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Pro-inflammatory S100A9 Protein as a Robust Biomarker Differentiating Early Stages of Cognitive Impairment in Alzheimer's Disease

Istvan Horvath,[†] Xueen Jia,[†,‡] Per Johansson,[§,∥] Chao Wang,[†] Roman Moskalenko,[†,⊥] Andreas Steinau,[†] Lars Forsgren,[#] Thomas Wågberg,[‡] Johan Svensson,[∥,▽] Henrik Zetterberg,[¶,○] and Ludmilla A. Morozova-Roche*[,†]

[†]Department of Medical Biochemistry and Biophysics, Umeå University, Umeå 901 87, Sweden

[‡]Department of Physics, Umeå University, Umeå 901 87, Sweden

[§]Department of Neuropsychiatry, Skaraborg Central Hospital, Falköping 521 85, Sweden

[∥]Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg 413 45, Sweden

[⊥]Department of Pathology, Sumy State University, Sumy 40000, Ukraine

[#]Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå 901 87, Sweden

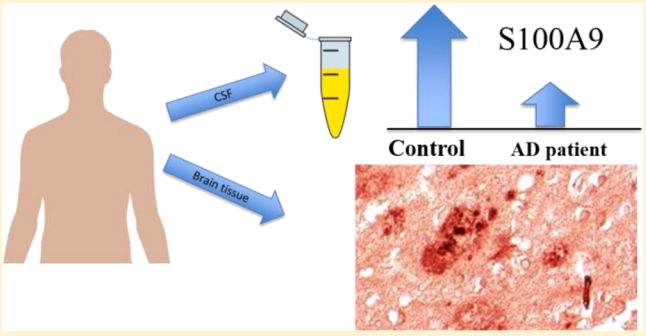
[▽]Department of Endocrinology, Skaraborg Central Hospital, Skövde 541 85, Sweden

[¶]Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, the Sahlgrenska Academy, University of Gothenburg, Mölndal 431 80, Sweden

[○]University College London, Institute of Neurology, Queen Square, London WC1E 6BT, United Kingdom

Supporting Information

ABSTRACT: Pro-inflammatory protein S100A9 was established as a biomarker of dementia progression and compared with others such as Aβ_{1-42} and tau-proteins. CSF samples from 104 stringently diagnosed individuals divided into five subgroups were analyzed, including nondemented controls, stable mild cognitive impairment (SMCI), mild cognitive impairment due to Alzheimer's disease (MCI-AD), Alzheimer's disease (AD), and vascular dementia (VaD) patients. ELISA, dot-blotting, and electrochemical impedance spectroscopy were used as research methods. The S100A9 and Aβ_{1-42} levels correlated with each other: their CSF content decreased already at the SMCI stage and declined further under MCI-AD, AD, and VaD conditions. Immunohistochemical analysis also revealed involvement of both Aβ_{1-42} and S100A9 in the amyloid-neuroinflammatory cascade already during SMCI. Tau proteins were not yet altered in SMCI; however their contents increased during MCI-AD and AD, diagnosing later dementia stages. Thus, four biomarkers together, reflecting different underlying pathological causes, can accurately differentiate dementia progression and also distinguish AD from VaD.



KEYWORDS: *Alzheimer's disease, mild cognitive impairment, cerebrospinal fluid, S100A9, Aβ_{1-42}, biomarkers, amyloid, inflammation*

Detection and quantification of disease-associated proteins in the cerebrospinal fluid (CSF) is increasingly important for the diagnosis, stratification, and consequent treatment of numerous neurodegenerative disorders, including Alzheimer's disease (AD) and vascular dementia (VaD). CSF is a sensitive indicator of hazardous pathological processes in the brain tissues, reflecting the delicate shifts in protein homeostasis.[1] This is particularly important because neurodegenerative syndromes may have potentially different underlying molecular and cellular causes. The detection of corresponding disease-associated proteins in CSF sheds light on the disease pathologies and enables us to distinguish them. Early detection of neurodegenerative pathology is particularly important because disease-modifying treatments have a higher potential to slow disease progression when initiated very early, in the predementia and potentially even presymptomatic phases before amyloid plaques and neurodegeneration become too widespread. This may provide a window of opportunity to limit or reverse these harmful processes. CSF biomarkers may also reflect the cellular and tissue responses to therapeutic treatments and enable us to monitor and guide the progress of therapeutic interventions.

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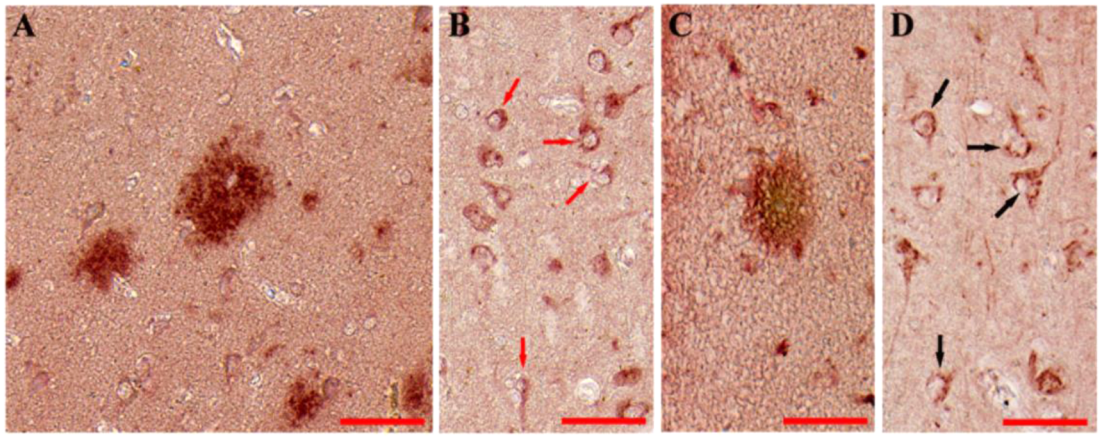

Figure 1. Immunohistochemical analysis of SMCI cerebellum by using $A\beta_{1-42}$ and S100A9 antibodies. Accumulation of $A\beta_{1-42}$ and S100A9 in plaques (A, C) and neurons (B, D). Scale bars are 50 μm.

Here we have conducted extensive studies on 104 CSF samples from nondemented controls and stringently diagnosed patients with different degrees of dementia, progressing from stable mild cognitive impairment (SMCI), to mild cognitive impairment due to AD (MCI-AD), and finally to AD or VaD. We have focused on the detection in CSF of pro-inflammatory S100A9 protein and compared its contents with an established AD biomarker triad including $A\beta_{1-42}$ (a major amyloidogenic peptide associated with AD), total human tau (H-tau), and tau phosphorylated at Thr181 (P-tau) (proteins, reflecting axonal degeneration and increased tau phosphorylation, respectively).[2,3] Indeed, Aβ peptides are viewed as major contributors to the amyloid cascade in AD. The brain tissues affected by AD contain soluble and insoluble amyloid assemblies of Aβ peptides, both of which may constitute an underlying cause of dementia.[4,5] H-tau and P-tau are microtubule-associated proteins expressed in neurons, and P-tau is a major component of abnormal intraneuronal aggregates observed in numerous tauopathies, including AD. To date, apart from Aβ and tau pathologies, inflammation is also considered to be at the core of major degenerative diseases of later life, including AD.[6,7] Even in the absence of specific pathological lesions, inflammatory gene expression increases during aging in humans and also in animal models.[8,9] Thus, inflammation may prove central to therapeutic interventions in neurodegenerative diseases as well as in general antiaging strategies. The role of inflammation in AD is supported by a sharp induction of inflammatory mediators in disease-affected brain tissues.[7] Recently it was found that a missense mutation in *TREM2* renders microglia overactive to brain Aβ pathology and increases risk of AD by 4-fold.[10] Epidemiological and experimental studies demonstrated that non-steroidal anti-inflammatory drugs markedly reduce the age-related prevalence of AD.[11,12] These drugs can slow amyloid deposition by mechanisms that are still not fully understood.

Pro-inflammatory S100 proteins are increasingly recognized as important contributors to inflammation-related neurodegeneration and aging.[9] The abundance of *S100a8* and *S100a9* mRNA was shown to be a robust feature of aging in mammalian tissues, including CNS.[9] Among them S100A9 specifically was implicated in neurodegenerative diseases, that is, significantly increased microglial expression of S100A9 was observed in the temporal cortex of both familial and sporadic AD compared with old and young controls.[13] S100A9 was also found in hippocampal and cortical neurons in AD, where it was colocalized with Aβ peptide.[14] Interestingly, S100A8, another protein from the S100 family, which is able to form a heterocomplex with S100A9, were not found to be involved in the neuroinflammatory cascade.[13,14] We have shown that S100A9 is intrinsically amyloidogenic and *in vitro* it forms cytotoxic amyloid oligomers, protofibrils, and fibrils as readily as $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides.[14] Importantly, S100A9 and Aβ are able to coaggregate with each other both *in vitro* and *in vivo*.[14] *In vitro*, depending on their molar ratio, they form together significantly larger micrometer-scale fibrillar, spherical, and nonstructured aggregates compared with each polypeptide incubated alone.[14] Consequently coaggregation can mitigate the amyloid cytotoxicity of both partners as well as S100A9 pro-inflammatory signaling properties. In the AD brain tissues, both Aβ and S100A9 were found to be colocalized in multiple amyloid plaques, indicating that due to its inherent amyloidogenicity S100A9 can contribute to amyloid plaque formation together with Aβ.[14] These emphasize the role of S100A9 in the AD amyloid−neuroinflammatory cascade and neurodegeneration.[14] Therefore, the comparison of the contents of S100A9, $A\beta_{1-42}$ and other biomarkers in CSF may reflect their involvement in pathological underlying mechanisms of AD and disease progression.

RESULTS AND DISCUSSION

S100A9 was implicated in pathology of advanced AD as discussed above,[14] and here we examined whether S100A9 is involved in the disease pathology already at the SMCI stage. Immunohistochemical analysis with S100A9 and $A\beta_{1-42}$ antibodies of the cerebellum tissues of a patient diagnosed with SMCI showed immunopositive plaques of both $A\beta_{1-42}$ peptide (Figure 1A) and S100A9 (Figure 1C). $A\beta_{1-42}$ and S100A9 were not colocalized, however, forming separate tissue deposits. Strong positive immunostainings with both $A\beta_{1-42}$ (Figure 1B) and S100A9 (Figure 1D) antibodies were detected also in numerous neuronal cells, demonstrating elevated presence of both polypeptides. The staining patterns indicating the colocalization of $A\beta_{1-42}$ and S100A9 were overlapped in some but not in all immunopositive neurons. By contrast, the presence of S100A8 extracellular and intracellular deposits was not detected in the same SMCI tissues (Figure S1A). Immunohistochemical analysis of the nondemented cerebellum using $A\beta_{1-42}$, S100A9, and S100A8 antibodies (Figure S1B−D)

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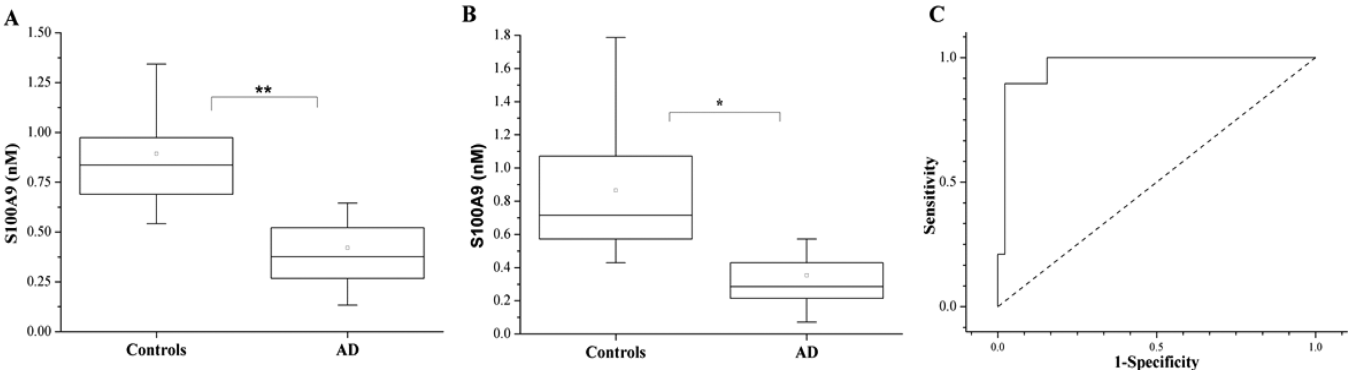

Figure 2. CSF S100A9 as a biomarker for AD. Levels of S1009 in CSF in AD patients and controls determined by dot-blot analysis (A) and EIS (B). Data is presented as box plots. Box-plots include from 25% to 75% of all measurements; central squares indicate the mean value for each group, the line drawn across the box represents the median value, and the whiskers indicate the distribution from 10% to 90%; $^{**}p < 0.001$, $^{*}p < 0.01$. (C) ROC curve based on dot-blot measurements to evaluate S100A9 as a biomarker for AD. Area under the curve is 0.92, 95% confidence interval width is 0.11, $p < 0.001$. Dashed line corresponds to the area under the curve equal to 0.5, indicative that the measured parameter has no diagnostic value.

did not reveal any specific immunostaining. This indicates that S100A9, but not S100A8, plays a role in the amyloid−neuroinflammatory cascade, accumulating both in the precursor plaques and intracellularly already at the SMCI stage. Therefore, in our further studies we have focused on S100A9 as a prospective MCI/AD biomarker linked to its underlying pathology, such as the amyloid−neuroinflammatory cascade.[14] To this end, we have evaluated how CSF changes of S100A9 and the core AD triad, $A\beta_{1-42}$, H-tau, and P-tau, reflect the progression of dementia. The CSF level of S100A9 was evaluated using two independent methods, dot-blot analysis and electrochemical impedance spectroscopy (EIS). Significantly lower S100A9 content was found by both methods in the AD group versus controls (Figure 2A,B). The receiver operating characteristic (ROC) curve analysis also confirmed that S100A9 has an excellent diagnostic potential in distinguishing AD from controls (Figure 2C).

Our next step was to differentiate the CSF content of S100A9 in the subgroups with various degrees of cognitive impairment starting from SMCI and proceeding to MCI-AD and AD. For this purpose, we have used dot-blot analysis, which demonstrated high sensitivity and consistency in evaluation and required a small amount of samples (Figure 3A). We have observed pronounced and statistically significant differences in the S100A9 levels between all demented groups and controls; the corresponding mean values are presented in Table 1. Importantly, the content of S100A9 was decreased significantly already in the SMCI group compared with controls. In the MCI-AD group, the S100A9 levels were decreased further, effectively reaching those characteristic for AD individuals. CSF of both AD and VaD patients were characterized by similar S100A9 levels, though broader distribution of values were observed in the VaD subgroup (Figure 3B). These results were further validated by ELISA measurements performed on the pooled CSF samples for each subgroup (Figure 3B). We observed effectively the same trend within the error of measurements as with the dot-blot analysis.

Moreover, the changes of both $A\beta_{1-42}$ and S100A9 levels followed the same trend throughout dementia stages (Figure 3). Similar to S100A9 measurements, the SMCI group showed intermediate values of $A\beta_{1-42}$ content compared with those for nondemented controls and AD patients (Figure 3C, Table 1). The MCI-AD, AD, and VaD subgroups were characterized by a similar range of $A\beta_{1-42}$ with the higher spread of values for VaD, resembling in this regard the corresponding observations for S100A9 (Figure 3B,C). The covariate analysis also demonstrated a correlation between the content of $A\beta_{1-42}$ and S100A9 across the whole CSF sample set with a correlation coefficient of 0.38, $p < 0.05$ (Figure 3F). The decrease of $A\beta_{1-42}$ was reported previously for a similar AD cohort.[3] The concomitant decrease of S100A9 and $A\beta_{1-42}$ in CSF during progressing dementia starting from SMCI could be related to their joint amyloid aggregation in the affected brain tissues,[14] which ultimately lead to their depletion from CSF. Because S100A9 is a pro-inflammatory cytokine, our findings indicate also that inflammatory processes play critical role in the development of cognitive impairment, starting from early stages such as SMCI.

Using Western blot analysis with S100A9 antibodies, we examined whether S100A9 is present in CSF in a low molecular weight or aggregated form (Figure S2). The S100A9 species in pooled CSF samples from the control, SMCI, AD, and VaD subgroups were compared with freshly dissolved recombinant S100A9 (Mw 13.2 kDa). Recombinant S100A9 was characterized by two bands corresponding by their molecular weight to monomer and dimer, respectively. By comparison, in the CSF samples S100A9 was present as a dimer, and this dimer displayed resistance to both SDS and reducing agent treatments. Because in AD CSF the amount of S100A9 was reduced compared with controls, the intensity of its Western blot band was at the limit of detection. The homodimer is the most common form of S100A9 in biological samples,[14] unless it forms heterodimer or heterotetramer with S100A8.[15]

Interestingly, the level of another inflammatory protein, S100A8, did not vary in CSF of all studied subgroups (Figure S3). Though S100A8 and S100A9 form heterocomplexes under various inflammatory conditions within the body,[15,16] our results imply that they can exist separately and fulfill independent functions under neurodegenerative conditions. While S100A9 was shown to be a critical factor in the inflammation-associated neurodegeneration cascade,[14] S100A8 is not apparently involved in this process.

By contrast, the CSF levels of H-tau and P-tau did not change in SMCI and showed an opposite trend compared with S100A9 and $A\beta_{1-42}$ at later stages of the dementia progression, that is, their levels increased significantly in the MCI-AD and AD subgroups but not in the subgroup with VaD (Figure 3C,D). In AD, the pathological accumulation of tau appears to

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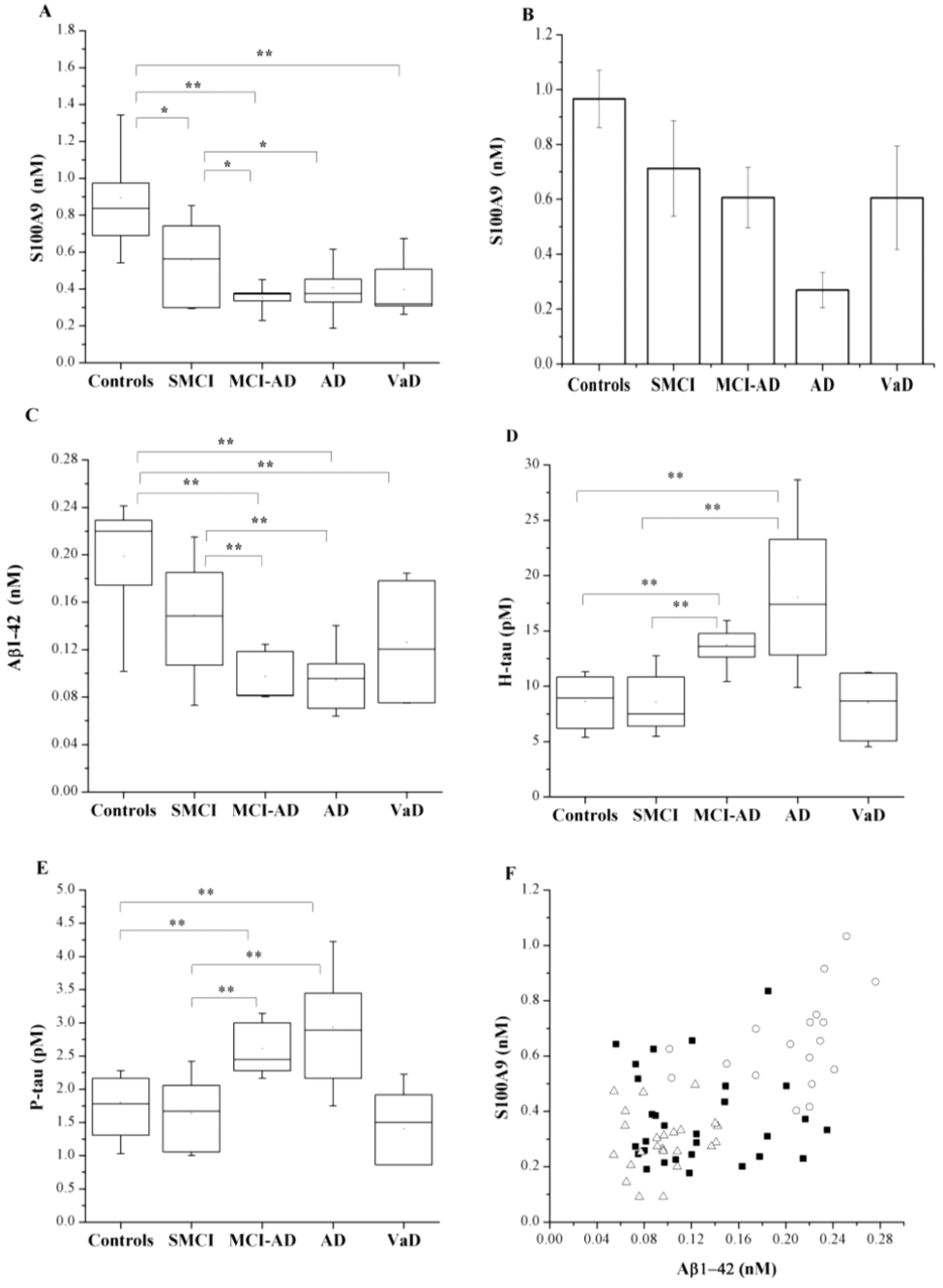

Figure 3. Measurements of CSF biomarkers during dementia progression from SMCI to MCI-AD, AD, and VaD. Levels of S100A9 measured by dot-blot analysis in individual samples (A) and by ELISA in the pooled samples (B). ELISA measurements of $A\beta_{1-42}$ (C), H-tau (D), and P-tau (E) contents in individual samples. Data is presented as box plots as described in Figure 1; $^{**}p < 0.0001$, $^{*}p < 0.005$. ELISA results for the pooled samples corresponding to each subgroup are presented as columns with error bars corresponding to standard deviations (SD). (F) Correlation between the levels of $A\beta_{1-42}$ and S100A9 across all studied samples. The values corresponding to healthy controls are shown by open circles, those to AD patients by open triangles, and those to SMCI, MCI-AD, and VaD by black squares.

Table 1. Mean Levels of Studied Biomarkers in CSF of Demented Patients and Controls

groups	S100A9 (SD), nM	$A\beta_{1-42}$ (SD), nM	H-tau (SD), pM	P-tau (SD), pM
controls	0.87 (0.29)	0.21 (0.06)	8.6 (2.5)	1.8 (0.6)
SMCI	0.55 (0.25)	0.16 (0.06)	8.6 (2.9)	1.6 (0.6)
MCI-AD	0.34 (0.08)	0.10 (0.02)	13.7 (1.9)	2.6 (0.4)
AD	0.40 (0.18)	0.10 (0.03)	18.1 (6.8)	2.9 (1.0)
VaD	0.39 (0.14)	0.14 (0.05)	8.5 (2.8)	1.4 (0.6)

be a downstream effect of $A\beta$ aggregation, though the relationship between $A\beta$ and tau pathologies can be more complex.[17,18] Intracellular tau aggregates are highly toxic leading to increasing neuronal cell loss, which may be reflected in the rise of their CSF level at the later dementia stages such as MCI-AD and AD. Summarizing, S100A9, $A\beta_{1-42}$, and tau proteins taken together can be used as highly potent biomarkers able to differentiate accurately various phases of dementia starting as early as SMCI. These biomarkers, reflecting different underlying pathological causes, can map

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dementia progression with a high accuracy and also potentially differentiate AD from VaD. It is important to note that none of these biomarkers, including the most studied Aβ peptide reflecting cerebral β-amyloidosis in MCI and AD, will be used in isolation to make a diagnosis of AD dementia, MCI, or "normal aging." Each biomarker is used to assess the corresponding underlying pathophysiology of subjects. Recent advances in research diagnostic criteria for AD provided better definition of its clinical phenotypes and also integrated biomarkers into the diagnostic process, covering all stages of the disease.[19−21] These criteria provided a new conceptual framework stating that AD could be diagnosed based on the combination of a clinical phenotype of episodic memory disturbances and one or more abnormal AD biomarkers including CSF biomarkers (Aβ and tau proteins), volumetric MRI, and amyloid PET. Here we suggest that S100A9, as the CSF biomarker of amyloid−neuroinflammatory cascade, should also enter this arena. Methodologies and diagnostic procedures described here can be effectively implemented in clinical practice to date. Our finding of S100A9 diagnostic potential indicates also that the neuroinflammatory proteins should be more widely explored for diagnostic purposes and especially in the early or even preclinical stages of neurodegeneration.

METHODS

Human Subjects. CSF samples from 104 individuals were analyzed. A selected cohort of 84 participants with different stages of dementia and nondemented controls was recruited at the Falköping Memory Clinic. They were all stringently diagnosed as described previously.[3] In addition, 20 nondemented participants treated as controls were recruited from the Umeå University Hospital. The controls were patients whose CSF samples were collected for analysis of different disorders other than neurodegenerative diseases, including headache, polyneuropathy, neurogenic pain, dizziness, and fatigue. The characteristics of human participants are presented in Table 2.

Table 2. Group Characteristics of Demented Patients and Controls

groups	number of participants	average age	age range	male/female (%)
controls	40	73.9	65−81	50/50
SMCI	11	71.3	65−77	39/61
MCI-AD	6	72.7	69−80	57/43
AD	40	74.0	66−80	48/52
VaD	7	74.0	67−78	71/29

Tissue Samples. The brain tissues were collected at the Medical Institute, Sumy State University, Ukraine. The cerebellum tissues from a SMCI patient, a 43 y.o. male deceased from liver cirrhosis, and from a neurologically healthy individual, a 48 y.o. female deceased from Wegener's granuloma, were analyzed. SMCI was diagnosed based on the respective clinical criteria and cognitive performances examined in a series of neuropsychological tests including mini-mental state examination.[22] All tissues were paraffin-embedded and microtome-sectioned to 4 μm thick slices.

CSF Collection. Lumbar puncture for collection of CSF samples was performed following the established procedure as described previously.[23] Each CSF sample was centrifuged at 2000*g* for 10 min; supernatants were frozen in 0.5 mL aliquots in polypropylene tubes and stored at −80 °C.

Immunohistochemistry. Immunohistochemistry on the tissue sections was performed as described previously.[14] The following antibodies were used: $A\beta_{1-42}$ (mouse monoclonal, ab11132, 1 in 200, Abcam), S100A8 (goat polyclonal, sc-8112, 1 in 100, St Cruz Biotechnology), and S100A9 (rabbit polyclonal, sc-20173, 1 in 100, St Cruz Biotechnology). The tissues were scanned by a Panoramic SCAN slide scanner 250 (3D Histech).

Dot-Blot Analysis. We used as little as 3 μL of CSF for analysis, taking all aliquots in triplicate. The contents of S100A8 and S100A9 were evaluated by dot-blot assay involving primary antibodies against human S100A8 (goat polyclonal, sc-8112, 1 in 2000, St Cruz Biotechnology) and human S100A9 (mouse monoclonal, MAB5578, 1 in 2000, R&D Biosciences), respectively.

EIS. Twenty AD and nine control CSF samples were subjected to EIS measurements. CSF volumes of 5 μL were analyzed in triplicate for each sample.

ELISA. CSF $A\beta_{1-42}$, H-tau, and P-tau concentrations were measured by Innotest ELISA kits (Fujirebio) as previously described.[23] The content of S100A9 was assessed by a newly developed sandwich ELISA.

Statistical Analysis. OriginPro 8.0 software was used for the statistical analysis. The comparison between groups was carried out by using a two-tailed Student's *t* test for unequal variance. Spearman's coefficient was used to evaluate the correlation between two variables. The level of significance was set at $p < 0.05$.

Ethical Approval. The study was conducted according to Declaration of Helsinki principles and approved by the medical ethics committees of University of Gothenburg, Umeå University, Sweden, and Medical Institute, Sumy State University, Ukraine. All subjects gave their written informed consent prior investigation. Participants were identified by number, not by name.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00265.

Detailed description of methods, including Western blot analysis, ELISA, and EIS methods, figures of immunohistochemistry of the SMCI cerebellum with S100A8 antibodies and immunohistochemistry of the nondemented cerebellum using $A\beta_{1-42}$, S100A9, and S100A8 antibodies, Western blot analysis with S100A9 antibodies of the CSF samples versus freshly dissolved recombinant S100A9, measurements of S100A8 levels in all studied subgroups, and calibration curves for S100A9 measurements by dot-blot (PDF)

AUTHOR INFORMATION

Corresponding Author

*Ludmilla A. Morozova-Roche. E-mail: ludmilla.morozova-roche@umu.se.

Author Contributions

I.H., X.J., A.S., R.M., and C.W. performed the experiments. P.J., J.S., H.Z., and L.F. collected samples and analyzed patient's data, T.W., H.Z., and L.A.M.-R. designed the project and analyzed experimental data. I.H. and L.A.M.-R. prepared the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Alzheimer's disease; EIS, electrochemical impedance spectroscopy; H-tau, total human tau; MCI-AD, mild cognitive impairment due to Alzheimer's disease; ROC, receiver operating characteristic; SMCI, stable mild cognitive impairment; P-tau, phosphorylated tau; VaD, vascular dementia

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