CSF SERPINA3 Levels Are Elevated in Patients With Progressive MS

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Abstract

Objective

To identify biomarkers associated with progressive phases of MS and with neuroprotective potential.

Methods

Combined analysis of the transcriptional and proteomic profiles obtained in CNS tissue during chronic progressive phases of experimental autoimmune encephalomyelitis (EAE) with the transcriptional profile obtained during the differentiation of murine neural stem cells into neurons. Candidate biomarkers were measured by ELISA in the CSF of 65 patients with MS (29 with relapsing-remitting MS [RRMS], 20 with secondary progressive MS, and 16 with primary progressive MS [PPMS]) and 30 noninflammatory neurologic controls (NINCs).

Results

Integrative analysis of gene and protein expression data identified 2 biomarkers, the serine protease inhibitor Serpina3n and the calcium-binding protein S100A4, which were upregulated in chronic progressive EAE and whose expression was induced during neuronal differentiation. Immunofluorescence studies revealed a primarily neuronal expression of S100A4 and Serpina3n during EAE. CSF levels of SERPINA3, the human ortholog of murine Serpina3n, and S100A4 were increased in patients with MS compared with NINCs (SERPINA3: 1,320 vs 838.6 ng/mL, $p = 0.0001$; S100A4: 1.6 vs 0.8 ng/mL, $p = 0.02$). Within the MS group, CSF SERPINA3 levels were significantly elevated in patients with progressive forms, mainly patients with PPMS compared with patients with RRMS (1,617 vs 1,129 ng/mL, $p = 0.02$) and NINCs (1,617 vs 838.6 ng/mL, $p = 0.0001$). Of interest, CSF SERPINA3 levels significantly correlated with CSF neurofilament light chain levels only in the PPMS group ($r = 0.62$, $p = 0.01$).

Conclusion

These results point to a role of SERPINA3 as a biomarker associated with the progressive forms of MS, particularly PPMS.

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Glossary

cDNA = complementary DNA; **EAE** = experimental autoimmune encephalomyelitis; **EDSS** = Expanded Disability Status Scale; **FDR** = false discovery rate; **GFAP** = glial fibrillary acid protein; **IFHC** = immunofluorescence histochemistry; **mRNA** = messenger RNA; **NINC** = noninflammatory neurologic control; **NFL** = neurofilament light chain; **NSCs** = neural stem cell; **PPMS** = primary progressive MS; **qPCR** = quantitative PCR; **RRMS** = relapsing-remitting MS; **SPMS** = secondary progressive MS.

MS is a chronic inflammatory, demyelinating, and neurodegenerative disease of CNS and a leading cause of neurologic disability in young adults.¹ The disease is characterized by an inflammatory component, which predominates in the initial relapsing-remitting phases, and a neurodegenerative component associated with the progressive stages of the disease, although it can be present early in the disease course.² Over the last decades, the immunologic aspects of MS have been extensively investigated; however, the mechanisms leading to neurodegeneration and tissue repair are not yet well understood.

Whereas currently approved MS therapies are highly effective to suppress the predominantly inflammatory component observed in patients with relapsing-remitting MS (RRMS), these therapies remain largely ineffective in patients with progressive forms of the disease in whom the neurodegenerative component dominates.^{3,4} Similarly, available treatments have shown limited neuroprotective efficacy. In this context, a better understanding of the mechanisms underlying neurodegeneration and neuroregeneration in MS may set the rationale for the design of effective therapies to stop disease progression and/or enhance neuroprotection.

In the present study, we pursued to identify biomarkers of disease progression that may have neuroregenerative potential. To this end, in a first phase of the study, we searched for biomarkers that, on the one hand, were upregulated in CNS tissue during the chronic progressive phases of experimental autoimmune encephalomyelitis (EAE) and, on the other hand, were upregulated during the differentiation of neural stem cells (NSCs) to neurons. In a second phase of the study, selected biomarkers were measured in CSF of patients with MS with different clinical forms of the disease to confirm its potential as biomarkers of progression.

Methods

Mice

All experiments were performed in 5- to 8-week-old female C57BL/6J mice (Harlan, Lesmo, Italy) in strict accordance with European Union and governmental regulations (Generalitat de Catalunya Decret 214/97 July 30). The Ethics Committee on Animal Experimentation of the Vall d'Hebron Research Institute approved all procedures described in the study (protocol numbers: 70/13, 81/17 CEEA).

EAE Induction

Anesthetized C57BL/6 mice (n = 4/group) were immunized with subcutaneous injections of myelin oligodendrocyte

glycoprotein (MOG)₃₅₋₅₅ (50 µg) (Peptide Synthesis Facility, Universitat Pompeu Fabra, Barcelona, Spain) in phosphate buffered saline (PBS) emulsified in complete Freund adjuvant (Sigma Chemical, St Louis, MO) and supplemented with 2 mg/mL *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI). Control animals (n = 4/group) received only PBS. All animals received an IV injection of 150 ng Pertussis toxin in 100 µL PBS on the day of immunization and another doses 48 hours later. Mice were weighted and examined daily for clinical signs of EAE, with the following scale: grade 0, no clinical disease; grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis; grade 3, hind leg paralysis; grade 4, paraplegia with forelimb weakness or paralysis; and grade 5, moribund state or death.⁵

Tissues

C57BL/6 mice immunized either with MOG (EAE group) or PBS (control group) were killed with carbon dioxide (>70%) at different stages of the disease: days (d) 8, 16, 36, 50, and 90 postimmunization (pi). Brain and spinal cords were dissected and divided into 2 parts, one was paraffin embedded and sectioned with 6-µm thickness and the other was snap-frozen in liquid N₂.

Immunofluorescence histochemistry

For immunofluorescence, we used the following antibodies at the dilution listed: goat polyclonal anti-Serpina3n antibody (1:5; purchased from R&D Systems, Minneapolis, MN, cat. No. AF4709); rabbit polyclonal anti-S100A4 antibody (1:10; purchased from Abcam, Cambridge, MA, cat. no. ab41532); antigial fibrillary acid protein (GFAP) Alexa Fluor-488 conjugated antibody (1:50; purchased from eBioscience, Vienna, Austria, cat. no. 53-9892-82); and chicken anti-β-Tubulin Isotype III polyclonal antibody (1:60; purchased from Abcam, cat.no. 117716). The following secondary antibodies were used: Alexa Fluor-568 rabbit anti-goat IgG (1:300; purchased from Invitrogen, Waltham, MA, cat. no. A11079); Alexa Fluor-594 goat anti-rabbit (1:300; purchased from Invitrogen, cat. no. A11012); and Alexa Fluor-488 goat anti-chicken (1:300; purchased from Invitrogen, cat. no. A11039). 4',6-diamidino-2-fenilindol (DAPI)-Pacific blue (1:30,000; purchased from Invitrogen, cat. no. D3571) was used for nuclei visualization.

Immunofluorescence histochemistry (IFHC) was performed on CNS tissue obtained on d16 and d50 pi. The staining procedure started with deparaffinization and rehydration followed by heat-induced epitope retrieval step with 10 mM

sodium citrate buffer pH 6.0. For S100A4 sections, slides were blocked in normal 5% goat serum. For *Serpina3n* sections, slides were blocked in 0.3% Triton X-100 and 0.24 g glycine. Slides were incubated overnight with primary antibodies (diluted in buffer containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100) at 4°C. Slides were afterward washed 3 times in 1xPBS-T for 5 minutes and incubated with secondary antibodies for 1 hour. Slides were then washed 3 times in 1xPBS-T for 3 minutes, and DAPI was added to the specimens for 10 minutes. After washing 3 times in 1xPBS-T for 1 minute, slides were mounted with ProLong Gold-antifade (Invitrogen) and air dried. Images were taken with ZEN-2011 software on Zeiss microscope (AXIO M2; Göttingen, Germany) with Zeiss (AxioCam) camera connected. Images were processed with Adobe Photoshop and Adobe Illustrator (Adobe Systems, San Jose, CA).

Generation of Mouse NSCs and Differentiation Into Neurons

NSCs were isolated from forebrain C57BL/6 mice ($n = 4$) E16–18. Briefly, the forebrain was dissected, cut into small fragments ($<1 \text{ mm}^3$), and digested with papain. The digested tissue fragments were passed through fire-polished fine tip Pasteur pipet to obtain a single cell suspension and cultured in DMEM/F12 medium (Invitrogen) containing B27 supplement, 20 ng/mL fibroblast growth factor basic (bFGF) (Invitrogen), 20 ng/mL epidermal growth factor (EGF) (Invitrogen), in the presence of FBS 5% and retinoic acid (0.05 μM). Neurons were characterized by immunofluorescence staining with MAP2 (microtubule-associated protein 2) and doublecortin (DCX).

Gene Expression Microarrays

Total messenger RNA (mRNA) and proteins (frozen at -80°C until used) from the brain and spinal cord were extracted using TRI reagent (Sigma Chemical) following the manufacturer's instructions. A similar protocol was followed to obtain total RNA from neurons and NSCs. Gene expression from mice tissue, NSCs, and neurons was evaluated with the Affymetrix Mouse Gene 1.0 Array using the Ambion WT Expression kit for target amplification (Applied Biosystems, Foster City, CA) and the target labeling with the WT Terminal Labeling kit (Affymetrix, Santa Clara, CA). The Affymetrix Expression Console software was used for gene-level log-scaled robust multiarray analysis. The linear models for microarray data (LIMMA) R package⁶ was used to identify differentially expressed genes between mice with EAE and control groups (PBS) and between NSCs and neurons. Differentially expressed genes in the 2-sample t -test with p value <0.05 were considered significant.

Determination of *Serpina3n* and *S100A4* Expression Levels in CNS by qPCR

mRNA expression levels for *Serpina3n* and *S100A4* were determined in CNS samples from 4 EAE and 4 control animals at days 8, 16, 36, 50, and 90 pi. Total RNA was taken from the same samples that had been used for the microarrays, and

complementary DNA (cDNA) synthesized using the High capacity cDNA Archive kit (Applied Biosystems). *Serpina3n* and *S100A4* transcripts were determined with TaqMan gene expression assays (Mm00776439_m1 and Mm00803372_g, respectively; Applied Biosystems). Obtained values were normalized according to the level of expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Assays were run on the ABI PRISM® 7900HT system (Applied Biosystems), and data were analyzed with the $2^{-\Delta\Delta\text{CT}}$ method.⁷ Results were expressed as fold change in gene expression in EAE mice compared with controls (calibrators).⁵

Mass Spectrometry Sample Preparation

Samples (10 μg) were reduced with dithiothreitol (30 nmol, 37°C , 60 minutes) and alkylated with iodoacetamide (60 nmol, 25°C , 30 minutes) in the dark. The resulting protein extract was diluted in 2 M urea with 200 mM ammonium bicarbonate for digestion with endoproteinase LysC (1:10 w: w, 37°C , o/n, Wako, Osaka, Japan), and afterward diluted 2-fold with 200 mM ammonium bicarbonate for trypsin digestion (1:10 w:w, 37°C , 8 hours, Promega, Madison, WI).⁸ After digestion, peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc., Southborough, MA) before Liquid chromatography (LC)-MS/MS analysis.

Chromatographic and Mass Spectrometric Analysis

Samples were analyzed using a LTQ-Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an EASY-nLC 1000 (Proxeon; Thermo Fisher Scientific, Denmark). Peptides were loaded directly onto the analytical column to be separated by reversed-phase chromatography by a 25-cm column with a 75 μm of inner diameter, packed with 1.9 μm C18 particles (Nikkyo Technos, Tokyo, Japan). Chromatographic gradients started at 97% buffer A (0.1% formic acid in water) and 3% buffer B (0.1% formic acid in acetonitrile) with a flow rate of 250 nL/minute for 5 minutes and gradually increased to 35% buffer B and 65% A in 120 minutes.

The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2 kV and source temperature at 275°C . Ultramark 1621 was used for external calibration of the FT mass analyzer prior the analyses, and an internal calibration was performed using the background polysiloxane ion signal at m/z 445.1200. The acquisition was performed in data-dependent acquisition mode, and full MS scans with 1 micro scans at resolution of 120,000 were used over a mass range of m/z 400–1,500 with detection in the Orbitrap mass analyzer. In each cycle of data-dependent acquisition analysis, following each survey scan, the most intense ions above a threshold ion count of 5,000 were selected for fragmentation. The number of selected precursor ions for fragmentation was determined by the Top Speed acquisition algorithm and a dynamic exclusion of 60 seconds. Fragment

ion spectra were produced via collision induced dissociation at normalized collision energy of 35%, and they were acquired in the ion trap mass analyzer.⁸ Auto gain control was set to 4E3, and an isolation window of 1.6 m/z and a maximum injection time of 300 ms were used. All data were acquired with Xcalibur software.

Digested bovine serum albumin (New England Biolabs) was analyzed between each sample to avoid sample carryover and to assure stability.⁹

Mass Spectrometry Data Analysis

Acquired spectra were analyzed using the Proteome Discoverer software suite (v1.4; Thermo Fisher Scientific) and the Mascot search engine v2.5, Matrix Science.¹⁰ The data were searched against a Swiss-Prot mouse database (as of April 2015) plus a list of common contaminants and all the corresponding decoy entries. For peptide identification, a precursor ion mass tolerance of 7 ppm was used for MS1 level, trypsin was chosen as enzyme, and up to 3 missed cleavages were allowed. The fragment ion mass tolerance was set to 0.5 Da for MS2 spectra. False discovery rate (FDR) for identification of peptides was set to a maximum of 1%.

Peptide quantification data were retrieved from the extracted ion chromatograms, and the obtained values used to perform protein relative quantitation using the R package MSstats v2.6. Protein abundance changes were considered significant when adjusted *p* value was below 0.05.

The raw proteomics data have been deposited to the PRIDE¹¹ repository with the data set identifier PXD018173.

Integrative Analysis of Gene and Protein Expression Data

Data sets of gene expression from transcriptome and protein relative abundances from proteome analyses were obtained. The most differentially expressed genes and proteins between the EAE and control groups (adjusted *p* value of 0.05) were selected on both data sets separately by a cutoff according to SD. Afterward, the top genes and proteins were matched in a common list and used for the integrative analysis. Finally, biomarkers of disease progression were identified by 2 complementary approaches. First, the Partial Least Squares (sPLS) canonical method using the “mixOmics” R package¹² was used to integrate both data sets for each time point, applying a cutoff threshold of 0.85 to restrict the analysis to stronger gene-protein associations. Second, each data set was analyzed along time with a 2-way backward regression with a 5% FDR with the maSigPro R package.¹³ sPLS looked at the correlation between genes and proteins at each given time point, and the time-course analysis focused at the relationship along each time point on a gene-by-gene and a protein-by-protein basis.

Patients

A cohort of 65 untreated patients with MS and 30 non-inflammatory neurologic controls (NINCs) was included

in the study. The MS group comprised 29 patients with RRMS, 20 with secondary progressive MS (SPMS), and 16 with primary progressive MS (PPMS). Diagnosis was based on 2005 and 2010 revised McDonald criteria.^{14,15} For patients with SPMS, progression was defined as a confirmed increase at 6 months in the Expanded Disability Status Scale (EDSS) of 1 point for EDSS < 5.5 and 0.5 points for EDSS ≥ 5.5. Table 1 summarizes demographic and clinical characteristics of patients with MS and controls included in the study.

Standard Protocol Approvals, Registrations, and Patient Consents

The project was approved by the Institutional Review Board of University of Belgrade School of Medicine (29/X-8), Medical University of Graz (17-046 ex 05/06), and Vall d'Hebron Hospital (EPA(AG)57/2013(3834)). Written informed consent was obtained from each participant.

Quantification of SERPINA3 and S100A4 Levels in CSF From Patients With MS and Controls by ELISA

CSF samples were collected by lumbar puncture and centrifuged for 5 minutes at 1,500 rpm to remove cells. Samples were aliquoted and frozen at -80°C until used. CSF levels of SERPINA3 (the human ortholog of murine Serpina3n, also known as α 1-antichymotrypsin) were measured with the human α 1-antichymotrypsin ELISA kit (Abnova, Taipei, Taiwan) following 1:40 dilution, and levels of S100A4 were determined with the human S100A4 ELISA kit (CycLex Co., Nagoya, Japan). All samples were measured in duplicate following the specific protocols provided by the manufacturers. The intra- and interassay coefficients of variation for SERPINA3 were 2.5% and 10.4%, respectively, and for S100A4 5.0% and 17.0%, respectively.

Quantification of CSF Neurofilament Light Chain Levels by Single Molecule Array (Simoa)

In patients with RRMS (*n* = 29) and PPMS (*n* = 16), CSF levels of neurofilament light chain (NFL) were measured using commercially available NFL immunoassay kits (Quanterix, Billerica, MA, cat#103186) run on the fully automated ultrasensitive Simoa HD-1 Analyzer (Quanterix). Samples were run in duplicate in accordance with manufacturers' instructions with appropriate standards and internal controls. The intra-assay and interassay coefficients of variation were 5% and 9%, respectively.

Statistical Analysis

Statistical analysis was conducted with the SPSS 17.0 package (SPSS Inc., Chicago, IL) for MS Windows. The significance of differences among experimental groups for microarrays, mass spectrometry, and quantitative PCR (qPCR) was assessed by the 2-tailed Student *t* test or Mann-Whitney *U* test. One-way analysis of variance followed by the Tukey post hoc test was applied for mean protein levels comparisons among groups. Partial correlations adjusting

Table 1 Demographic and Baseline Clinical Characteristics of Patients With MS and Controls Included in the Study

Baseline characteristics	NINCs	MS ^a		
		RRMS	SPMS	PPMS
N	30	29	20	16
Age (y)	37.5 (12.0)	34.2 (10.3)	52.7 (10.1)	51.9 (11.6)
Female/male (% women)	26/4 (86.7)	18/11 (62.1)	13/7 (65.0)	10/6 (62.5)
Duration of disease (y)	—	3.1 (4.0)	15.7 (12.3)	4.9 (2.1)
EDSS^b	—	2.4 (2.0–3.5)	5.3 (4.0–6.6)	5.2 (4.5–6.1)
Number of Gd-enhancing lesions	—	0.8 (1.4)	1 (1.9)	0.2 (0.4)

Abbreviations: EDSS = Expanded Disability Status Scale; Gd = gadolinium; NINC = noninflammatory neurologic control; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS.

Data are expressed as mean (SD) unless otherwise stated.

NINCs include 26 patients with headache, 1 patient with cerebral small vessel disease, 1 patient with lumbosacral polyradiculopathy, 1 patient with conversion disorder, and 1 patient with idiopathic intracranial hypertension.

^a Refers to the whole MS group.

^b Data are expressed as median (interquartile range).

for age were used to assess linear association between CSF levels of NFL and CSF levels of SERPINA3 or S100A4. Quantitative data are presented as mean values $\pm$ standard error of mean, unless otherwise stated. Differences were considered statistically significant when *p* values were below 0.05.

Data Availability

All data analyzed during this study will be shared anonymized by request of a qualified investigator to the corresponding author.

Results

Selection of Differentially Expressed Genes and Proteins in EAE and Upregulated Genes in Neuronal Differentiation

A flowchart summarizing the main steps of the study design and analysis is shown in figure 1. To identify biomarkers associated with disease progression, chronic EAE was induced with MOG, and CNS tissue obtained at different chronic stages of the disease (figure 1A). By applying a combination of gene expression microarray and mass spectrometry analyses, we identified 7,600 genes and 5,700 proteins that were differentially expressed between EAE and control mice, of which 1,763 molecules were found in both studies (figure 1B). Of these, 441 genes and 227 proteins showing highest differential expression through the respective gene and protein data sets were selected as potential markers for further analysis (see Methods; figure 1B).

We also analyzed the transcriptional profiling of murine NSCs and neurons using microarrays. Differentiation of mouse NSCs into neurons was associated with upregulation of 1,705 genes, which were selected for further analysis (figure 1C).

Integrative Omics Analysis Reveals Upregulation of *Serpina3n* and *S100A4* During Chronic Progressive EAE and Neurogenesis

Two multivariate approaches (sPLS and 2-way backward regression) were used to integrate genomics and proteomics data sets to assess what molecules identified at both the expression and translational levels could better explain the chronic progressive phase of EAE in comparison to controls and use these candidates as potential biomarkers. Although sPLS looked at the correlation on a gene-by-protein basis at each given time point separately, the time-course analysis focused at the relationship along each time point on a gene-by-gene and a protein-by-protein basis. As shown in figure 1D, the sPLS analysis identified a total of 380 genes and proteins showing similarly upregulated expression at each time point of the study. Time-course analysis by 2-way backward regression identified 227 genes and proteins showing a similar pattern of expression. Intersection between the 3 sets of results (i.e., sPLS analysis, time-course analysis, and neuronal differentiation) revealed 2 molecules, *Serpina3n* and *S100A4*, that showed a high expression in the progressive phases of EAE and were upregulated during neurogenesis (figure 1D). Tables 2 and e-1 (links.lww.com/NXI/A366) show the list of candidate molecules scoring 2 or 3 in the different approaches used for analysis.

Clinical disease score after EAE induction is represented in figure 2A. Figure 2B shows the time-course gene and protein expression for *Serpina3n* and *S100A4* in CNS tissue. Gene expression levels and protein abundance of *Serpina3n* and *S100A4* were significantly increased in the chronic progressive phases of EAE compared with the reference time point (d8) and with control mice (figure 2B). We also performed qPCR for *Serpina3n* and *S100A4* to validate microarray findings. qPCR also included an additional time point at d16 to discriminate between acute and chronic EAE phases.

Figure 1 Schematic Representation of the Strategy Used to Identify Biomarkers Associated With Progression and With Neuroregenerative Potential

(A) EAE was induced by immunization with MOG₃₅₋₅₅ (EAE group) or PBS (control group) in C57BL/6 mice. On days 8, 36, 50, and 90 postimmunization, mice were killed and CNS tissue obtained. (B) Total RNA and proteins extracted from CNS were quantified by means of microarrays and mass spectrometry, respectively. Gene and protein expression profiling allowed to identify 7,600 genes and 5,700 proteins differentially expressed between EAE and control mice. Of these, 1,763 genes and proteins matched in both data sets (transcriptomic and proteomic) and were used for further analysis. A total of 441 genes and 227 proteins showing the highest variations were selected for integrative analysis. (C) NSCs were differentiated into neurons, and total RNA was obtained from both cell types. Gene expression was determined by microarrays, and a total of 1,705 genes were differentially expressed between NSCs and neurons. (D) Integrative data analysis. Venn diagrams show the intersection between, on the one hand, the number of biomarkers of disease progression identified by applying the R/Bioconductor packages sPLS ("mixOmics" package) and time-course ("maSigPro" package) and, on the other hand, the number of differentially expressed genes identified in the differentiation from NCS to neurons. EAE = experimental autoimmune encephalomyelitis; NSC = neural stem cell.

As shown in figure 2C, mRNA expression levels of *Serpina3n* measured by qPCR over time faithfully mirrored those obtained with gene expression microarrays, with higher levels in chronic EAE. Although a similar gene expression pattern was also observed for *S100A4* over time, mRNA expression levels were more pronounced at d16 and d36 compared with later chronic time points, suggesting that *S100A4* expression is strongly induced by inflammation (figure 2C). Finally, as shown in figure 2D, gene expression levels for *Serpina3n* and *S100A4* were significantly upregulated in neurons compared with NSCs.

Histology Studies Reveal a Primarily Neuronal Expression of *Serpina3n* and *S100A4* During EAE

Cellular expression of *Serpina3n* and *S100A4* was determined with double IFHC in EAE during the course of the disease. CNS tissue from both acute inflammatory (d16) and chronic progressive (d50) time points was included for comparison purposes. Mouse cerebellum, hippocampus, and spinal cord slides were stained with antibodies against *Serpina3n* or *S100A4*. β -III-Tubulin and GFAP were used as neuronal and astrocytic markers, respectively. Both at d16 and d50, *Serpina3n* colocalized with β -III-Tubulin in cerebellar Purkinje

Table 2 Score of Molecules Upregulated in the Chronic Progressive Phase of Experimental Autoimmune Encephalomyelitis and Upregulated During Neurogenesis

Entrez ID	Symbol	Description	NSCs vs neurons	sPLS network	Time course	Sum
20716	Serpina3n	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	1	1	1	3
20198	S100a4	S100 calcium-binding protein A4	1	1	1	3
11430	Acox1	Acyl-Coenzyme A oxidase 1, palmitoyl	1	1	0	2
13030	Ctsb	Cathepsin B	1	0	1	2
66259	Camk2n1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	1	1	0	2
69786	Tprkb	Tp53rk-binding protein	1	1	0	2
14469	Gbp2	Guanylate-binding protein 2	1	1	0	2
19682	Rdh5	Retinol dehydrogenase 5	1	1	0	2
11816	ApoE	Apolipoprotein E	1	0	1	2
20615	Snapin	SNAP-associated protein	1	1	0	2
27883	Tango 2	Transport and golgi organization 2	1	1	0	2
56188	fxyd1	FXD domain-containing ion transport regulator 1	1	1	0	2
11303	Abca1	ATP-binding cassette, subfamily A (ABC1), member 1	1	0	1	2
12266	C3	Complement component 3	1	0	1	2
12268	C4b	Complement component 4B	1	0	1	2
12527	CD9	CD9 antigen	1	0	1	2
13010	Cst3	Cystatin C	1	0	1	2
16854	Lgals3	Lectin, galactose binding, soluble 3	1	0	1	2
17035	Lxn	Latexin	1	0	1	2
17096	Lyn	LYN proto-oncogene, Src family tyrosine kinase	1	0	1	2
18301	Fxyd5	FXD domain-containing ion transport regulator 5	1	0	1	2
20148	Dhrs3	Dehydrogenase/reductase (SDR family) member 3	1	0	1	2
21356	Tapbp	TAP-binding protein	1	0	1	2
26362	Axl	AXL receptor tyrosine kinase	1	0	1	2
74011	Slc25a27	Solute carrier family 25, member 27	1	0	1	2
74559	elovl7	ELOVL family member 7, elongation of long chain fatty acids (yeast)	1	0	1	2
214944	Mob3b	MOB kinase activator 3B	1	0	1	2
243906	Zfp14	Zinc finger protein 14	1	0	1	2
321007	Serac1	Serine active site containing 1	1	0	1	2

Abbreviation: NSC = neural stem cell.

Overlapping molecules between the 3 bioinformatics approaches used for analysis. Only molecules upregulated during the differentiation of NSCs to neurons with a p value < 0.001 are shown. Entrez ID = identifier for a gene per the NCBI Entrez database (<http://www.ncbi.nlm.nih.gov/gene>).

cells and neurons of the granular layer (figure 3A), hippocampal pyramidal neurons of the CA1 region (figure 3B), and spinal cord neurons (figure e-1A, links.lww.com/NXI/A365) of CNS tissue from EAE mice but not from PBS-immunized mice. Of interest, for all tissue sections, neuronal expression of Serpina3n was more evident in the chronic time point

compared with the acute time point, findings that are in agreement with the qPCR data suggesting that Serpina3n expression is not significantly induced during the inflammatory insult (figure 3, A and B, figure e-1A). No colocalization was observed in CNS sections of EAE or control mice stained for Serpina3n and GFAP (figure 3, C and D,

Figure 2 Serpina3n and S100A4 Levels Are Significantly Upregulated in Chronic EAE and During Neurogenesis

(A) Disease course after EAE induction. Only time points chosen for analyses are annotated. (B) Gene expression levels and protein abundance for *Serpina3n* and *S100A4* were determined by means of microarrays and mass spectrometry, respectively, in CNS samples obtained at d8, d36, d50, and d90 postimmunization ($n = 4$). (C) messenger RNA expression levels for *Serpina3n* and *S100A4* determined by quantitative PCR in CNS from EAE mice or control mice during disease course. Gray bars indicate the additional time point during the inflammatory phase of the disease (d16 postimmunization). Glyceraldehyde-3-phosphate dehydrogenase was used as endogenous control. Results are expressed as fold change in gene expression in EAE mice relative to control mice (calibrators). (D) Gene expression levels for *Serpina3n* and *S100A4* determined by means of microarrays in neural stem cells and differentiated neurons. For (A–D), depicted values represent the mean $\pm$ standard error of the mean. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. EAE = experimental autoimmune encephalomyelitis; NSC = neural stem cell.

Figure 3 Serpina3n Expression Is Restricted to Neurons During EAE

Representative images of Serpina3n expression in neurons (A–B) and astrocytes (C–D) of PBS (controls) or EAE mice at d16 and d50 postimmunization. Tissues were stained for Serpina3n (red), β -III-Tubulin (neuronal marker, green), and DAPI (blue) (A–B). Colocalization of Serpina3n in astrocytes was evaluated by staining for Serpina3n (red), GFAP (astrocyte marker, green), and DAPI (blue) (C–D). EAE = experimental autoimmune encephalomyelitis; GFAP = glial fibrillary acid protein.

figure e-1B), indicating that Serpina3n expression is restricted to neurons during EAE course.

Similar to the pattern observed for Serpina3n, cerebellum slides showed colocalization of S100A4 and β -III-Tubulin in Purkinje cells, as well as neurons from the granular region of EAE animals, whereas no expression was observed in control mice (figure 4A). At d50 pi, S100A4 expression was not only observed in Purkinje cells but also in neurons from the molecular region of the cerebellum of EAE mice compared with the PBS group (figure 4A). Hippocampal CA1 pyramidal neurons clearly showed S100A4 expression in EAE (figure 4B). By contrast, no S100A4 expression was found in neurons from the spinal cord neither in EAE nor in PBS (figure e-1C, [links.lww.com/NXI/A365](https://www.lww.com/NXI/A365)). Double staining for S100A4 and GFAP revealed no major expression of S100A4 in astrocytes in any of the CNS analyzed regions, although occasional

double-positive cells were detected in the hippocampus (figure 4, C and D, figure e-1D).

SERPINA3 Levels Are Increased in CSF From Patients With Progressive forms of MS

To investigate whether selected biomarkers of progression with neuroregenerative potential identified in EAE could also play a role in MS, levels of SERPINA3 and S100A4 were measured in CSF samples from a cohort of patients with MS and neurologic controls. As shown in figure 5A, CSF SERPINA3 and S100A4 levels were significantly increased in the whole group of patients with MS compared with NINCs. Stratification of patients with MS into different clinical forms revealed that the highest CSF SERPINA3 levels were observed in patients with progressive MS, particularly in patients with PPMS, and differences were statistically significant for patients with SPMS and PPMS compared with NINCs, and

Figure 4 S100A4 Expression in CNS Tissue During EAE

Representative images are shown for S100A4 expression in neurons (A–B) and astrocytes (C–D) of PBS (controls) or EAE mice at d16 and d50 post-immunization. Tissues were stained for S100A4 (red), β-III-Tubulin (neuronal marker, green), and DAPI (blue) (A–B). Colocalization of S100A4 in astrocytes was evaluated by staining for S100A4 (red), GFAP (astrocyte marker, green), and DAPI (blue) (C–D). EAE = experimental autoimmune encephalomyelitis; GFAP = glial fibrillary acid protein.

also for patients with PPMS compared with patients with RRMS. In contrast, although CSF S100A4 levels were slightly increased in patients with RRMS, no statistically significant differences were observed across MS groups and NINCs (figure 5B). CSF levels of SERPINA3 and S100A4 did not correlate with EDSS scores at the time of sample collection or the number of gadolinium-enhancing lesions in brain MRI scans performed in proximity to lumbar puncture (data not shown). Similarly, segregation of SERPINA3 and S100A4 CSF levels into high and low according to a cutoff value based on median protein levels did not reveal significant differences between patients with MS with high and low SERPINA3 and S100A4 CSF levels and EDSS scores or the number of contrast-enhancing lesions (data not shown).

CSF SERPINA3 Levels Correlate With CSF NFL Levels in Patients With PPMS

To investigate whether SERPINA3 and S100A4 are biomarkers associated with disease progression or instead may have

neuroprotective roles in MS, we measured CSF levels of NFL in patients with RRMS and PPMS. As shown in figure 6A, CSF levels of NFL and SERPINA3 significantly correlated in the whole group of patients ($r = 0.56$, $p = 0.001$). Of interest, association was driven by the PPMS group inasmuch as statistical significance remained significant only in patients with PPMS following segregation into clinical forms ($r = 0.62$, $p = 0.01$; figure 6A). In contrast, CSF NFL levels did not correlate with CSF S100A4 levels neither in the whole group of patients nor after segregation into RRMS and PPMS clinical forms (figure 6B). These findings point to SERPINA3 as a biomarker associated with disease progression in patients with PPMS.

Discussion

In the present study, we aimed to identify molecules associated with disease progression that may have

Figure 5 Dot Plots Showing Levels of SERPINA3 and S100A4 in CSF Samples of Patients With MS and Controls

As described in Methods, CSF levels of SERPINA3 and S100A4 were determined by ELISA and represented as ng/mL in the whole MS group and NINCs (A) and in patients with MS classified according to the different clinical forms of MS (B). For the sake of clarity, only significant *p* values are shown in the graphs. Number of individuals included in the study is shown in parentheses. One-way analysis of variance followed by multiple comparisons test was performed comparing all groups. ****p* < 0.001, ***p* < 0.01, **p* < 0.05. NINC = noninflammatory neurologic control; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS.

neuroregenerative capacity. To this end, we used gene expression microarrays combined with mass spectrometry analysis to determine which genes and proteins were significantly modulated using a chronic progressive EAE model. Statistical analysis of transcriptional and proteomic data sets from different phases of the disease identified a series of molecules that were upregulated in the chronic progressive phases of EAE. In parallel, these molecules were matched with genes upregulated during the differentiation process from NSCs to neurons. Our results showed a selective increase in gene and protein expression of Serpina3n and S100A4 in the progressive phases of EAE and upregulation of both markers during the differentiation of NSCs to neurons. Serpina3n and S100A4 showed a primarily expression in neurons that, particularly for Serpina3n, was increased in the chronic progressive stages of the disease. These observations in the EAE mouse model were further substantiated for Serpina3n in CSF samples of patients with MS, where SERPINA3 levels, the human ortholog of murine Serpina3n, were increased in patients with the progressive forms of MS (SPMS and PPMS) compared with patients with RRMS and NINCs, postulating Serpina3n/SERPINA3 as a biomarker of disease progression with potential for neuroregeneration.

Gene and protein expression profiling during the course of EAE offers a powerful approach for understanding the molecular changes that characterize the disease, which in turn provide a starting point to identify new molecules involved in the disease. Previous reports applied either transcriptional or proteomic profiling analyses to identify new biomarkers and therapeutic candidates in the context of MS.^{16–18} In those analyses, each feature from each technology (transcripts and proteins) was analyzed independently through univariate statistical methods; however, such analyses ignored relationships between the different features and may have missed crucial biological information. Indeed, biological features act together to control biological systems and signaling pathways. Multivariate approaches, which model features as a set, can offer a more insightful picture of a biological system and complement results obtained from univariate methods. In this regard, omics integration analyses have been developed to analyze large amounts of biological data to identify molecular signatures across multiple data sets.¹⁹

We performed differentially expressed analysis by means of gene expression microarrays and mass spectrometry at different stages of EAE and identified 1,763 common molecules.

Figure 6 Correlation Plots Between CSF SERPINA3 and S100A4 Levels and CSF NFL Levels

Analysis was adjusted by age and graphs show biomarker levels corrected for age. (A) Correlation plots between SERPINA3 and NFL levels in the CSF of patients with MS, patients with PPMS, and patients with RRMS. (B) Correlation plots between S100A4 and NFL levels in the CSF of patients with MS and patients with PPMS and RRMS. MS = includes patients with RRMS and PPMS. NFL = neurofilament light chain; PPMS = primary progressive MS; r = correlation coefficient; RRMS = relapsing-remitting.

To select the molecules showing the highest differences with respect to the entire data set, only 441 genes and 227 proteins were chosen for further bioinformatic analysis that allowed us to focus only on those molecules whose expression was highly modified as the cause of the disease. As we were particularly interested in markers associated with the progressive phase of EAE, we chose 2 bioinformatic analyses, sPLS that investigates gene-protein associations, and a time-course study using a 2-way backward regression to select molecules displaying a pattern of low expression at the initial stages and a clear increase in the progressive phases of the disease. We identified 47 molecules, at the gene and protein level, that met these parameters. Based on these observations, it was reasonable to think that in addition to biomarkers of disease progression, those molecules could also be involved in neurodegeneration. Nevertheless, the mere increase of the levels of certain molecules in the CNS does not prove their pathogenicity in EAE progression, as previously illustrated in different EAE models, in which transcriptional studies identified molecules whose expression was increased in late phases of EAE, such as anti-inflammatory cytokines, growth factors, and oligodendroglial progenitor and neuroglial regeneration markers.^{16,20}

In parallel to our findings on biomarkers upregulated during the chronic progressive phases of EAE, we aimed to explore whether these molecules were also upregulated during neuronal differentiation. To this end, microarray analysis was performed in NSCs differentiated into neurons, resulting in a total of 1,705 genes upregulated in neurons compared to NSCs. Then, we examined the overlapping of identified biomarkers of disease progression, together with molecules upregulated during neuronal differentiation. As a result, we observed that 2 markers, Serpina3n and S100A4, matched with the biomarkers of disease progression previously identified in the EAE model. According to several publications, new functional neurons are constantly generated from NSCs throughout life,²¹ and stem cells with potential to give rise to new neurons reside in many different regions of the mammalian brain.^{22,23} Hence, neurogenesis occurs and persists in the adult brain, where it may contribute to repair and recovery after injury.

Gene expression profiles determined by microarrays revealed that Serpina3n and S100A4 levels are low at early EAE (d8 pi), similar to the control group, but then their expression was

highly increased during disease progression. Likewise, at the protein level, almost no expression for both markers was detected in the inflammatory phase of the disease, indicating that both markers are associated with the progressive phases of EAE. To validate microarray data and to determine whether levels of both markers were induced by inflammation, gene expression experiments by means of qPCR included an additional time point (d16 pi). At the inflammatory phase of the disease (d16 pi), S100A4 levels were significantly increased and remained high at later time points, suggesting that S100A4 could also be a biomarker strongly induced by inflammation. In contrast, expression levels for Serpina3n were increasingly elevated during EAE course and peaked at chronic time points (from d36 onward), findings that point to Serpina3n as a biomarker of disease progression that is not significantly influenced by the inflammatory process. In the context of EAE, no publications have determined the expression levels of Serpina3n during the disease. Conversely, a proteomic approach identified S100A4 among other 3 proteins whose levels were increased during EAE course of relapse-remitting and chronic models of the disease.²⁴

Serpina3n is a secreted peptidase inhibitor of the serpin family,²⁵ whose expression is induced by inflammation and nerve injury.^{26,27} S100A4 is a Ca-binding protein, member of the S100 family of proteins, which plays an important role in tumor progression,²⁸ but is also overexpressed in the damaged human and rodent brain.²⁹ We examined the expression of both markers by immunofluorescence staining in cerebellum, hippocampus, and spinal cord tissues of EAE mice. Our findings showed a strong colocalization of Serpina3n with β -III-Tubulin⁺ neurons in EAE at d16, but not in control mice. In addition, levels of Serpina3n in neurons were increased at d50 pi, showing a correlation with the chronic progressive phases of the disease. On the other hand, no double staining Serpina3n/GFAP was observed neither at d16 nor at d50 pi in EAE and PBS-immunized mice, indicating that Serpina3n is not detected in astrocytes during the EAE course. β -III-Tubulin⁺ neurons were coimmunostained for S100A4 in EAE but not in control animals, whereas no differences were observed between d16 and d50 pi. GFAP⁺ astrocytes showed sporadic expression of S100A4 in the hippocampus. Immunostaining of CNS tissues correlated with gene expression levels observed during EAE, suggesting that S100A4 is strongly induced by inflammation, and Serpina3n is a marker associated with the progressive phases of the disease.

It is important to mention that previous reports have described both Serpina3n and S100A4 as secreted markers of reactive astrocytes^{30,31} that, after brain injury, have been detected in human and mouse neurons.^{32–34} In this context, our present observations suggest that Serpina3n and S100A4 may be produced by reactive astrocytes at early EAE, and later, both markers are secreted to neurons where they may participate in repairing mechanisms. In this regard, a previous

work by Haile et al.³⁵ demonstrated that in vivo treatment with Serpina3n attenuated EAE severity through inhibition of the enzymatic activity of the granzyme B released by activated T cells, which protected from neuronal death. Similarly, in models of brain injury, increased levels of S100A4, either released by reactive astrocytes or after treatment with recombinant S100A4, induced neuroprotection by rescuing neurons from death and stimulating neuronal differentiation.^{29,36}

In the present study, we found that patients with MS showed increased CSF levels of SERPINA3 and S100A4 compared with neurologic controls. Within the MS group, differences were only observed for SERPINA3 in the progressive forms of the disease, SPMS and PPMS, compared with controls and RRMS. Other studies have also demonstrated elevated levels in CSF samples from patients with MS compared with controls.^{37,38} However, in those studies, no comparisons were performed between the different clinical forms. In other pathologic conditions, SERPINA3 was found to be increased in the CSF of patients with Alzheimer disease,^{39,40} and levels correlated with the degree of disease severity. In this context, SERPINA3 has been associated with hyperphosphorylation and tau aggregation leading to neurodegeneration.⁴¹ These results most likely reflect the different roles that Serpina3n/SERPINA3 may be playing depending on the nature of the CNS insult⁴² and certainly underscore the need of future studies to gain more insight into the complex and diverse Serpina3n/SERPINA3 functions. However, the finding of a significant correlation between CSF SERPINA3 levels and CSF NFL levels in patients with PPMS suggests that SERPINA3 is a biomarker associated with disease progression rather than neuroprotection.

Because of the abundance of SERPINA3 in blood, a limitation of the study is the lack of SERPINA3 determinations in serum of patients to rule out a peripheral origin of the increased CSF SERPINA3 levels observed in patients with progressive MS. Although future studies are needed in this direction, the fact that blood-brain barrier permeability is decreased in patients with progressive MS⁴³ makes this possibility unlikely.

In conclusion, we propose Serpina3n/SERPINA3 as a biomarker associated with the progressive forms of MS, particularly PPMS. Additional studies in large cohorts of patients with relapsing and progressive MS are needed to confirm SERPINA3 association with disease progression in patients with PPMS.

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Disclosure

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References

- Compston A, Coles A. Multiple sclerosis. *Lancet* 2008;372:1502–1517.
- Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* 2008;31:247–269.
- Frieze MA, Schattling B, Fugger L. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. *Nat Rev Neurol* 2014;10:225–238.
- Loleit V, Biberacher V, Hemmer B. Current and future therapies targeting the immune system in multiple sclerosis. *Curr Pharm Biotechnol* 2014;15:276–296.
- Fissolo N, Costa C, Nurdinor R, et al. Treatment with MOG-DNA vaccines induces CD4+CD25+FoxP3+ regulatory T cells and up-regulates genes with neuroprotective functions in experimental autoimmune encephalomyelitis. *J Neuroinflammation* 2012;9:139.
- Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004;3:Article3.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 2001;25:402–408.
- Espadas G, Borràs E, Chiva C, Sabidó E. Evaluation of different peptide fragmentation types and mass analyzers in data-dependent methods using an Orbitrap Fusion Lumos Tribrid mass spectrometer. *Proteomics* 2017;17.
- Chiva C, Olivella R, Borràs E, et al. QCloud: a cloud-based quality control system for mass spectrometry-based proteomics laboratories. *PLoS One* 2018;13:e0189209.
- Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999;20:3551–3567.
- Vizcaino JA, Csordas A, Del-Toro N, et al. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res* 2016;44:D447–D456.
- Rohart F, Gautier B, Singh A, Lê Cao K. mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLoS Comput Biol* 13(11): e1005752.
- Conesa A, Nueda MJ, Ferrer A, Talón M. maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. *Bioinformatics* 2006;22:1096–1102.

14. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005;58:840–846.
15. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011;69:292–302.
16. Sevastou I, Pryce G, Baker D, Selwood DL. Characterisation of transcriptional changes in the spinal cord of the progressive experimental autoimmune encephalomyelitis biozzi ABH mouse model by RNA sequencing. *PLoS One* 2016;11:e0157754.
17. Comabella M, Martin R. Genomics in multiple sclerosis—current state and future directions. *J Neuroimmunol* 2007;187:1–8.
18. Liguori M, Qualtieri A, Tortorella C, et al. Proteomic profiling in multiple sclerosis clinical courses reveals potential biomarkers of neurodegeneration. *PLoS One* 2014;9:e103984.
19. Rohart F, Gautier B, Singh A, Lê Cao KA. mixOmics: an R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol* 2017;13:e1005752.
20. Espejo C, Penkowa M, Demestre M, Montalban X, Martínez-Cáceres EM. Time-course expression of CNS inflammatory, neurodegenerative tissue repair markers and metallothioneins during experimental autoimmune encephalomyelitis. *Neuroscience* 2005;132:1135–1149.
21. Lie DC, Song H, Colamarino SA, Ming G, Gage FH. Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol* 2004;44:399–421.
22. Van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in adult hippocampus. *Nature* 2002;415:1000–1034.
23. Picard-Riera N, Nait-Oumesmar B, Baron-Van Evercooren A. Endogenous adult neural stem cells: limits and potential to repair the injured central nervous system. *J Neurosci Res* 2004;76:223–231.
24. Turvey ME, Koudelka T, Comerford I, et al. Quantitative proteome profiling of CNS-infiltrating autoreactive CD4+ cells reveals selective changes during experimental autoimmune encephalomyelitis. *J Proteome Res* 2014;13:3655–3670.
25. Gettins P. Serpin structure, mechanism, and function. *Chem Rev* 2002;102:4751–4803.
26. Takamiya A, Takeda M, Yoshida A, Kiyama H. Inflammation induces serine protease inhibitor 3 expression in the rat pineal gland. *Neuroscience* 2002;113:387–394.
27. Gesase AP, Kiyama H. Peripheral nerve injury induced expression of mRNA for serine protease inhibitor 3 in the rat facial and hypoglossal nuclei but not in the spinal cord. *Ital J Anat Embryol* 2007;112:157–168.
28. Boye K, Maelandsmo GM. S100A4 and metastasis: a small actor playing many roles. *Am J Pathol* 2010;176:S28–S35.
29. Dmytriyeva O, Pankratova S, Owczarek S, et al. The metastasis-promoting S100A4 protein confers neuroprotection in brain injury. *Nat Commun* 2012;3:1197.
30. Zamanian JL, Xu L, Foo LC, et al. Genomic analysis of reactive astrogliosis. *J Neurosci* 2012;32:6391–6410.
31. Aberg F, Kozlova EN. Metastasis-associated mts1 (S100A4) protein in the developing and adult central nervous system. *J Comp Neurol* 2000;424:269–282.
32. Lin X, Antalffy B, Kang D, Orr HT, Zoghbi HY. Polyglutamine expansion downregulates specific neuronal genes before pathologic changes in SCA1. *Nat Neurosci* 2000;2:157–163.
33. Sergi D, Campbell FM, Grant C, et al. SerpinA3N is a novel hypothalamic gene upregulated by a high-fat diet and leptin in mice. *Genes Nutr* 2018;13:28.
34. Kiryushko D, Novitskaya V, Soroka V, et al. Molecular mechanisms of Ca(2+) signaling in neurons induced by the S100A4 protein. *Mol Cell Biol* 2006;26:3625–3638.
35. Haile Y, Carmine-Simmen K, Olechowski C, Kerr B, Bleackley RC, Giuliani F. Granzyme B-inhibitor serpinA3n induces neuroprotection in vitro and in vivo. *J Neuroinflammation* 2015;12:157.
36. Pedersen MV, Köhler LB, Grigorian M, et al. The Mts1/S100A4 protein is a neuroprotectant. *J Neurosci Res* 2004;77:777–786.
37. Häggmark A, Byström S, Ayoglu B, et al. Antibody-based profiling of cerebrospinal fluid within multiple sclerosis. *Proteomics* 2013;13:2256–2267.
38. Ottervald J, Franzén B, Nilsson K, et al. Multiple sclerosis: identification and clinical evaluation of novel CSF biomarkers. *J Proteomics* 2010;73:1117–1132.
39. Nielsen HM, Minthon L, Londo E, et al. Plasma and CSF serpins in Alzheimer disease and dementia with Lewy bodies. *Neurology* 2007;69:1569–1579.
40. DeKosky ST, Ikonovic MD, Wang X, et al. Plasma and cerebrospinal fluid alpha1-antichymotrypsin levels in Alzheimer's disease: correlation with cognitive impairment. *Ann Neurol* 2003;53:81–90.
41. Padmanabhan J, Levy M, Dickson DW, Potter H. Alpha1-antichymotrypsin, an inflammatory protein overexpressed in Alzheimer's disease brain, induces tau phosphorylation in neurons. *Brain* 2006;129:3020–3034.
42. Aslam MS, Yuan L. SerpinA3n: potential drug and challenges, mini review. *J Drug Target* 2020;28:368–378.
43. Correale J, Gaitán MI, Ysraelit MC, Fiol MP. Progressive multiple sclerosis: from pathogenic mechanisms to treatment. *Brain* 2017;140:527–546.

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CSF SERPINA3 Levels Are Elevated in Patients With Progressive MS

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In the article “CSF SERPINA3 Levels Are Elevated in Patients With Progressive MS” by N. Fissolo et al.,¹ Dr. Nicolás Fissolo should be listed as a corresponding author in addition to Dr. Manuel Comabella Lopez. Dr. Fissolo can be reached at nicolas.fissolo@vhir.org. The authors and editorial staff regret the omission.

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