

FEATURED ARTICLE

Cerebrospinal fluid proteomic profiling of individuals with mild cognitive impairment and suspected non-Alzheimer's disease pathophysiology

Aurore Delvenne¹  | Johan Gobom^{2,3} | Betty Tijms⁴ | Isabelle Bos^{1,4} | Lianne M. Reus⁴ | Valerija Dobricic⁵ | Mara ten Kate^{4,6} | Frans Verhey¹ | Inez Ramakers¹ | Philip Scheltens⁴ | Charlotte E. Teunissen⁷ | Rik Vandenberghe^{8,9} | Jolien Schaevebeke^{8,9} | Silvy Gabel^{8,9} | Julius Popp^{10,11} | Gwendoline Peyratout¹⁰ | Pablo Martinez-Lage¹² | Mikel Tainta¹² | Magda Tsolaki¹³ | Yvonne Freund-Levi^{14,15,16} | Simon Lovestone¹⁷ | Johannes Streffer^{18,19} | Frederik Barkhof^{6,20} | Lars Bertram^{5,21} | Kaj Blennow^{2,3} | Henrik Zetterberg^{2,3,22,23} | Pieter Jelle Visser^{1,4,24} | Stephanie J. B. Vos¹

¹Department of Psychiatry and Neuropsychology, Alzheimer Centrum Limburg, School for Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands

²Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

³Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

⁴Alzheimer Center Amsterdam, Department of Neurology, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam UMC, the Netherlands

⁵Lübeck Interdisciplinary Platform for Genome Analytics, University of Lübeck, Lübeck, Germany

⁶Department of Radiology and Nuclear Medicine, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam, the Netherlands

⁷Neurochemistry Laboratory, Department of Clinical Chemistry, Amsterdam University Medical Centers (AUMC), Amsterdam Neuroscience, the Netherlands

⁸Neurology Service, University Hospitals Leuven, Leuven, Belgium

⁹Laboratory for Cognitive Neurology, Department of Neurosciences, KU Leuven, Leuven, Belgium

¹⁰Old Age Psychiatry, University Hospital Lausanne, Lausanne, Switzerland

¹¹Department of Geriatric Psychiatry, Psychiatry University Hospital Zürich, Zürich, Switzerland

¹²Fundación CITA-Alzhéimer Fundazioa, San Sebastian, Spain

¹³1st Department of Neurology, AHEPA University Hospital, Medical School, Faculty of Health Sciences, Aristotle University of Thessaloniki, Makedonia, Thessaloniki, Greece

¹⁴Department of Neurobiology, Caring Sciences and Society (NVS), Division of Clinical Geriatrics, Karolinska Institutet, Stockholm, Sweden

¹⁵Department of Psychiatry in Region Örebro County and School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

¹⁶Department of Old Age Psychiatry, Psychology & Neuroscience, King's College, London, UK

¹⁷University of Oxford, Oxford, United Kingdom (currently at Johnson and Johnson Medical Ltd.), London, UK

¹⁸Institute Born-Bunge, Reference Center for Biological Markers of Dementia (BIODEM), Institute Born-Bunge, University of Antwerp, Belgium

¹⁹UCB Biopharma SPRL, Brain-l'Alleud, Belgium

²⁰Institutes of Neurology & Healthcare Engineering, UCL London, London, UK

²¹Center for Lifespan Changes in Brain and Cognition, Department of Psychology, University of Oslo, Oslo, Norway

²²Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK

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²³UK Dementia Research Institute at UCL, London, UK

²⁴Department of Neurobiology, Care Sciences and Society, Division of Neurogeriatrics, Karolinska Institutet, Stockholm, Sweden

Correspondence

Aurore Delvenne, Department of Psychiatry and Neuropsychology, Alzheimer Centrum Limburg, School for Mental Health and Neuroscience, Maastricht University, P.O. Box 616, 6200 MD Maastricht, the Netherlands. E-mail: a.delvenne@maastrichtuniversity.nl

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Abstract

Background: Suspected non-Alzheimer's disease pathophysiology (SNAP) is a biomarker concept that encompasses individuals with neuronal injury but without amyloidosis. We aim to investigate the pathophysiology of SNAP, defined as abnormal tau without amyloidosis, in individuals with mild cognitive impairment (MCI) by cerebrospinal fluid (CSF) proteomics.

Methods: Individuals were classified based on CSF amyloid beta (A β)1-42 (A) and phosphorylated tau (T), as cognitively normal A-T- (CN), MCI A-T+ (MCI-SNAP), and MCI A+T+ (MCI-AD). Proteomics analyses, Gene Ontology (GO), brain cell expression, and gene expression analyses in brain regions of interest were performed.

Results: A total of 96 proteins were decreased in MCI-SNAP compared to CN and MCI-AD. These proteins were enriched for extracellular matrix (ECM), hemostasis, immune system, protein processing/degradation, lipids, and synapse. Fifty-one percent were enriched for expression in the choroid plexus.

Conclusion: The pathophysiology of MCI-SNAP (A-T+) is distinct from that of MCI-AD. Our findings highlight the need for a different treatment in MCI-SNAP compared to MCI-AD.

KEYWORDS

Alzheimer's disease, biomarkers, cerebrospinal fluid, mild cognitive impairment, pathophysiology, proteomics, suspected non-Alzheimer's disease pathophysiology, tau

1 | BACKGROUND

Suspected non-Alzheimer's disease pathophysiology (SNAP) is a biomarker concept describing individuals with normal biomarkers of amyloid beta (A β) accumulation, but abnormal neuronal injury biomarkers. SNAP can be defined based on abnormal tau (A-T+) or other Alzheimer's disease (AD)-associated neuronal injury, such as hypometabolism or atrophy in AD-specific regions (A-N+).¹⁻⁴ In the present paper, SNAP is defined as normal A β but abnormal tau (A-T+). Overall, SNAP (A-T+/N+) is common in individuals with mild cognitive impairment (MCI; 20%–40%)^{3,5} and associated with cognitive decline.⁶ It is unclear whether SNAP (A-T+) is a disease entity in itself or reflects an atypical presentation of AD. SNAP could be atypical AD characterized by a different temporal evolution of AD biomarkers, in which A β only becomes abnormal at a later stage. Alternatively, SNAP could be atypical AD with a higher production or lower clearance of A β 42, leading to A β aggregation without falling below the cut-off threshold.

MCI-SNAP (A-T+/N+) has been associated with a lower apolipoprotein E (APOE) ϵ 4 frequency compared to MCI individuals with abnormal A β and neuronal injury markers (MCI-AD).^{3,7} Previous research has shown that MCI-SNAP (A-T+) has a higher

risk of progression to AD dementia compared to MCI biomarker-negative and cognitively normal (CN) groups, but a lower risk compared to MCI-AD.⁶ Moreover, MCI-SNAP has a higher likelihood to progress to non-AD dementia compared to MCI-AD.⁶ Several non-AD pathologies may be associated with SNAP (A-T+), including primary age-related tauopathy,⁷⁻⁹ argyrophilic grain disease,⁸⁻¹⁰ and Lewy body disease.⁸⁻¹¹ Nonetheless, the pathophysiology of SNAP is still unclear and could be studied using cerebrospinal fluid (CSF) proteomics. Proteomic studies allow the identification and quantification of proteins in tissues or biological fluids. Because of its direct contact with the brain, CSF uniquely reflects ongoing biochemical and metabolic changes in the brain.¹²

In the present study, we aimed to investigate the pathophysiology of SNAP using large-scale CSF proteomics. We compared the proteomic profile of MCI-SNAP (normal CSF A β 42 and abnormal phosphorylated tau [p-tau]) to those of CN individuals (normal A β 42 and p-tau; controls) and MCI individuals with typical AD (abnormal A β 42 and p-tau; MCI-AD). We characterized dysregulated proteins by using Gene Ontology (GO) analysis as well as cell type- and brain region-specific gene expression. In addition, we investigated in MCI-SNAP the vascular and atrophy patterns on imaging as well as the genetic risk for AD using AD polygenic risk scores (PGRS).

2 | METHODS

2.1 | Participants

We included 232 participants from the Maastricht BioBank Alzheimer Center Limburg cohort (BB-ACL, $n = 23$) memory clinic study¹³ and the European Medical Information Framework for Alzheimer's Disease Multimodal Biomarker Discovery study (EMIF-AD MBD, $n = 209$).¹⁴ The EMIF-AD MBD study gathered data and samples collated from pre-existing studies, as described in detail elsewhere.¹⁴ In short, the study included individuals aged ≥ 50 years with normal cognition, MCI, or dementia with available data on $A\beta$ status as well as magnetic resonance imaging (MRI) scans, plasma, CSF, or DNA samples (at least two of the modalities).

For the present study, we included individuals without dementia (CN/subjective cognitive decline and MCI) with availability of CSF $A\beta_{42}$ and p-tau data, APOE genotype, and CSF samples at baseline. Informed consent for research was provided by all the participants. All centers approved participation in this study after local medical ethics committee approval.

2.2 | Clinical and cognitive assessment

Clinical and cognitive data were collected, including the Mini-Mental State Examination (MMSE) and neuropsychological tests assessing several cognitive domains. Neuropsychological tests differed between centers but most common tests were the Consortium to Establish a Registry for Alzheimer's Disease wordlist for delayed memory, the Trail Making Test A for attention, the Trail Making Test B for executive functioning, and Animal Fluency for language. Information about the neuropsychological tests and calculation of Z-scores is described elsewhere.¹⁴ Cognition was defined as normal if neuropsychological test performance ranged within 1.5 standard deviations (SDs) of the average for age, sex, and education. Diagnosis of MCI was according to the criteria of Petersen.^{14,15}

2.3 | CSF protein analysis

CSF was collected using lumbar puncture, centrifuged, and stored at -80°C in polypropylene tubes. Targeted analyses were centrally performed for well-established CSF markers, that is, $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, p-tau, total-tau (t-tau), neurofilament light (NfL), neurogranin (Ng), and YKL-40. $A\beta_{42}$, t-tau, and p-tau levels were measured locally with INNOTEST enzyme-linked immunosorbent assays (ELISAs; Fujirebio) and for a subset with AlzBio3 xMAP Luminex ($n = 26$) to classify individuals in groups. CSF samples were shipped on dry ice to the Neurochemistry Lab of University of Gothenburg in Mölndal, Sweden, where central analyses of $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$ (using V-PLEX Plus $A\beta$ Peptide Panel 1 [6E10] Kit from Meso Scale Discovery [MSD]), NfL (using NF-light® ELISA, UmanDiagnostics), Ng (using an in-house

RESEARCH IN CONTEXT

- 1. Systematic review:** Suspected non-Alzheimer's disease pathophysiology (SNAP) is a biomarker concept that encompasses individuals with neurodegeneration but without amyloidosis. SNAP is common in individuals with mild cognitive impairment (MCI). Little is known about the pathophysiology of A-T+ SNAP and it is not yet clear whether SNAP is a disease entity in itself or reflects an atypical presentation of Alzheimer's disease (AD).
- 2. Interpretation:** Compared to cognitively normal (CN) and MCI-AD, MCI-SNAP showed decreased cerebrospinal fluid (CSF) levels of proteins associated with extracellular matrix, hemostasis, immune system, lipids, protein processing/degradation, and synapses, and with a predominant expression in the choroid plexus. This study supports that A-T+ MCI-SNAP is a distinct disease entity that differs from MCI-AD.
- 3. Future directions:** Our findings highlight the need for a different treatment in MCI-SNAP compared to MCI-AD. Further research is needed to unravel the causes and consequences of the dysregulated processes as well as the longitudinal cognitive outcomes of MCI-SNAP.

HIGHLIGHTS

- Individuals with suspected non-Alzheimer's disease pathophysiology (SNAP) and mild cognitive impairment (MCI), A-T+, showed decreased cerebrospinal fluid levels of proteins compared to cognitively normal individuals and individuals with MCI and Alzheimer's disease.
- The decreased proteins in MCI-SNAP were related to pathways linked with extracellular matrix, hemostasis, immune system, lipids, protein processing/degradation, and synapses.
- The decreased proteins in MCI-SNAP showed a predominant expression in the choroid plexus.

immunoassay), and YKL-40 were performed (using human chitinase-3 quantikine ELISA kit [R&D Systems, Inc.]).

Untargeted central proteomic and peptidomic analyses were performed using the tandem mass tag (TMT) technique with 10+1 plexing as described elsewhere, using high-pH reverse phase high-performance liquid chromatography for peptide prefractionation¹⁶ to quantify ≈ 500 proteins as well as endogenous peptides in the same CSF sample aliquot. We used spectral clustering for matching liquid chromatography-tandem mass spectrometry (LC-MS) data sets of

TMT-labeled peptides, as this makes it possible to detect biomarker candidates that cannot be identified by conventional database searching in the first step. In a second step, retrieved candidates of interest have been identified by manual spectra interpretation or by further targeted experiments. A total of 2535 proteins were quantified centrally using tandem tag mass 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. Out of the 2535 proteins quantified, 1440 proteins fit these criteria and were included in the study.

All analyses were performed according to the manufacturer's instructions in one occasion and using one batch of reagents by board-certified laboratory technicians who were blinded to clinical information.

2.4 | Genetic analysis

Protocols for *APOE* genotyping are described elsewhere.^{14,17} Participants were classified as *APOE* $\epsilon 4$ carrier or non-carrier.

Polygenic risk score analyses were available for a subset of patients of the EMIF-AD MBD study (CN = 63, MCI-SNAP = 17, MCI-AD = 61) on genome-wide single nucleotide polymorphism (SNP) genotyping data generated with the Global Screening Array (Illumina, Inc.) using PRSice (v2.3).¹⁶ PGRS were calculated by adding the sum of each allele weighted by the strength of its association with AD risk as calculated previously by the largest genome-wide association study (GWAS) on AD.¹⁸ Clumping was performed prior to calculating PGRS, to remove SNPs that are in linkage disequilibrium ($r^2 > 0.1$) within a sliding 1M bp window. After clumping, PGRS were computed using various SNP inclusion thresholds (i.e., $P \leq 1e-30$, $P \leq 1e-8$, $P \leq 1e-5$, $P \leq .01$, $P \leq .02$, $P \leq .03$, $P \leq .04$, $P \leq .1$, $P \leq .2$, $P \leq .3$, $P \leq .4$, $P \leq .5$).¹⁶ Analyses without *APOE* region were also performed.

2.5 | Image analysis

Imaging data were used to describe our study population. At each site, imaging data were acquired using local protocols. MRI scans were assembled centrally, visual quality check was performed and visually rated by a single rater for EMIF-AD MBS and for Maastricht. T1-weighted and, when available, fluid-attenuated inversion recovery (FLAIR) and/or T2*/SWI images were used for qualitative visual rating.¹⁹ Medial temporal lobe atrophy (MTA), global cortical atrophy (GCA), and parietal atrophy were assessed using scales ranging from 0 (no atrophy) to 4 (end-stage atrophy). White matter hyperintensities were evaluated using the 4-point Fazekas scale (none, punctuate, early confluent, and confluent). Microbleeds were defined as small (<10 mm) rounded hypointense homogeneous foci in brain parenchyma. Lacunes were defined as deep lesions (3–15 mm) with CSF-like signal. Cerebrovascular disease was defined as Fazekas ≥ 2 and/or microbleeds ≥ 1 and/or lacunes ≥ 1 .

2.6 | Participant classification

We used CSF A β 42 as a measure for A β (A) and p-tau as a measure of tau (T). Cohort-specific cut-offs were used to define abnormal biomarker levels (Table S1 in supporting information). Because centers used different methodologies to determine A β 42 cut-offs, these cut-offs were redefined for each cohort using unbiased Gaussian mixture modeling.²⁰ Individuals were classified as CN with normal A and T (CN, $n = 81$), MCI with normal A and abnormal T (MCI-SNAP, $n = 22$), and MCI with abnormal A and T (MCI-AD, $n = 77$). For post hoc analysis, we included individuals with MCI and with normal A and T as additional control group (MCI A-T-, $n = 52$).

2.7 | Pathway enrichment analysis

GO enrichment analysis was performed using PANTHER (Protein Analysis Through Evolutionary Relationships, v15.0)²¹ to identify the biological processes, cellular components, and molecular functions related to the increased or decreased proteins of each group comparison. This tool used Fisher's exact test with false discovery rate (FDR) and only reports pathways with a FDR-corrected P -value $< .05$. The FDR is calculated by the Benjamini-Hochberg procedure²² and is generally considered a great choice in enrichment analysis to reduce the proportion of false positive results.^{23,24} To reduce redundancy and facilitate interpretation, we clustered related GO terms in broader categories. We validated these pathways and categories using the online database STRING version 11.0²⁵ and ClueGO, a Cytoscape plug-in.²⁶ We selected a subset of pathways for each category for visualization while all the enriched GO terms for each comparison are reported in the supporting information (Tables S2 and S4).

2.8 | Brain cell expression analysis

We used the online database Brain RNA-Seq (<http://www.brainrnaseq.org/>) to investigate whether proteins that differed between groups were associated with specific brain cell types. This database includes human transcript expression levels in fragments per kilobase of transcript per million mapped fragments (FPKM) for central nervous system cell types, including fetal and mature astrocytes, neurons, oligodendrocytes, microglia/macrophages, and endothelial cells.²⁷ Proteins were considered expressed by a cell type if the FPKM value was higher than 1.²⁸ For proteins that differed between groups, we calculated the percentage of proteins associated with each cell type as well as the median FPKM and the interquartile range (IQR) taking into account only the proteins expressed by a cell type (FPKM < 1).

2.9 | Gene set expression enrichment in adult human brain regions

We explored whether proteins that differed between groups were enriched for specific brain regions. We primarily focused on tau-related

brain regions, selected through previous research papers.^{29–34} The regions included were the anterior group of nuclei of the thalamus, hippocampal areas CA1 and CA3, the central nucleus of amygdala, the basal nucleus of Meynert, the inferior colliculus, the pars compacta of substantia nigra, the locus coeruleus, the fusiform gyrus, the inferior temporal, the temporal pole, the occipitotemporal region, the parahippocampal gyrus, the tuberomammillary nucleus, the cingulate gyrus, the pars triangularis, and the frontal pole. The choroid plexus was another region of interest, as it produces most of the CSF in the central nervous system.³⁵ High expression of proteins in these brain regions was identified based on the online database Allen Brain Atlas³⁶ through Harmonizome.³⁷ Additionally, we investigated the whole adult brain regions by performing expression enrichment analysis using the R package ABAEnrichment. This tool uses two human brain expression datasets (adult and developing human brain) provided by the Allen Brain Atlas.³⁸

2.10 | Statistical analysis

Clinical and imaging measures were compared between groups using analyses of covariance (ANCOVA) corrected for age, sex, and APOE $\epsilon 4$ carriership for continuous variable and Chi-square for categorical variables. PGRS were compared between groups using linear models. For the targeted CSF and imaging markers, the normality of distributions was tested using the Shapiro–Wilk test and visual inspection of data. For the markers with a non-normal distribution, log transformation (for targeted CSF markers) or square root transformation (for imaging scores) was performed. CSF protein levels were normalized according to the mean and standard deviation of the control group and compared between groups using ANCOVA corrected for age, sex, and APOE $\epsilon 4$ carriership. Post hoc, these ANCOVA analyses were also performed without APOE $\epsilon 4$ correction. Statistical analyses were performed using R 3.6.2 and IBM SPSS Statistics version 26. Two-sided statistical significance was used and set at $P < .05$.

3 | RESULTS

3.1 | Sample characteristics

Sample characteristics are presented in Table 1. MCI-SNAP and MCI-AD were older and had lower education than CN. Sex distribution was similar between groups. MCI-SNAP and CN were less often APOE $\epsilon 4$ carriers and more often APOE $\epsilon 2$ carriers compared to MCI-AD. MCI-SNAP and MCI-AD had lower scores for MMSE, attention, executive functioning, and memory compared to CN. MCI-SNAP performed better on executive functioning than MCI-AD.

CSF A β 38 and A β 40 levels were higher in MCI-SNAP compared to CN and MCI-AD. CSF A β 42 levels were higher in MCI-SNAP compared to MCI-AD and tended to be higher compared to CN ($P = .069$). The A β 42/40 ratio did not differ between MCI-SNAP and CN, and was lowest in MCI-AD. NfL, Ng, and YKL-40 levels were higher in MCI-SNAP and MCI-AD groups compared to CN. P-tau and t-tau levels were, by

definition, higher in MCI-SNAP and MCI-AD compared to CN, and p-tau levels were lower in MCI-SNAP compared to MCI-AD.

MCI-SNAP and MCI-AD both showed higher MTA scores compared to CN. MCI-AD showed higher GCA scores and more parietal atrophy and cerebrovascular pathology compared to CN.

3.2 | CSF proteomic profiles in CN, MCI-SNAP, and MCI-AD

The number and patterns of decreased and increased CSF proteins in CN, MCI-SNAP, and MCI-AD groups are presented in Figure S1 in supporting information and described below.

3.2.1 | MCI-SNAP versus CN

Thirteen proteins were increased and 136 decreased in MCI-SNAP compared to CN (Figure 1A, Table S2A). The decreased proteins were slightly more expressed by microglia (40%, median 8.7 FPKM; Figure 1B). Sixty-five decreased proteins showed a trend for enriched expression in the choroid plexus (48%, ABAenrichment $P = .061$). The decreased proteins in MCI-SNAP were enriched for biological processes related to extracellular matrix (ECM), immune system, protein processing and degradation, hemostasis, lipids, and synapse pruning. The biological, molecular, and cellular processes are described in Figure 1C–E and Table S2B. Increased proteins were not associated with specific cells, brain regions, or pathways.

3.2.2 | MCI-AD versus CN

In MCI-AD compared to CN, 185 proteins were increased and 10 decreased (Figure 2A, Table S2A). Increased proteins were most expressed by neurons (69%, median 20.0 FPKM; Figure 2B) and were not enriched for a specific brain region (data not shown). These increased proteins were enriched with biological GO terms linking to energy metabolism, immune response, oxidative stress, neurons, axons, synaptic transmission, and plasticity and cytoskeleton (Figure 2C–E, Table S2C). Decreased proteins were not associated with specific cells, brain regions, or pathways.

3.2.3 | MCI-SNAP versus MCI-AD

Twenty-three proteins were increased and 243 decreased in MCI-SNAP compared to MCI-AD (Figure 3A–C, Table S2A). Eighty-six decreased proteins were enriched for expression in the choroid plexus (36%, ABAenrichment $P = .002$). Several other brain regions also showed enrichment, with the most significant regions being the arcuate nucleus of medulla, medial habenular nucleus, and the central gray of the pons (data not shown). The decreased proteins in MCI-SNAP were enriched for biological GO terms related to ECM, immune system, nervous system, energy metabolism, hemostasis, protein processing and degradation, oxidative stress, and lipids. The

TABLE 1 Sample characteristics

	CN n = 81	MCI-SNAP n = 22	MCI-AD n = 77	P MCI-SNAP vs. CN	P MCI-AD vs. CN	P MCI-SNAP vs. MCI-AD
Age, years	65.3 (8.0)	69.9 (6.5)	70.8 (6.1)	.016	<.001	.546
Female (%)	40 (49)	14 (64)	43 (56)	.235	.416	.514
Education, years	12.8 (3.5)	10.0 (3.4)	11.2 (3.5)	.001	.004	.171
APOE ε4 carriers (%)	22 (27)	3 (14)	56 (73)	<.001	.189	<.001
APOE genotype (%)	ε2/ε3 = 9 (11) ε2/ε4 = 2 (2) ε3/ε3 = 49 (60) ε3/ε4 = 20 (25) ε4/ε4 = 1 (1)	ε2/ε3 = 5 (23) ε2/ε4 = 0 (0) ε3/ε3 = 14 (64) ε3/ε4 = 1 (4) ε4/ε4 = 2 (9)	ε2/ε3 = 1 (1) ε2/ε4 = 1 (1) ε3/ε3 = 20 (26) ε3/ε4 = 39 (51) ε4/ε4 = 16 (21)	.052*	<.001 †	<.001 ‡
MMSE	28.9 (1.2)	26.6 (2.2)	26.3 (2.7)	<.001	<.001	.465
Memory, Z score	0.4 (0.9)	−1.3 (1.0)	−1.4 (1.2)	<.001	<.001	.227
Attention, Z score	0.3 (1.1)	−0.6 (2.0)	−0.6 (1.7)	.046	<.001	.281
Executive functioning, Z score	0.4 (1.1)	−0.4 (1.8)	−0.5 (1.6)	.024	<.001	.009
Language, Z score	0.0 (1.1)	−1.0 (0.7)	−0.9 (0.8)	.060	<.001	.705
CSF Aβ38, pg/ml	2416 (741)	3324 (929)	2815 (921)	.002	.246	.031
CSF Aβ40, pg/ml	5560 (1572)	7400 (1786)	6442 (1797)	.001	.099	.046
CSF Aβ42, pg/ml	517 (173)	670 (272)	294 (146)	.069	<.001	<.001
CSF Aβ42/40 ratio	0.09 (0.01)	0.09 (0.20)	0.04 (0.01)	.164	<.001	<.001
CSF NfL, pg/ml	667 (291)	1575 (1106)	1278 (1042)	<.001	<.001	.338
CSF Ng, pg/ml	82 (83)	171 (119)	195 (138)	.011	<.001	.371
CSF YKL40, ng/ml	130 (40)	213 (72)	194 (63)	<.001	.001	.183
CSF p-tau, Z-score	0.3 (0.5)	−1.1 (1.3)	−1.7 (1.1)	<.001	<.001	.035
CSF t-tau, Z-score	0.2 (0.5)	−1.1 (1.7)	−1.6 (1.0)	<.001	<.001	.285
MTA (score 0–4)	0.2 (0.5)	1.0 (0.8)	0.8 (0.8)	.003	.036	.465
GCA (score 0–3)	0.3 (0.4)	0.5 (0.5)	0.8 (0.6)	.692	.015	.181
Parietal atrophy (score 0–3)	0.3 (0.5)	0.6 (0.7)	0.9 (0.8)	.730	.011	.267
WMH (score 0–3)	0.9 (0.8)	1.1 (0.6)	1.2 (0.9)	.910	.516	.455
Microbleeds >1 (%)	3 (16)	4 (27)	16 (26)	.436	.350	.973
Lacunes >1 (%)	4 (13)	3 (25)	11 (18)	.335	.508	.425
CeVD (%)	7 (19)	4 (25)	26 (39)	.616	.037	.302

Note: Values represent mean (standard deviation) or number (percentages). Significant *P*-values (<.05) are bold. The sample size was smaller for some variables: A total of 25 values are missing for attention, 31 for executive functioning, 21 for language, 4 for memory. Twenty-three values are missing for Aβ38, Aβ40, Aβ42, Aβ42/40 ratio, and YKL40. Twenty-four values are missing for NfL and 22 values for Ng. Fifty-eight values are missing for MTA, GCA, and parietal atrophy; 70 values for WMH; 85 values for microbleeds; 77 values for lacunes; and 60 for CeVD. T-tau and p-tau values were measured locally and so are presented as Z-scores with controls within each data set as a reference. Abnormal number of microbleeds and lacunes are defined as ≥1. CeVD is defined by Fazekas-score ≥2 and/or lacunes ≥1 and/or cerebral microbleeds ≥1. Results for comparison of APOE genotypes were: * = The percentage of ε3/ε4 carrier is lower in MCI-SNAP compared to CN (*P* = .038). † = The percentage of ε2/ε3 carriers is lower in MCI-AD compared to CN (*P* = .011); The percentage of ε3/ε3 carriers is lower in MCI-AD compared to CN (*P* < .001); The percentage of ε3/ε4 carriers is higher in MCI-AD compared to CN (*P* = .001); The percentage of ε4/ε4 carriers is higher in MCI-AD compared to CN (*P* < .001). ‡ = The percentage of ε2/ε3 carriers is higher in MCI-SNAP compared to MCI-AD (*P* < .001); the percentage of ε3/ε3 carriers is higher in MCI-SNAP compared to MCI-AD (*P* = .001); The percentage of ε3/ε4 is lower in MCI-SNAP compared to MCI-AD (*P* < .001).

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; APOE, apolipoprotein E; CeVD, cerebrovascular disease; CN, cognitively normal; CSF, cerebrospinal fluid; GCA, global cortical atrophy; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MTA, medial temporal lobe atrophy; NfL, neurofilament light; Ng, neurogranin; *P*, *P*-value; p-tau, phosphorylated tau; SNAP, suspected non-Alzheimer's pathophysiology; t-tau, total tau; WMH, white matter hyperintensities.

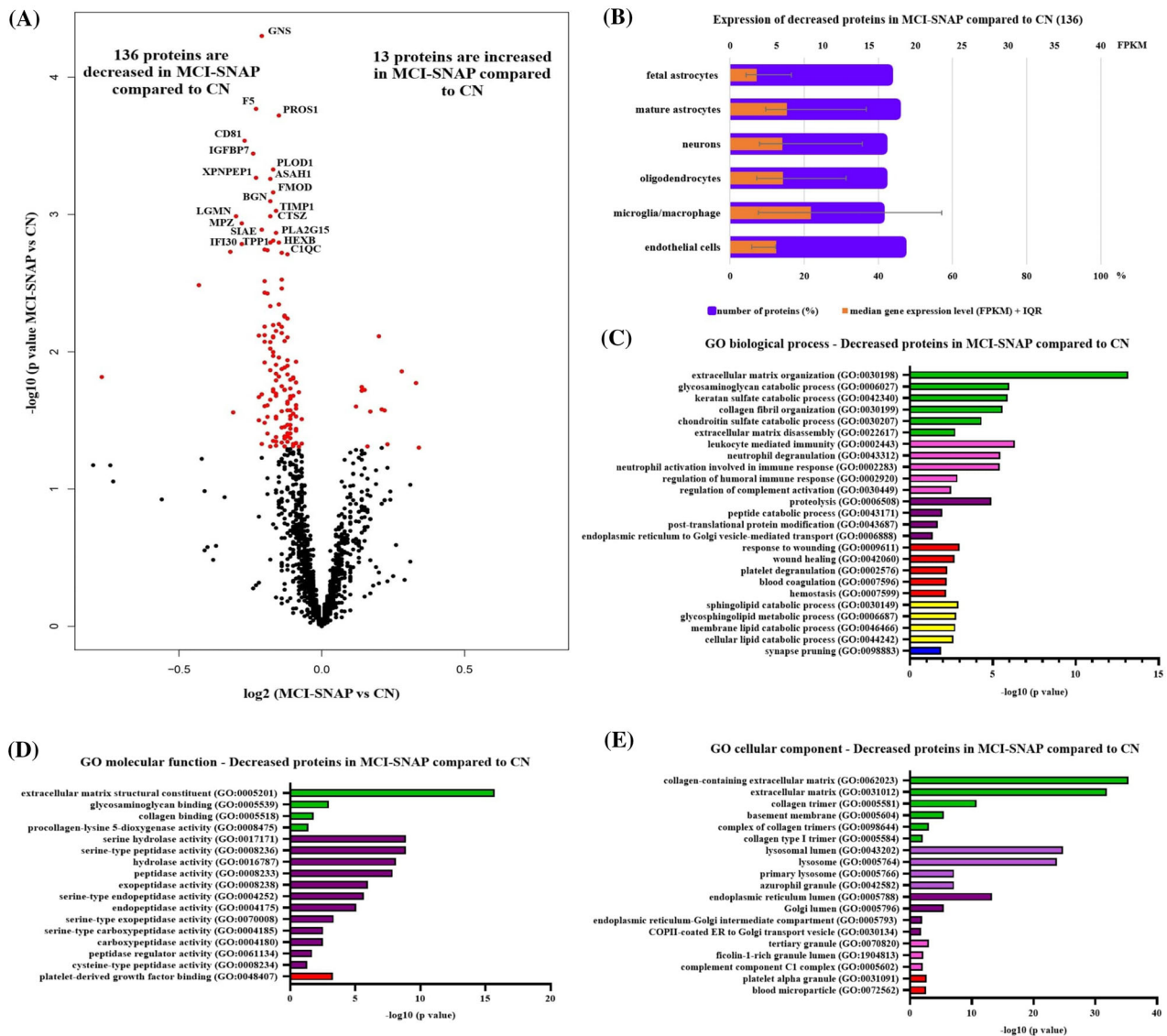


FIGURE 1 Cerebrospinal fluid (CSF) proteomics in mild cognitive impairment-suspected non-Alzheimer's pathophysiology (MCI-SNAP) versus cognitively normal (CN). A, Volcano plot displaying the log₂ fold-change against the -log₁₀ statistical P-value. Significantly different proteins are red. The top 20 proteins are named. B, Brain cell type protein expression. The graphs present the number of proteins expressed (% in blue) and the gene expression levels (fragments per kilobase of transcript per million mapped fragments [FPKM], in orange) for expressed proteins in fetal astrocytes, mature astrocytes, neurons, oligodendrocytes, macrophages/microglia, and endothelial cells for decreased proteins. D-I, Selected Gene Ontology (GO) terms including biological process (C), molecular function (D), and cellular component (E) for decreased proteins. Pathways linked with extracellular matrix (ECM) are green, immune system pathways are pink, hemostasis-linked pathways are red, protein-linked pathways are in dark purple, lipid-linked pathways are yellow, lysosome-linked pathways are light purple, and pathways related to nervous system are blue. IQR, interquartile range

increased proteins were enriched for immune processes and fibrinolysis (Figure 3D-I, Table S2D).

3.3 | CSF proteomics identify unique underlying processes in MCI-SNAP

To identify the uniquely dysregulated proteins and pathways in MCI-SNAP, we selected the overlapping dysregulated proteins from MCI-SNAP versus CN and MCI-SNAP versus MCI-AD comparisons.

Five proteins were increased and 96 decreased in MCI-SNAP compared to CN and MCI-AD (Figure S1, Table S2A). Decreased proteins were slightly more expressed by microglia (44%, median 10.0 FPKM; Figure 4A). Forty-nine decreased proteins were enriched for expression in the choroid plexus (51%, ABAenrichment $P = .018$). The decreased proteins in MCI-SNAP were enriched for biological processes related to ECM, immune system, hemostasis, protein processing and degradation, lipids, and synaptic pruning (Figure 4B-D, Table S2E). The decreased proteins enriched for expression in the choroid plexus were related to ECM, hemostasis, and neutrophils.

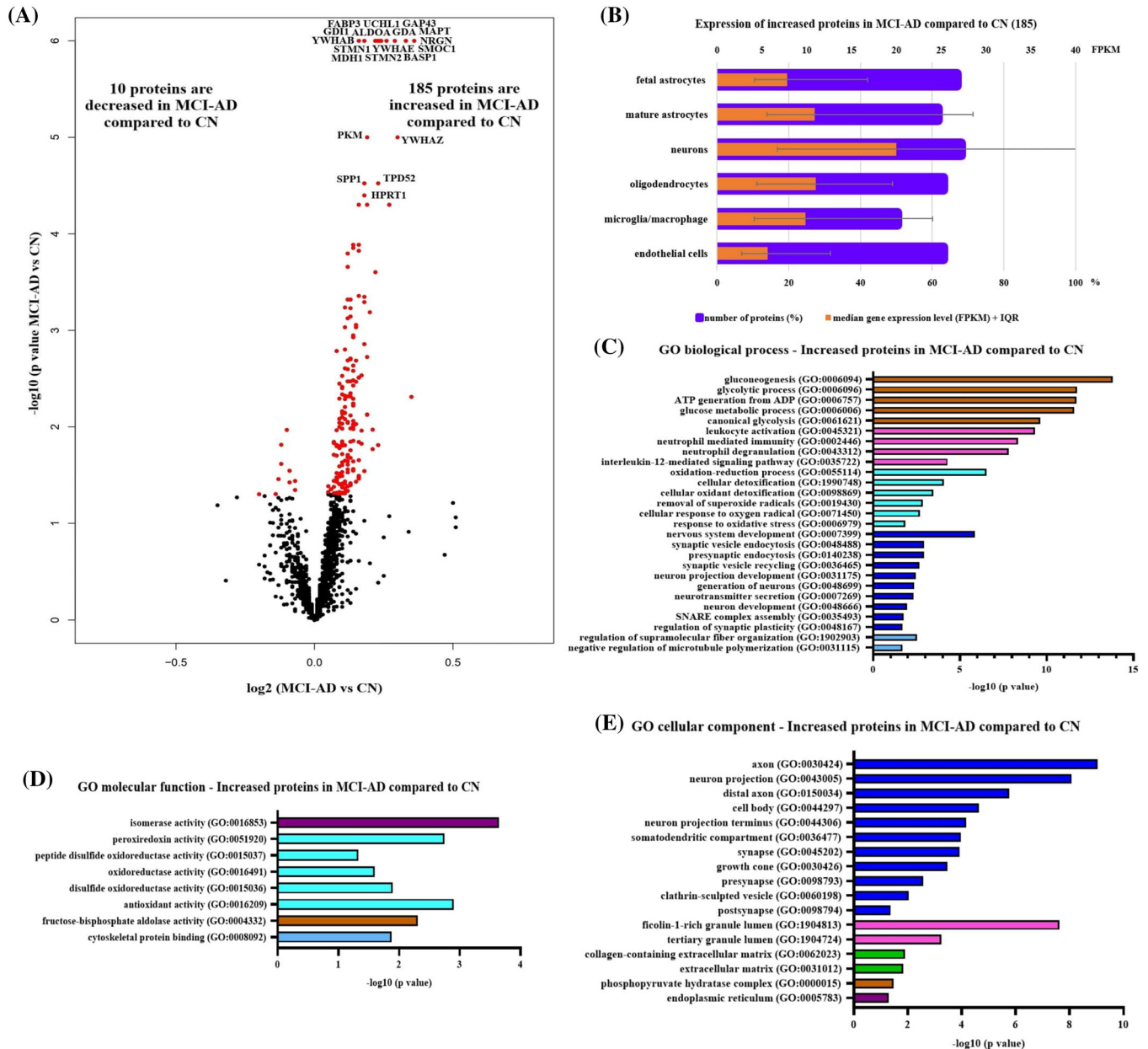


FIGURE 2 Cerebrospinal fluid (CSF) proteomics in mild cognitive impairment–Alzheimer's disease (MCI-AD) versus cognitively normal (CN). A, Volcano plot displaying the log₂ fold-change against the -log₁₀ statistical P-value. Significantly different proteins are red. The top 20 proteins are named. B, Brain cell type protein expression. The graphs present the number of proteins expressed (% in blue) and the gene expression levels (fragments per kilobase of transcript per million mapped fragments [FPKM], in orange) for expressed proteins in fetal astrocytes, mature astrocytes, neurons, oligodendrocytes, macrophages/microglia, and endothelial cells for increased proteins. C–E, Selected Gene Ontology (GO) terms including biological process (C), molecular component (D), and cellular component (E) for increased proteins. Energy metabolism pathways are brown, immune system pathways are pink, pathways related to nervous system are dark blue, oxidative stress pathways are sea-green, pathways associated with cytoskeleton are light blue, protein-linked pathways are purple, and pathways linked with extracellular matrix (ECM) are green. IQR, interquartile range

3.4 | AD polygenic risk scores in MCI-SNAP

Next, we examined the differences in AD genetic risk profile between the groups. We found significantly higher AD PGRS for MCI-AD compared to CN for the threshold P-value of 1e-30, 1e-8, 5e-8, and 1e-5, reflecting risk genes with a high association with AD, which confirmed

validity of our data and approach. MCI-AD showed significantly higher AD PGRS compared to MCI-SNAP, for the threshold P-value of 1e-30, 1e-8, 5e-8, and 1e-5. These differences between the groups are driven by APOE, as shown in Figure 5. No significant PGRS differences were found between MCI-SNAP and CN. Age and sex correction did not change the results (Table S3 in supporting information).

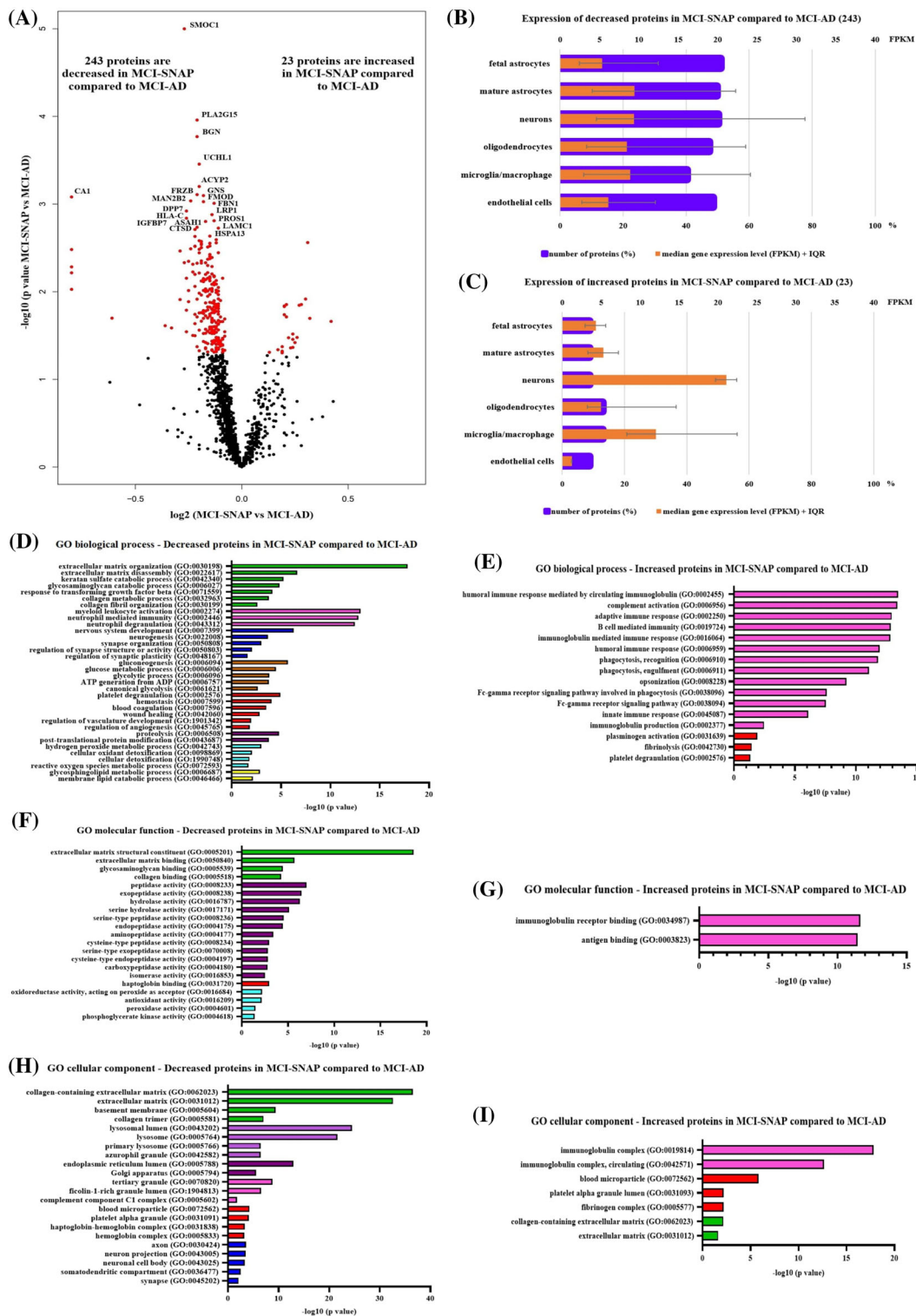


FIGURE 3 Cerebrospinal fluid (CSF) proteomics in mild cognitive impairment–suspected non-Alzheimer’s pathophysiology (MCI-SNAP) versus mild cognitive impairment–Alzheimer’s disease (MCI-AD). A, Volcano plot displaying the log2 fold-change against the -log10 statistical P-value. Significantly different proteins are red. The top 20 proteins are named. B,C, Brain cell type protein expression. The graphs present the number of proteins expressed (% in blue) and the gene expression levels (fragments per kilobase of transcript per million mapped fragments [FPKM], in orange) for expressed proteins in fetal astrocytes, mature astrocytes, neurons, oligodendrocytes, macrophages/microglia, and endothelial cells for (B) decreased proteins and (C) increased proteins. D–I, Selected Gene Ontology (GO) terms including biological process (D,E), molecular component (F,G), and cellular component (H,I) for decreased (D,F,H) and increased (E,G,I) proteins. Pathways linked with extracellular matrix (ECM) are green, immune system pathways are pink, nervous system linked pathways are blue, hemostasis-linked pathways are red, energy metabolism pathways are brown, protein-linked pathways are dark purple, oxidative stress pathways are sea-green, lipid-linked pathways are yellow, and lysosome-linked pathways are light purple. IQR, interquartile range

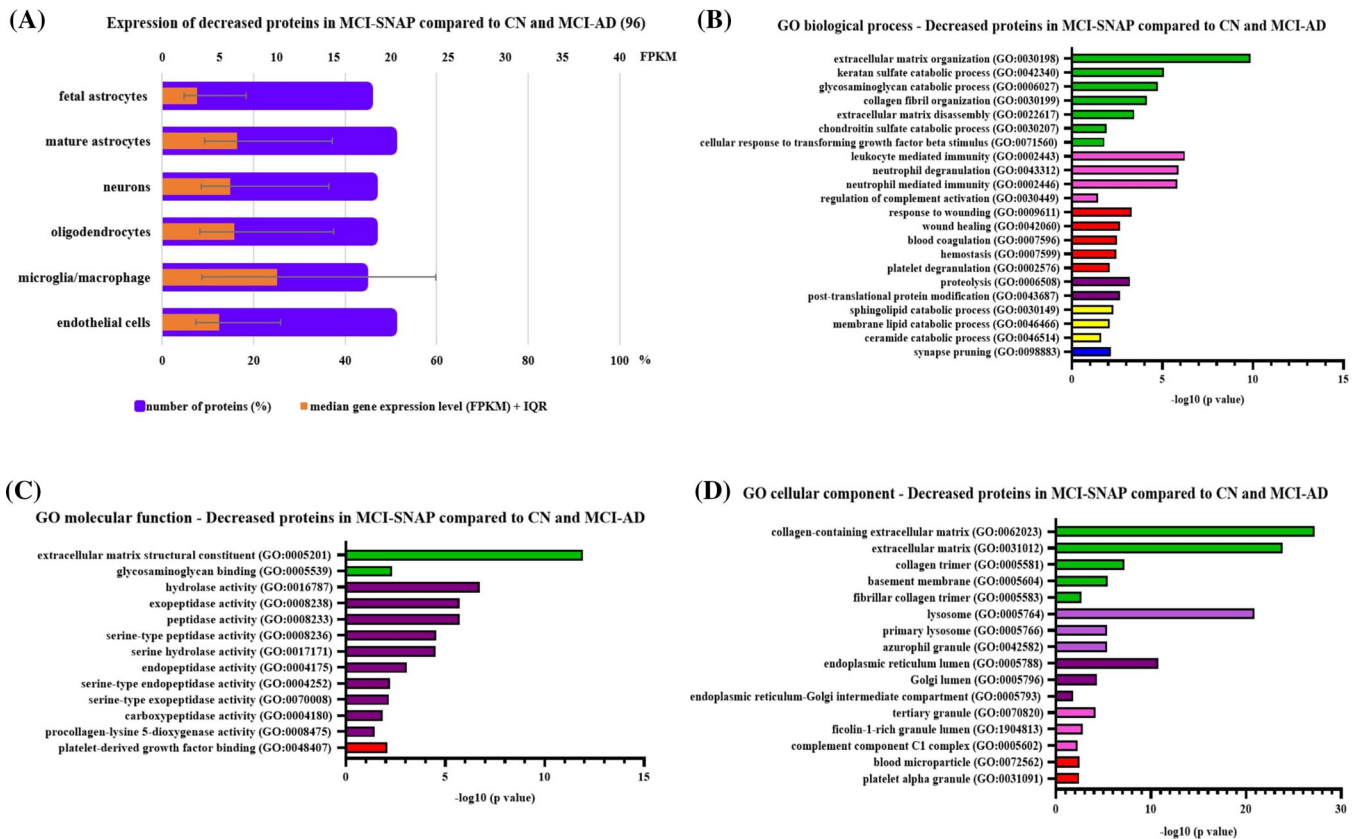


FIGURE 4 Decreased cerebrospinal fluid (CSF) proteins in mild cognitive impairment-suspected non-Alzheimer's pathophysiology (MCI-SNAP) versus cognitively normal (CN) and mild cognitive impairment-Alzheimer's disease (MCI-AD). A, Brain cell type protein expression. The graph presents the number of proteins expressed (% in blue) and the gene expression levels (fragments per kilobase of transcript per million mapped fragments [FPKM], in orange) for expressed proteins in fetal astrocytes, mature astrocytes, neurons, oligodendrocytes, macrophages/microglia, and endothelial cells. B,C, Selected Gene Ontology (GO) terms including biological process (B), cellular component (C), and molecular component (D). Pathways linked with extracellular matrix (ECM) are green, immune system pathways are pink, hemostasis-linked pathways are red, protein-linked pathways are dark purple, lipid-linked pathways are yellow, lysosome-linked pathways are light purple, and nervous system linked pathways are blue. IQR, interquartile range

3.5 | Post hoc analysis

To better understand the influence of *APOE* ϵ 4 on our CSF findings, we also performed analyses without *APOE* ϵ 4 correction, which did not appreciably change the results (Figure S2, Table S4).

We also performed the CSF analyses with correction for education, as MCI-SNAP and MCI-AD had a lower educational level than CN. This did not substantially change the results (data not shown).

To assess whether the proteomic differences between MCI-SNAP and CN were dependent on cognitive impairment, we also investigated the proteomic profile of individuals with MCI and normal $A\beta$ 42 and p-tau levels (MCI A-T-, $n = 52$). This MCI A-T- group showed generally a similar proteomic pattern as the CN A-T- group (data not shown). Eleven increased and 144 decreased proteins were found in MCI-SNAP compared to MCI A-T-. This confirms a unique CSF proteomic profile for MCI-SNAP among individuals with MCI.

4 | DISCUSSION

CSF proteomics provide a unique opportunity to investigate the pathophysiology of MCI-SNAP. Compared to CN and MCI-AD, individuals with MCI-SNAP showed decreased CSF levels of proteins associated with ECM, hemostasis, immune system, lipids, protein processing/degradation and synapses, and with a predominant expression in the choroid plexus. In PGRS analyses based on AD risk GWAS data, MCI-SNAP was indistinguishable from controls. MCI-AD showed higher PGRS compared to controls and MCI-SNAP. These differences were driven by *APOE*.

Relative to controls, MCI-SNAP showed mostly decreased levels of proteins, while MCI-AD showed mainly increased protein levels, with minimal overlap between the proteins that were changed in MCI-SNAP and MCI-AD. Together this indicates that MCI-SNAP and MCI-AD are different entities. Dysregulated pathways in MCI-AD are consistent with previous studies.³⁹⁻⁴¹ The differences between MCI-SNAP and MCI-AD could be partially explained by previous studies that showed

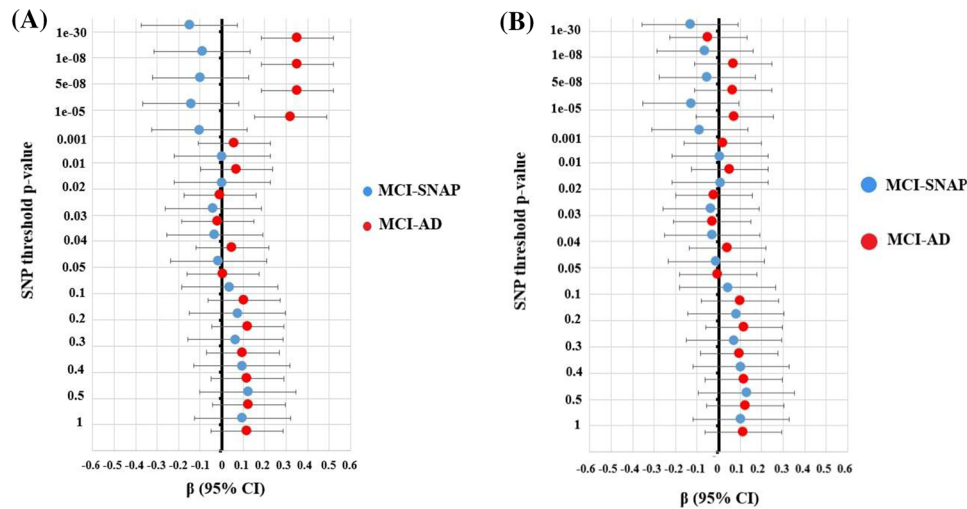


FIGURE 5 Polygenic risk score (PGRS) across single nucleotide polymorphism (SNP) thresholds. Differences in Alzheimer's disease (AD) PGRS (based on de Rojas et al.¹⁸) for different SNP threshold *P*-values. The black line indicates PGRS of controls, the blue dots represent PGRS of mild cognitive impairment–suspected non-Alzheimer's pathophysiology (MCI-SNAP), and the red dots represents PGRS of mild cognitive impairment–Alzheimer's disease (MCI-AD). A, AD PGRS with apolipoprotein E (APOE) region. B, AD PGRS without APOE region

an association between tau-only pathology (like in MCI-SNAP) and cellular hypoactivity, while $A\beta$ -only pathology may be associated with cellular hyperactivity.⁴² When there is both tau and $A\beta$ pathology, cellular hyperactivity is expected in earlier stages of the disease, such as seen in MCI-AD, likely followed by hypoactivity at later stages of the disease.⁴² Evidence from in vitro and mice studies has shown that tau pathology, in the absence of $A\beta$, is related to a decrease of ribosomal proteins synthesis and sequestration of ribosomal components, which may progressively lead to a dysfunction of the entire protein biosynthesis machinery^{43,44} and hence decreased protein levels as observed in MCI-SNAP. Furthermore, it could be that increased tau levels in MCI-SNAP and MCI-AD may differ in tau isoforms.⁴⁵

Interestingly, a large percentage of the decreased proteins in MCI-SNAP was enriched for expression in the choroid plexus. The choroid plexus is located inside the brain ventricles and is responsible for the production of CSF; transport of ions, proteins, nutrients, and metabolic precursors across the epithelium to the CSF; and clearance of proteins from the CSF.³⁵ Reduced structural integrity of the choroid plexus might explain the dysregulated synthesis or transport of CSF proteins in MCI-SNAP⁴⁶ and may be caused by conditions such as chronic inflammation and immune dysregulation, stress, or sleep disorders.⁴⁷

The decreased proteins enriched for expression in the choroid plexus in MCI-SNAP were related to the ECM, hemostasis, platelets, and immune system. The choroid plexus is composed of a basement membrane, a layer of the ECM. It is a highly vascular structure that creates the blood–CSF barrier.⁴⁸ A lack of homeostasis of ECM components in the vessel wall could lead to a rupture of the vessel wall and hemostasis.⁴⁸ Possibly, MCI-SNAP individuals could present vascular injury in choroid plexus blood vessels, with activation of clotting factors.⁴⁸ The choroid plexus is also a gateway for immune cell entry in the central nervous system, including neutrophils and lymphocytes.³⁵ So, choroid plexus disruption could affect the migration of immune cells

to the CSF. Similar patterns have also been observed in transcriptomics studies of major depressive disorder and amyotrophic lateral sclerosis (ALS). In major depressive disorder, decreased proteins enriched for expression in the choroid plexus were related to ECM⁴⁹ and, in ALS, decreased proteins enriched for expression in the choroid plexus were related to hemostasis and platelets.⁴⁸ This suggests that choroid plexus dysfunction may play a role in several diseases.

We observed higher levels of $A\beta_{38}$ and $A\beta_{40}$ in MCI-SNAP compared to CN and MCI-AD groups. This may point toward disturbed $A\beta$ metabolism. However, MCI-SNAP likely does not represent atypical AD as we found lower AD PGRS scores in MCI-SNAP compared to MCI-AD and no evidence of $A\beta$ aggregation given the similar $A\beta_{42/40}$ ratio in MCI-SNAP and controls. Dysfunction of choroid plexus could impair $A\beta$ clearance and cause increased $A\beta$ levels.⁵⁰ The lower frequency of *APOE* $\epsilon 4$ carriers and higher frequency of *APOE* $\epsilon 2$ carriers in MCI-SNAP compared to MCI-AD is consistent with previous studies^{3,6} and could explain the absence of $A\beta$ aggregation in MCI-SNAP.⁵¹

We also found few proteins increased in both MCI-SNAP and MCI-AD, that is, NfL (axonal degeneration), Ng (synapse dysfunction), YKL-40 (astrocyte activation), STMN1/2 (cytoskeleton regulation), and GAP43 (axonal regeneration), all related to tau pathology.^{52–55}

MCI-SNAP and MCI-AD both had a lower educational level than CN. Nonetheless, this difference in education between groups did not impact our proteomics findings as correction for education resulted in similar findings.

While our overall sample size for proteomic analyses was large, the MCI-SNAP group was relatively small. This could have limited our statistical power. Furthermore, we had to rely on a single tau biomarker, while different definitions of SNAP may give different pathophysiological profiles. Future studies should validate our findings and investigate SNAP based on other biomarkers, such as hippocampal volume or tau positron emission tomography imaging. Further research is also

needed to unravel the causes and consequences of the dysregulated processes as well as the longitudinal cognitive outcomes of MCI-SNAP.

Together, our findings suggest that MCI-SNAP is an entity in itself rather than atypical AD. Our results are the first step toward a definition of non-A β pathophysiology in MCI-SNAP. Choroid plexus dysfunction may play a role in MCI-SNAP, as well as pathways linked with ECM, immune system, hemostasis, proteolysis, lipids, and synapses. Our findings have implications for trial design, as they highlight the need for a different treatment in individuals with MCI-SNAP compared to individuals with MCI-AD.

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CONFLICTS OF INTEREST

The present study was supported by the Memorabel program of ZonMw (the Netherlands Organization for Health Research and Development) grant number 733050502, Janssen Pharmaceutica N.V., and EMIF-AD. J.G. has received in the past 36 months grants or contracts from Åhléns-stiftelsen (150000 SEK), Gothenburg University Alzheimerfonden (500000 SEK), and Gothenburg University FoU VGR (50000 SEK). B.T. has received in the past 36 months grants or contracts from ZonMW VID1 (#09150171910068). B.T. has a patent on CSF proteomic subtypes in AD (#19165795.6 / #PCT/NL2020/050216; Applicant: Stichting Vumc). V.D. has received in the past 36 months a grant (SEQ1122) from DFG. F.V. has, in the past 36 months, other financial or non-financial interests as a member of Dutch advisory board Biogen. I.R. has received in the past 36 months grants or contracts from EIT Health Institution and Janssen Institution. P.S. has received consultancy fees (paid to the institution) from AC Immune, Alker-

mes, Alnylam, Alzheon, Anavex, Biogen, Brainstorm Cell, Cortexyme, Denali, EIP, ImmunoBrain Checkpoint, GemVax, Genentech, Green Valley, Novartis, Novo Nordisk, PeopleBio, Renew LLC, Roche. He is PI of studies with AC Immune, CogRx, FUJI-film/Toyama, IONIS, UCB, and Vivoryon. He is a part-time employee of Life Sciences Partners Amsterdam. He serves on the board of Brain Research Center and New Amsterdam Pharma. P.S. has, in the past 36 months, held a leadership or fiduciary role in Trustee of the World Dementia Council. C.E.T. has received in the past 36 months grants or contracts from Weston brain institute, ZonMW, Alzheimer Nederland, and European Union and is part of the editorial board of *Alzheimer Research and Therapy*, and *Neurology*. R.V. has received in the past 36 months clinical trial agreements with Roche, AbbVie, J&J, UCB, NovoNordisk. R.V.'s institution has received payment for consultancy agreement Cytox and for Data Safety Monitoring Board AC Immune and Novartis. J.S. has received in the past 36 months a personal grant of the Research Foundation Flanders (FWO)(12Y1620N). P.M. has received in the past 36 months grants or contracts from Carlos III Institute, Ministry of Health of Spain, for the EPAD and AMYPAD projects, and from the Department of Health, Government of the Basque Country. P.M. has received in the past 36 months consulting fees from Biogen, Esteve, Zambon, and Roche (to himself); payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from Biogen, Roche, Zambon, Grifols, and Nutricia (to himself); and support for attending meetings and/or travel from Nutricia (to himself). S.L. has been on the Dementia Discovery Fund advisory board member on behalf of Janssen, has held stock options as part of remuneration from Janssen Medical Ltd, and has been an employee of Janssen Medical Ltd (UK). F.B. is a consultant to Biogen, Roche, Merck, Novartis, IXICO, and Combinostics; has participated on a Data Safety Monitoring Board or Advisory Board for Merck, Biogen, and NIA. L.B. has received in the past 36 months grants or contracts ERC (institution), DFG (institution), Cure Alzheimer Foundation (institution). K.B. is supported by the Swedish Research Council (#2017-00915), the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615), the Swedish Alzheimer Foundation (#AF-742881), Hjärnfonden, Sweden (#FO2017-0243), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), and the National Institute of Health (NIH), USA, (grant #1R01AG068398-01). K.B. has served as a consultant or at advisory boards for Abcam (payment to himself), Axon (payment to himself), Biogen (payment to himself), JOMDD/Shimadzu (payment to himself), Lilly (payment to himself), MagQu (payment to himself), Prothena (payment to himself), Roche Diagnostics (payment to himself), and Siemens Healthineers (payment to himself). K.B. has served on data monitoring committees for Julius Clinical (payment to himself) and Novartis (payment to himself). K.B. is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. H.Z. is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931), the Alzheimer Drug

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Author disclosures are available in the supporting information.

ORCID

Aurore Delvenne  <https://orcid.org/0000-0001-8724-3010>

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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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