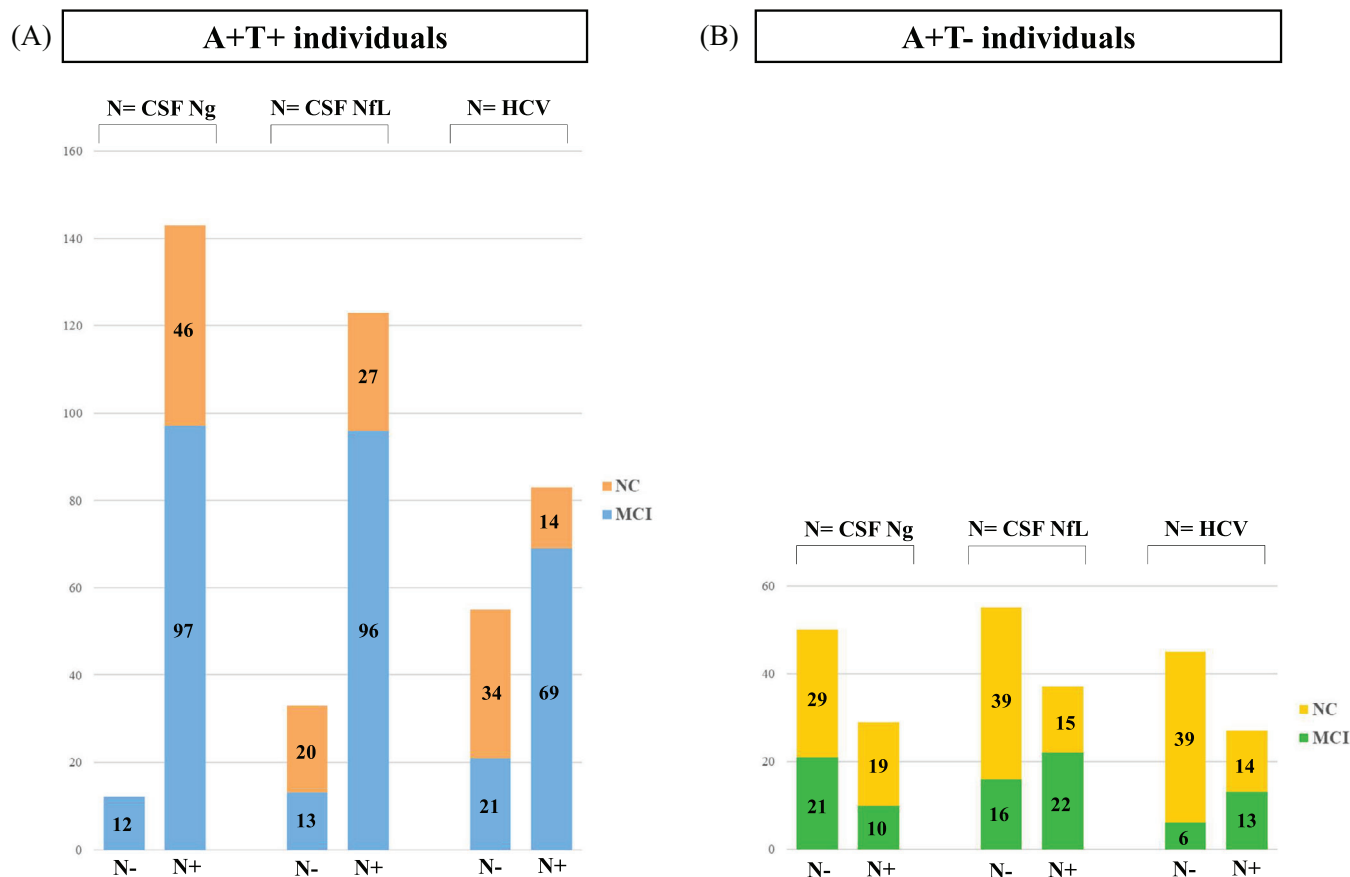


FIGURE 1 A, Stacked bar graph showing the numbers of N+ and N- participants within the A+T+ group for the three neurodegeneration markers (i.e., CSF neurogranin, CSF neurofilament light, and hippocampal volume). The number of individuals with normal cognition (NC; orange) and mild cognitive impairment (MCI; blue) is also shown. B, Stacked bar graph showing the numbers of N+ and N- participants within the A+T- group for the three neurodegeneration markers (i.e., CSF neurogranin, CSF neurofilament light, and hippocampal volume). The number of individuals with NC (yellow) and MCI (green) is also shown. A-, absence of amyloid pathology determined by CSF A β 42 level below cut point; A+, presence of amyloid pathology determined by CSF A β 42 level above cut point; T-, normal CSF p-tau level below cut point; T+, abnormal CSF p-tau level above cut point; N-, absence of neurodegeneration or neuronal injury determined by CSF neurogranin, CSF neurofilament light, or hippocampal volume; N+, presence of neurodegeneration or neuronal injury determined by CSF neurogranin, CSF neurofilament light, or hippocampal volume. A β , amyloid beta; CSF, cerebrospinal fluid; HCV, hippocampal volume; MCI, mild cognitive impairment; NC, normal cognition; NfL, neurofilament light; Ng, neurogranin; p-tau, phosphorylated tau

3.1.2 | CSF neurofilament light as a neurodegeneration marker

Using NfL as N marker, 33 participants were classified as A+T+N- and 123 as A+T+N+ (Figure 1A). A+T+N+ individuals were older, more often males, and more often diagnosed with MCI than A+T+N-. A+T+N+ individuals had higher levels of A β 40, p-tau, t-tau, and Ng; a lower A β 42/40 ratio; and smaller HCV compared to A+T+N- (Table 1). No significant AD PGRS differences were found between A+T+N- and A+T+N+ (Figure S2A).

CSF proteomic analysis showed that 13 proteins were increased and 144 were decreased in A+T+N+ compared to A+T+N- (Figure 3A, Table S3A). No pathways were associated with the increased proteins. Decreased proteins were related to biological pathways linked with cell adhesion and migration, nervous system development, extracel-

lular matrix (ECM), lipids, protein processing, hemostasis and blood vessel development, transforming growth factor β signaling, and lysosome organization (Figure 3B,C, Table S3B). Fifty-eight decreased proteins were enriched for expression in the ChP (40%, ABAenrichment $P = 0.034$).

In A+T- individuals (Figure 1B), similar proteomic pattern was found as in A+T+ individuals, with mainly decreased proteins in N+ compared to N- (Supplementary Results, Figure S4A in supporting information, Table S3C). Nonetheless, biological pathways were distinct from those in A+T+. In A+T-, increased proteins were enriched for biological pathways related to the immune system, while decreased proteins were enriched for ion homeostasis, cell migration, immune system and inflammation, protein degradation, and lipids and epithelial cells (Figure S4B,C, Table S3D). Decreased proteins were also enriched for expression in the ChP (41%, ABAenrichment $P = 0.001$).

TABLE 1 Sample characteristics of A+T+ individuals classified using different neurodegeneration (N) biomarker.

A. Neurodegeneration biomarker (N) = neurogranin (Ng)				
	Controls <i>n</i> = 145	A+T+N− <i>n</i> = 12	A+T+N+ <i>n</i> = 143	<i>P</i> value N+ vs. N−
Age, years	64.6 (8.6)	71.6 (5.9)	71.1 (6.8)	0.798
Female (%)	83 (57.2)	7 (59.3)	80 (55.9)	0.873
Education, years	13.2 (3.6)	10.9 (3.1)	12.4 (3.9)	0.226
APOE ε4 carriers (%)	28 (24.1)	6 (54.5)	94 (67.1)	0.395
MCI diagnosis	/	12 (100.0)	97 (67.8)	0.019
MMSE	28.9 (1.2)	26.3 (3.1)	26.6 (2.8)	0.749
Memory, <i>Z</i> score	0.2 (0.9)	−1.0 (1.5)	−1.9 (1.4)	0.798
CSF Aβ40, <i>Z</i> score	0.3 (1.2)	−1.0 (1.1)	0.1 (1.8)	0.025
CSF Aβ42, <i>Z</i> score	0.5 (1.3)	−1.6 (0.4)	−0.9 (1.2)	0.031
CSF Aβ42/40 ratio, <i>Z</i> score	0.9 (1.0)	−1.7 (0.6)	−1.7 (0.8)	0.808
CSF p-tau, <i>Z</i> score	−0.4 (0.5)	0.99 (0.43)	1.8 (1.3)	0.049
CSF t-tau, <i>Z</i> score	−0.3 (0.5)	0.8 (0.7)	1.6 (1.3)	0.050
CSF Ng, <i>Z</i> score	−0.3 (0.7)	−0.9 (0.3)	0.6 (1.1)	<0.001
CSF NfL, <i>Z</i> score	0.0 (1.5)	0.6 (1.8)	1.3 (4.0)	0.530
HCV, <i>Z</i> score	0.1 (1.3)	−0.7 (1.5)	−1.7 (0.7)	0.151
B. Neurodegeneration biomarker (N) = neurofilament light (NfL)				
	Controls <i>n</i> = 145	A+T+N− <i>n</i> = 33	A+T+N+ <i>n</i> = 123	<i>P</i> value N+ vs. N−
Age, years		68.1 (6.9)	72.0 (6.5)	0.003
Female (%)		26 (78.8)	61 (49.6)	0.003
Education, years		13.2 (4.0)	12.1 (3.8)	0.136
APOE ε4 carriers (%)		19 (59.4)	83 (69.2)	0.295
MCI diagnosis		13 (39.4)	96 (78.0)	<0.001
MMSE		27.1 (3.1)	26.4 (2.8)	0.212
Memory, <i>Z</i> score		−0.6 (1.4)	−1.0 (1.4)	0.488
CSF Aβ40, <i>Z</i> score		−0.7 (0.8)	0.2 (1.8)	<0.001
CSF Aβ42, <i>Z</i> score		−0.7 (1.0)	−1.0 (1.2)	0.373
CSF Aβ42/40 ratio, <i>Z</i> score		−1.5 (0.7)	−1.8 (0.8)	0.007
CSF p-tau, <i>Z</i> score		1.3 (0.9)	1.8 (1.4)	0.008
CSF t-tau, <i>Z</i> score		1.0 (0.9)	1.7 (1.3)	0.001
CSF Ng, <i>Z</i> score		0.2 (0.8)	0.6 (1.2)	0.032
CSF NfL, <i>Z</i> score		−0.4 (0.7)	1.7 (4.3)	0.013
HCV, <i>Z</i> score		−1.0 (1.6)	−1.8 (1.7)	0.018
C. Neurodegeneration biomarker (N) = hippocampal volume (HCV)				
	Controls <i>n</i> = 145	A+T+N− <i>n</i> = 55	A+T+N+ <i>n</i> = 83	<i>P</i> value N+ vs. N−
Age, years		68.8 (6.6)	72.0 (5.9)	0.004
Female (%)		26 (47.3)	49 (59.0)	0.174
Education, years		13.6 (3.8)	12.0 (3.5)	0.018
APOE ε4 carriers (%)		32 (59.3)	60 (74.1)	0.070
MCI diagnosis		21 (38.2)	69 (83.1)	<0.001
MMSE		27.8 (2.2)	26.2 (2.9)	0.001
Memory, <i>Z</i> score		−0.1 (1.2)	−1.4 (1.4)	<0.001
CSF Aβ40, <i>Z</i> score		0.0 (1.6)	0.3 (1.8)	0.161
CSF Aβ42, <i>Z</i> score		−0.5 (1.3)	−1.0 (1.0)	0.026

(Continues)

TABLE 1 (Continued)

C. Neurodegeneration biomarker (N) = hippocampal volume (HCV)			
	Controls <i>n</i> = 145	A+T+N– <i>n</i> = 55	A+T+N+ <i>n</i> = 83
CSF Aβ42/40 ratio, Z score		–1.6 (0.9)	–1.8 (0.7)
CSF p-tau, Z score		1.3 (0.9)	2.0 (1.5)
CSF t-tau, Z score		1.3 (1.0)	1.9 (1.5)
CSF Ng, Z score		0.5 (1.0)	0.6 (1.2)
CSF NfL, Z score		1.3 (5.4)	1.1 (1.9)
HCV, Z score		0.1 (0.9)	–2.6 (1.2)
			<i>P</i> value N+ vs. N–

Notes: Controls were individuals with normal cognition and normal levels of Aβ42 and p-tau. A+T+N– individuals were non-demented (NC+MCI) individuals with abnormal levels of Aβ42 (A) and p-tau (T) and normal levels of neurodegeneration marker (N, either Ng, NfL, or HCV). A+T+N+ individuals were non-demented (NC+MCI) individuals with abnormal levels of Aβ42 (A), p-tau (T), and neurodegeneration marker (N, either Ng, NfL, or HCV). Values represent mean (standard deviation) or number (percentages). Significant *P* values (< 0.05) are bold. The sample size was smaller for some variables: A total of 13 values are missing for education, 36 for APOE ε4, and 54 for memory. Twenty-three values are missing for Aβ40, Aβ42, and HCV; 37 for NfL; 45 for Ng; and 74 for HCV. Aβ40, Aβ42, t-tau, p-tau, Ng, and NfL values are presented as Z scores with controls as a reference.

Abbreviations: APOE, apolipoprotein E; Aβ, amyloid beta; CSF, cerebrospinal fluid; HCV, hippocampal volume; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NC, normal cognition; NfL, neurofilament light; Ng, neurogranin; p-tau, phosphorylated tau; t-tau, total tau.

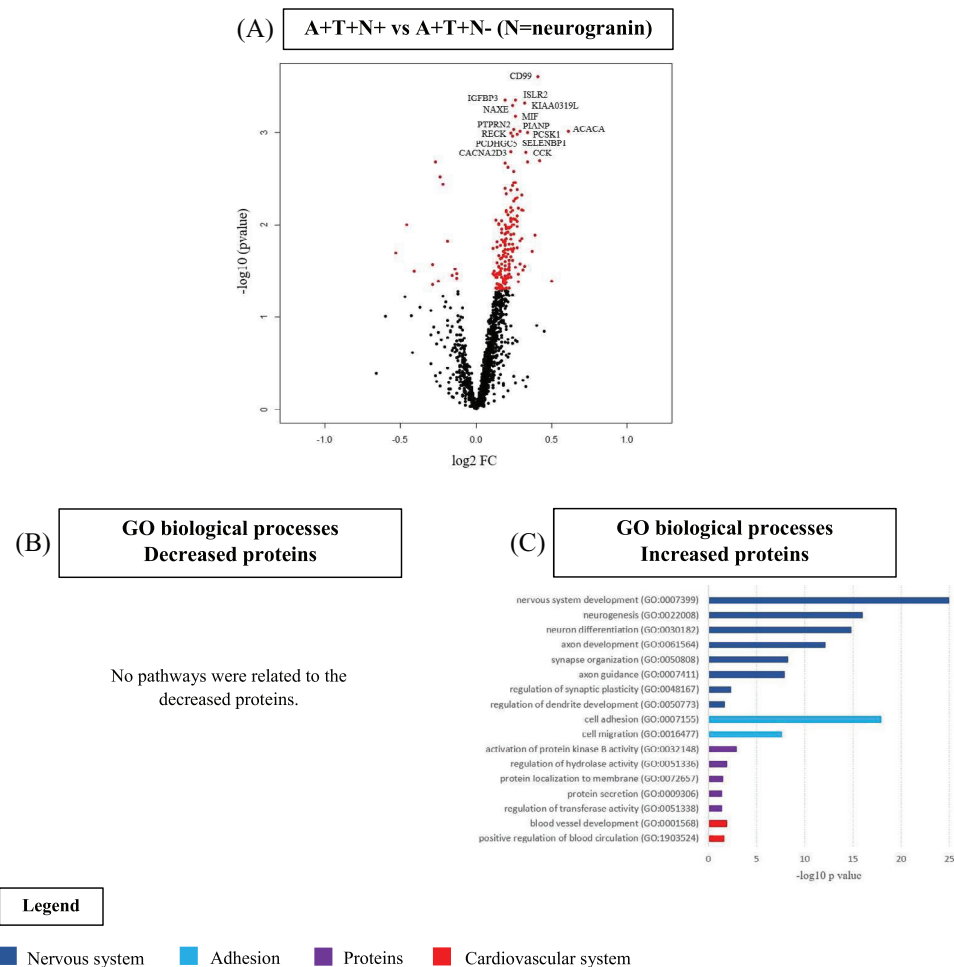


FIGURE 2 Cerebrospinal fluid (CSF) proteomics in A+T+ individuals without dementia by neurogranin status. A, Volcano plot displaying the log2 fold-change against the $-\log_{10}$ statistical *P* value for the comparison of A+T+N+ versus A+T+N– (N = neurogranin). Significantly different proteins are red. The top 15 proteins are named. B, C, Selected biological processes Gene Ontology (GO) terms for decreased (B) and increased (C) proteins in the comparison of A+T+N+ versus A+T+N–

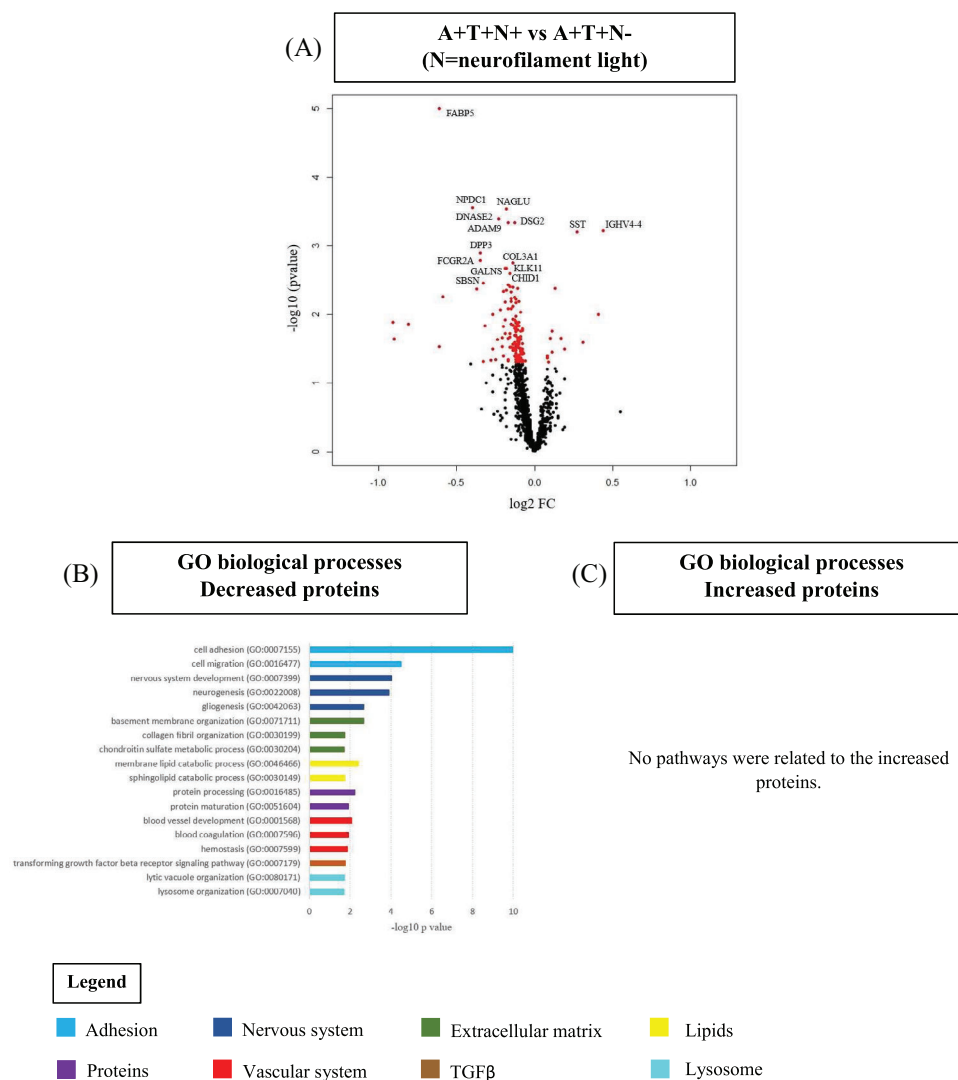


FIGURE 3 CSF proteomics in A+T+ individuals without dementia by neurofilament light status. A, Volcano plot displaying the log₂ fold-change against the $-\log_{10}$ statistical *P* value for the comparison of A+T+N+ versus A+T+N- (N = neurofilament light). Significantly different proteins are red. The top 15 proteins are named. B, C, Selected biological processes Gene Ontology (GO) terms for decreased (B) and increased (C) proteins in the comparison A+T-N+ versus A+T-N-. CSF, cerebrospinal fluid; TGFβ, transforming growth factor beta

3.1.3 | Hippocampal volume as a neurodegeneration marker

Using HCV as N marker, 55 participants were classified as A+T+N- and 83 as A+T+N+ (Figure 1A). A+T+N+ individuals were older and more often diagnosed with MCI, and had lower levels of Aβ₄₂, a lower Aβ₄₂/40 ratio, and higher p-tau and t-tau levels than A+T+N- (Table 1). No significant PGRS differences were found between A+T+N- and A+T+N+ (Figure 2A).

CSF proteomic profiling showed that 7 proteins were increased and 22 were decreased in A+T+N+ compared to A+T+N- (Figure 4A, Table S4A). No biological pathways were associated with the increased proteins. Decreased proteins were associated with oxidative stress (Figure 4B,C, Table S4B).

Similar proteomic results were found in A+T- (Figure 1B, Supplementary Results, Figure S5 in supporting information, Table S4C,D).

3.2 | Comparison of proteomic profiles of distinct N markers

Next, we compared the proteomic results of the N+ versus N- groups for the three N markers in A+T+ individuals. NfL+ was mainly associated with decreased proteins, while we found mainly increased proteins for Ng+. There was limited overlap in these proteins (4% to 5%; Figure 5A). Yet, top biological pathways were overlapping and associated with neuronal plasticity (Figure 5B), suggesting hyperplasticity in Ng+ and hypoplasticity in NfL+. HCV+ showed limited overlap with protein changes in Ng+ and NfL+ groups (between 2% and 35% of overlap; Figure 5A). Nonetheless, A+T+HCV+ showed overlap in the top 10 dysregulated GO biological pathways with A+T-Ng+, related to oxidative stress (Figure 5B). Comparison results for A+T- individuals can be found in the Supplementary Results.

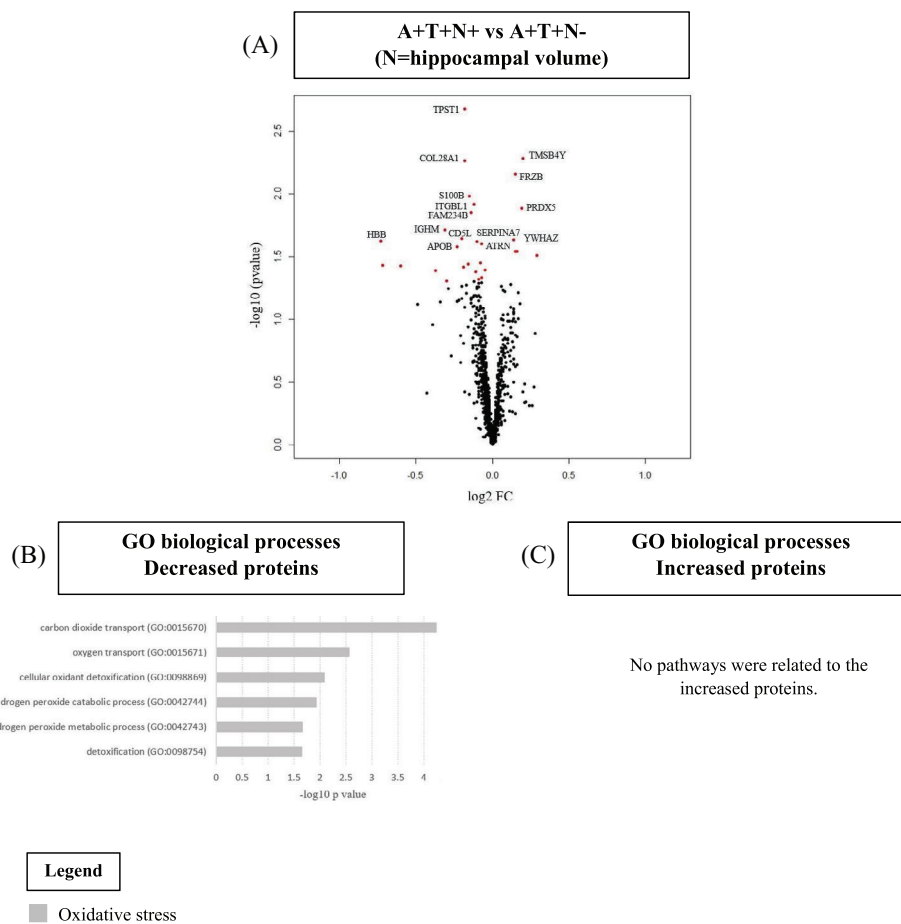


FIGURE 4 Cerebrospinal fluid (CSF) proteomics in A+T+ individuals without dementia by hippocampal volume status. A, Volcano plot displaying the log2 fold-change against the $-\log_{10}$ statistical P value for the comparison A+T+N+ versus A+T+N- (N = hippocampal volume). Significantly different proteins are red. The top 15 proteins are named. B, C, Selected biological processes Gene Ontology (GO) terms for decreased (B) and increased (C) proteins in the comparison of A+T-N+ versus A+T-N-

3.3 | Correlations between ATN markers

Figure 6A displays the correlation between ATN biomarkers. In a subset of 128 A+T+ individuals with availability of the three N markers, Ng was positively correlated with p-tau, t-tau, NfL, A β 40, and A β 42 and negatively correlated with the ratio A β 42/40. NfL was positively correlated with t-tau, p-tau, Ng, and A β 40, and negatively correlated to HCV. HCV was positively correlated with the ratio A β 42/40 and A β 42 and negatively with p-tau, t-tau, and NfL. In a subset of 58 A+T- individuals with availability of the three N markers, we found that Ng was positively correlated with p-tau and t-tau, and negatively correlated with the ratio A β 42/40, while NfL was positively correlated with t-tau.

Most of the A+T+ participants had more than one abnormal N marker, including most often Ng (Figure 6B). In A+T-, most individuals presented only one abnormal N marker (Figure 6C).

3.4 | Post hoc CSF proteomic analyses

In post hoc analyses, proteomic profiles were studied separately in individuals with NC and MCI (Table S6A-F in supporting information). In

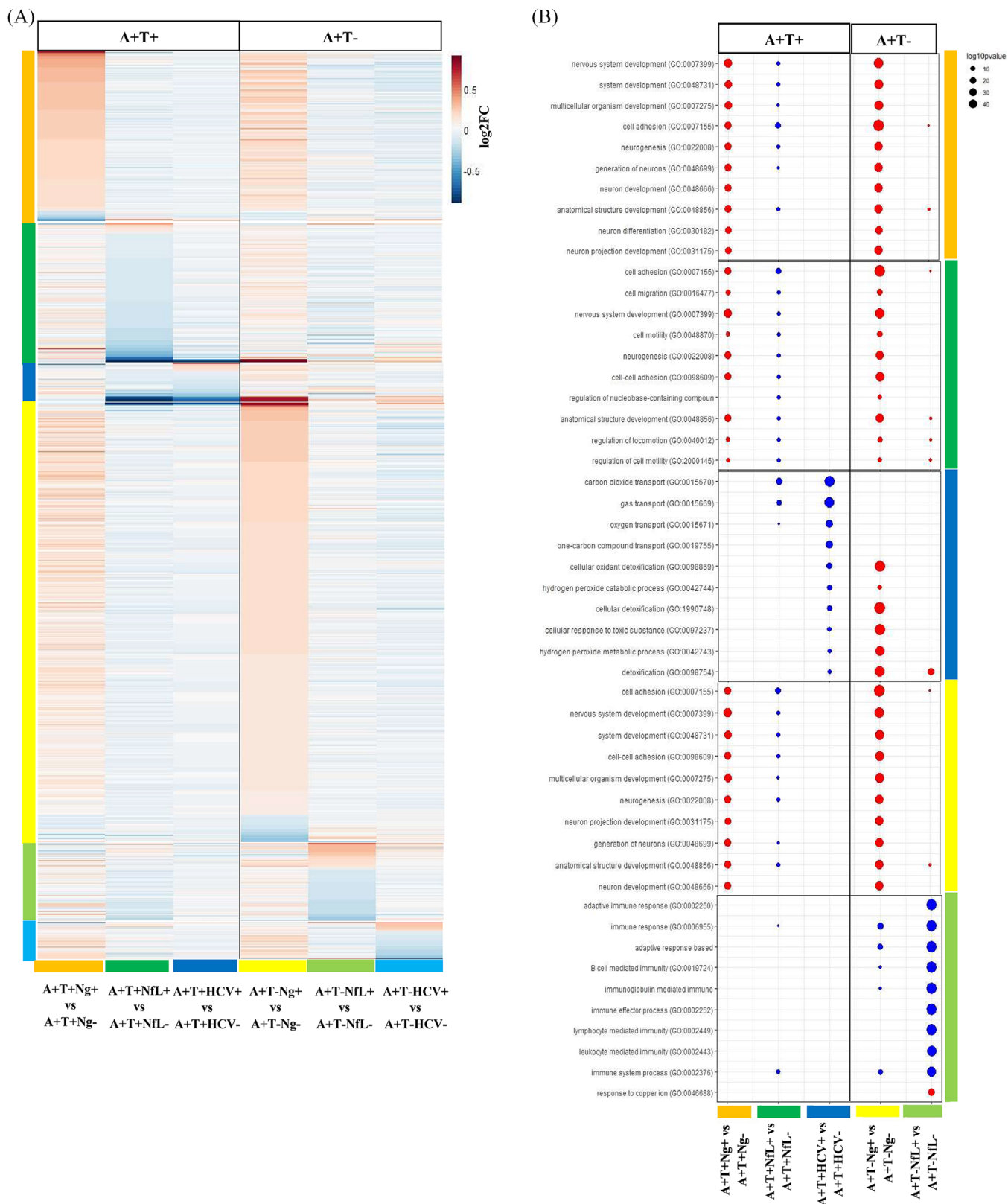
A+T+, results remained similar between individuals with NC and MCI for all N markers (Table S6A-C). In A+T-, results remained similar when Ng was used as the N marker, but were somewhat different in MCI A+T- when NfL or HCV were used as N markers (Supplementary Results and Table S6D-F).

As a sensitivity analysis, we corrected our main analysis for batch effects, which resulted in similar findings. We also compared the key demographic and clinical characteristics between participants with all three N markers to those with at least one N marker, and found no statistically significant differences, indicating similar sample characteristics between the groups.

Finally, as there is an age-dependent increase of CSF NfL, we determined post hoc an age-adjusted cut-off of ≥ 2 SD (see Materials and Methods). The use of this age-adjusted cut-off resulted in similar results.

4 | DISCUSSION

Comparing neurodegeneration markers in AD individuals without dementia, we found that CSF Ng, CSF NfL, and HCV were each asso-



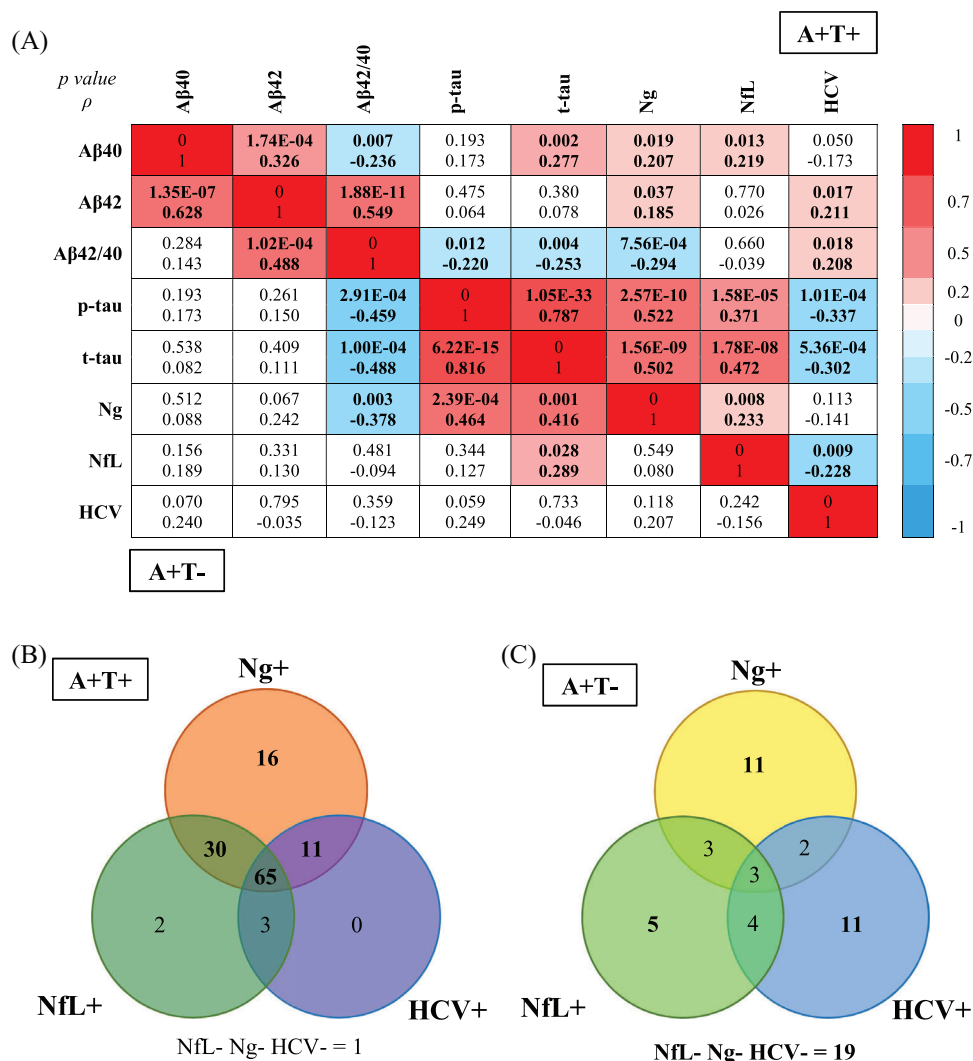


FIGURE 6 Association between the different neurodegeneration biomarkers. A, Correlation matrix for AD biomarkers using the Spearman rank correlation test in A+T+ individuals with no dementia (upper part of the graph) and in A+T- individuals with no dementia (lower part of the graph). The figure presents the *P* value and correlation coefficient (*ρ*). The color scale depicts the strength of the Spearman correlation coefficient in the significant correlations. B, Venn diagram depicting the distribution of the three neurodegeneration biomarkers in a subset of 128 A+T+ individuals and with availability of the three N markers. C, Venn diagram depicting the distribution of the three neurodegeneration biomarkers in a subset of 58 A+T- individuals and with availability of the three N markers. Aβ, amyloid beta; AD, Alzheimer's disease; HCV, hippocampal volume; NC, normal cognition; NfL, neurofilament light; Ng, neurogranin; p-tau, phosphorylated tau; t-tau, total tau

ciated with distinct CSF proteomic profiles. Yet, Ng+ and NfL+ protein changes showed overlap in top biological pathways, which were associated with neuroplasticity, though in opposite directions. HCV+ was associated with relatively few proteomic changes that were related to oxidative stress. Overall, our results show that different N markers represent distinct pathophysiological mechanisms in AD and cannot be used interchangeably.

A high number of increased proteins were associated with Ng+ compared to Ng-. Those increased proteins were related to aberrant neuroplasticity.³⁰ This is consistent with the global function of Ng in the central nervous system and with its role as a biomarker of synaptic dysregulation in AD. Findings could be also partially linked to tau, as CSF t-tau is linked in AD with changes in proteins associated with neuronal plasticity.³⁰ Other dysregulated pathways associated with

Ng were linked to angiogenesis. Cerebral hypoperfusion is a key hallmark of AD and a previous study reported a high positive correlation between CSF Ng levels and CSF levels of vascular endothelial growth factor, a protein involved in modulation of vascular remodeling, permeability, and angiogenesis. Angiogenesis could also be part of the neuronal plasticity response, as previously reported.^{30,53} A high percentage of the decreased proteins related to Ng+, in both A+T- and A+T+, showed an association with BBB functioning (39% to 41%). This is in line with a study on traumatic brain injury reporting increased CSF Ng levels as a result of damage to the BBB.⁵⁴ The proteomic profile of Ng+ was similar across AD pathology stages (A+T+ and A+T-), as well as across clinical stages (NC or MCI). This suggests that Ng reflects Aβ-dependent degeneration¹⁰ independently of the clinical stage and tau status. Still, it should be noted that the levels of Ng were higher

in A+T+N+ compared to A+T–N+. T-tau, p-tau, and Ng incongruency was uncommon, which is in line with the high correlation between Ng and tau (both p- and t-tau) in our study, as also previously reported.⁵⁵ Along with the shared associations of Ng and tau with aberrant neuroplasticity, this suggests that Ng may serve more as a tau-related marker, rather than a neurodegeneration marker.

A high number of decreased proteins were associated with NfL+ compared to NfL–. The proteomic profile of NfL+ was different across AD pathology stages (A+T– and A+T+), and for the A+T– also across clinical stages (NC or MCI). This could be explained by the fact that NfL reflects neurodegeneration independently of A β pathology.¹⁰ Hypoplasticity pathways in A+T+, including downregulated pathways related to neurogenesis and nervous system development, might be related to the degeneration of neurons associated with the increased levels of NfL in the CSF.^{2,6,7} The opposite profile of Ng and NfL, in which Ng is associated with hyperplasticity and NfL with hypoplasticity, may reflect different temporal dynamics in AD pathology. Changes in Ng levels may occur earlier in the disease progression, reflecting synaptic dysfunction^{2–5} and hyperplasticity, and potentially precede neurodegenerative processes. Conversely, alterations in NfL levels may occur later, reflecting axonal degeneration and hypoplasticity. A high percentage of the decreased proteins in NfL+ were highly expressed by the ChP (40% to 41%). The ChP is located inside the brain ventricles and is responsible for the production of CSF; transport of ions, proteins, nutrients, lipids, and metabolic precursors across the epithelium to the CSF; and clearance of proteins from the CSF.⁵⁶ Hence, pathways dysregulated in NfL+ could be linked with ChP dysfunction, that is, pathways associated with ion homeostasis, immune system, lipids, and ECM.^{57,58} The ChP seems to also play an important role in neuroplasticity and synaptic functions.⁵⁹ Yet, the implication of ChP functioning in AD in relation to CSF NfL changes warrants further investigation. Pathways associated with protein degradation and processing were also downregulated in NfL+, which could partially explain the downregulation of proteins.

Contrary to our expectations, HCV+ in AD was not associated with many protein changes in CSF. In A+T+, we found that decreased proteins were associated with oxidative stress, while in A+T– we found no pathways associated with the significant proteins. Oxidative stress is one of the earliest events occurring in AD and the hippocampus is a brain structure highly sensitive to oxidative stress.^{60,61} Oxidative stress in the hippocampus alters neurogenesis, dendritic complexity, and learning and could lead to atrophy.⁶² As we only found few proteomic changes in HCV, it might be that the pathophysiological mechanisms associated with hippocampal atrophy are not directly reflected in the CSF, but maybe more in the tissue itself.⁶³ Alternatively, as hippocampal atrophy is a relatively late marker in AD, it might be difficult to identify HCV changes using dichotomization in early AD stages, especially in preclinical AD. Yet, several previous studies have successfully used dichotomized HCV measures in early AD.^{64,65} Another hypothesis could propose that, because lower HCV are not exclusive to AD but also occur in other neurodegenerative diseases,^{66,67} each of these conditions might exhibit its own distinct proteomic profile. In cases in which multiple pathologies coexist, the

proteomic signatures associated with each pathology could potentially be diluted or obscured by the presence of others.

The non-interchangeability between N markers is in line with conclusions from previous studies.^{12,68} Nonetheless, it should be noted that most of the A+T+ participants showed abnormalities in more than one N marker, and almost all had abnormal levels of Ng. This is in line with the strong correlation that we observed between tau and Ng levels, consistent with previous findings.¹⁰ Moreover, NfL showed a positive correlation with Ng and negative correlation with HCV. In A+T–, the levels of the three N markers were not significantly correlated and most of the A+T– individuals had only one abnormal N marker. This indicates that, whenever only abnormal levels of A β 42 are found, distinct neurodegeneration mechanisms can occur in different persons with AD. The N markers can represent AD but also non-AD pathologies, as N markers are not AD specific.¹

Our study has several strengths and limitations. To the best of our knowledge, this is the first study reporting CSF proteomic profiling of distinct neurodegeneration biomarkers in AD. Our results are a first step toward a better pathophysiological characterization of the distinct neurodegeneration processes happening in AD. Another main strength of this study is that our overall sample size for proteomic analyses was large, encompassing 407 individuals. Yet, for some subgroups, the sample size was rather small. While this reflects lower frequency of subgroups, this could have reduced our statistical power. Furthermore, to define biomarker abnormality, we had to calculate cut-offs for the neurodegeneration markers, as no reference values were available. Future studies should validate our cut-points in independent or larger datasets. Furthermore, the methodology to quantify ATN biomarkers varied between centers, potentially introducing variability into the results. However, we defined center- or study-specific cutoffs to account for this. Further research is also needed to unravel the causes and consequences of the dysregulated processes associated with the distinct N markers. Longitudinal studies are needed to investigate the cognitive outcomes of each neurodegeneration marker in relation to proteomic profiles.

Together, our findings suggest that N markers cannot be used interchangeably, as Ng, NfL, and HCV markers measure largely different neurodegenerative processes in AD. Yet, Ng+ and NfL+ showed some overlap in biological pathways related to neuroplasticity. Ng might not be a good N marker given its close associations with tau pathophysiology. HCV+ was associated with fewer proteomic differences. The use of different N markers may refine disease staging beyond amyloid and tau by providing complementary information, which has implications for clinical trial design.

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CONFLICT OF INTEREST STATEMENT

A.D. received funding from Alzheimer Nederland (grant No. WE.15-2022-01). J.G. has nothing to disclose. S.E.S. has analyzed data provided by C2N Diagnostics to Washington University. She has served on scientific advisory boards for Eisai. M.K. has nothing to disclose. L.M.R. has nothing to disclose. V.D. has nothing to disclose. B.M.T. has nothing to

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CONSENT STATEMENT

All human subjects provided informed consent for research.

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SUPPORTING INFORMATION

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

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