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Microstructural and functional brain abnormalities in multiple sclerosis predicted by osteopontin and neurofilament light

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ABSTRACT:

Background: Osteopontin (OPN) is a proinflammatory biomarker, and neurofilament light chain (NFL) levels reflect axonal damage. Resting-state functional MRI (rs-fMRI) defines brain networks during wakeful rest.

Objective: To examine, if levels of OPN and NFL are associated on the long term with (i) lesion evolution, (ii) changes in normal-appearing white matter (NAWM) microstructure and (iii) functional connectivity in multiple sclerosis (MS).

Methods: Concentration of NFL and OPN in the blood and CSF were related to MRI findings 10.3±2.8 years later in 53 patients with MS. NFL was examined by Simoa method, OPN by ELISA. Lesion volume in the brain and cervical spinal cord was examined by 3D FLAIR images. Voxel-wise images of fractional anisotropy (FA), axial diffusivity (AD), mean diffusivity (MD), and radial diffusivity (RD) were examined by tract-based spatial statistics corrected for gender, age and lesion volume. Metabolites were examined by single-voxel MR-spectroscopy in the NAWM. Fifty-five default mode network connections were examined by rs-fMRI corrected for gender, age, MS subtype and current therapy as covariates.

Results: While NFL in paired serum and CSF positively correlated (p=0.019), there was no correlation between serum and CSF OPN. Higher OPN levels in the CSF but not in the serum showed association with increased brain WM lesion volume (p=0.009) in 10.3±2.8 years. Higher OPN in the CSF was associated with reduced FA, increased MD, and reduced RD in different NAWM areas 10.3±2.8 years later. Higher OPN in the serum and CSF were associated with increased connectivity strength between the medial prefrontal cortex (MPFC) and other regions except with inferior parietal lobule. NFL in the CSF and in the serum was associated with decreased connectivity strength except for ventral MPFC-hippocampal formation. Neither serum OPN nor NFL at the time of the MRI were associated with functional connectivity changes.

Conclusion: While serum NFL levels reflects CNS production, OPN in serum and CSF may have different cellular sources. OPN within the CSF but not in the serum may forecast development of lesions and microstructural abnormalities in 10 years, indicating the detrimental role of CNS inflammation on the long-term. Although both OPN and NFL in the CSF were associated with functional connectivity changes in 10 years, NFL was associated with decreased strength possibly indicating general axonal loss. In contrast, the positive association of OPN

levels in the CSF with increased connectivity strength in 10 years may point to adaptive re-organization due to inflammatory WM lesions and microstructural changes.

1. INTRODUCTION

MRI is a sensitive tool for detecting tissue abnormalities in the central nervous system (CNS) related to multiple sclerosis (MS). The brain-related wide-spread aspects include focal lesions of the white matter (WM) and gray matter (GM), diffuse microstructural abnormalities of the WM, irreversible tissue loss, metabolic changes of the normal-appearing white matter (NAWM), and functional abnormalities measured by functional MRI (fMRI) (Poloni et al., 2011; Rocca et al., 2020).

The functional connectivity within the brain is defined as the coherence in the activity between different cerebral regions under a specific task or in rest. Connectivity analysis based on resting-state functional MRI (rs-fMRI) shows several patterns, defining resting state brain networks (Rosazza and Minati, 2011). One such network is the default mode network (DMN). The main components of the DMN are the posterior cingulate cortex/precuneus, medial prefrontal, and inferior parietal cortices (Broyd et al., 2009). Focal damage to the WM and GM in MS patients is likely to disrupt brain network connections within cortical and subcortical networks (Basile et al., 2014). Default-mode network was reported to be affected by MS pathology both structurally and functionally (Rocca et al., 2010). DMN disruption in MS was associated with a reduced cognitive performance (Rocca et al., 2010), depression (Bonavita et al., 2017), and fatigue (Høgestøl et al., 2019).

Osteopontin (OPN) produced by various immune cells promotes pro-inflammatory cytokine production of Th1 cells, regulates Th17 responses, and inhibits IL-10 production and Th2 polarization, all indicated in the pathogenesis of MS (Rittling and Singh, 2015). Enhanced OPN expression was found in active MS lesions, in the WM surrounding the lesions, and OPN levels in blood and cerebrospinal fluid (CSF) of MS patients are increased (Agah et al., 2018; Chabas et al., 2001).

Neurofilament light chain (NFL) is an emerging biomarker in MS: increased CSF concentrations are reflected by elevated levels in the blood if measured by a sensitive method, Simoa (Kuhle et al., 2016). NFL concentration is elevated at the time of diagnosis and increases during relapse and when new lesions are detected by MRI (Disanto et al., 2017a). Treatment with disease modifying therapies (DMTs) reduces NFL in the CSF and blood (Kuhle et al., 2019; Sejbaek et al., 2019).

In this study, we examined, if OPN and NFL in the serum and CSF predict microstructural, metabolic, and functional abnormalities of the CNS in 10 years measured by MRI; and if the serum level of these biomarkers correlates with such MRI changes in a cross-sectional study.

2. MATERIALS AND METHODS

2.1 Subjects and samples. Fifty-three patients (34 females, age range 20 – 68 years) diagnosed with MS according to the 2017 modified McDonald diagnostic criteria (Thompson et al., 2018) participated in the study (**Table 1**).

CSF (n=33) and serum (n=22) samples were collected 10.3±2.8 years before MRI, and aliquots were kept at −80°C. A new serum sample was taken at the day of MRI from each patient (n=53).

The study was conducted according to the World Medical Association Declaration of Helsinki and approved by the Regional Ethical Committee of the University of Pecs (7068-PTE 2018). All patients signed written informed consent.

***2.2 Measurement of osteopontin in serum and CSF*.** We used a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (Human Osteopontin DuoSet ELISA, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Serum samples were diluted 1:25 and CSF samples were diluted 1:100 for OPN analysis. All samples were run in duplicates. Optical density was detected at 450 nm using an iEMS MF microphotometer (Thermo Labsystem, Beverly MA, USA). The detection limit for the assay was 62.5 pg/mL.

***2.3 Measurement of NFL in serum and CSF*.** A commercially available NFL kit (Quanterix©, Lexington, MA, USA) for the Single Molecule Array (Simoa) HD-1 Analyzer (Quanterix) was used to quantify NFL light chain in the serum and CSF according to the manufacturer's procedure. In-house serum and CSF pools were used as internal controls and included in each assay for evaluating assay performance. The total coefficient of variation was <12%. Lower limit of detection was 0.038 pg/mL and lower limit of quantification was 0.174pg/mL.

2.4 Magnetic resonance imaging. All subjects were scanned using the same 3T MRI scanner (MAGNETOM Prisma^{Fit}, Siemens Healthineers, Erlangen, Germany). The prospective MRI study protocol included the following sequences: 3D T1 magnetization-prepared rapid

acquisition with gradient echo (MPRAGE), 3D fluid-attenuated inversion recovery (FLAIR), diffusion tensor imaging (DTI), single voxel Point RESolved Spectroscopy (MRS), phase-sensitive inversion recovery (PSIR) imaging of the cervical spine region and resting-state functional MRI (rs-fMRI) with field mapping to reduce image distortions due to B0 inhomogeneities. Sequence parameters are detailed in the Supplementary Materials.

2.5 Tract-Based Spatial Statistics (TBSS) analysis. Voxel-wise images of fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) were calculated using FMRIB's diffusion toolbox by fitting a diffusion tensor model to the pre-processed diffusion data at each brain voxel (Smith et al., 2004). Voxel-wise statistical analyses of diffusion data were carried out using TBSS v1.2 (Smith et al., 2006). The analysis steps of DTI pre-processing and TBSS are detailed in the Supplementary Materials.

2.6 Segmentation of cerebral white matter and cervical spine lesions. Cerebral white matter lesions were segmented on 3D FLAIR images using the lesion prediction algorithm (Schmidt, 2017) as implemented in the LST toolbox version 3.0.0 (Lesion Segmentation Toolbox, www.statistical-modelling.de/lst.html) for SPM (SPM12). Cervical spine lesions were manually segmented on sagittal PSIR images using 3DSlicer (4.10.2 r28257). Level tracing option was used within the editor and the resulting labels were manually corrected. Label statistics were used to export the number of lesions and the volume of each lesion for statistical evaluation.

2.7 rs-fMRI evaluation. rs-fMRI data were pre-processed using DPARSF 5.0, part of DPABI (V4.3_200401, http://rfmri.org/dpabi) (Yan et al., 2016) and SPM 12. Functional connectivity was automatically calculated between the 11 regions of interest (ROIs) of the Default Mode network (DMN) based on the Andrews-Hannah DMN atlas (Andrews-Hanna et al., 2010) along with the amplitude of low-frequency fluctuation (ALFF) maps for all subjects. The z-standardized functional connectivity values between the predefined ROIs were exported for further statistical analyses. Fifty-five connections were examined altogether ($\frac{n*(n-1)}{2}$). See **Table 2** for the names, abbreviations, and MNI coordinates of DMN ROIs and Supplementary Materials for further details on evaluation.

2.8 Single-voxel MR spectroscopy. Water-scaled spectroscopy data were evaluated using

LCModel Version 6.2 (Provencher, 1993). The absolute metabolite concentrations of total N-Acetylaspartate (tNAA), total Choline (tCho), total Creatine (tCr), and myo-inositol (Ins) were calculated. Further details are discussed in Supplementary Materials.

2.9 Statistical analysis. For TBSS and ALFF analyses, voxel-wise statistics were performed using a permutation-based non-parametric analysis.

All other statistical analyses were performed using SPSS (IBM Corp., Version 25.0. Armonk, NY). The statistical analysis is detailed in the Supplementary materials.

3. RESULTS

3.1 Patient demographics

During the time between CSF collection and MRI, EDSS has significantly increased ($p=0.046$, Wilcoxon Signed Rank Test) in the patient population, and 44% of patients showed disability progression. The mean number of relapses during the same period, was 3.72±2.49. At the time of MRI, 66% of the patients had relapsing-remitting MS (RRMS), 21% secondary progressive MS (SPMS), and 13% primary progressive MS, but no patients had clinical and/or MRI activity.

3.2 No correlation between CSF and serum OPN concentrations in contrast to NFL

We found correlation between the median NFL level in the CSF (1406 pg/mL, 447-3163) and the paired serum (17.85 pg/mL, 9.4-28.8) (Spearman's $\rho=0.576$, $p=0.019$). Median NFL level in serum at the time of MRI was 9.3 (7.8-16.1) pg/mL.

The median OPN level in the CSF (246.36 ng/mL, 164.5-439.5), and paired serum (12.52 ng/mL, 6.8-16.7) showed no correlation. Median OPN serum level at the time of MRI was 9.8 (6.2-18.8) ng/mL.

3.3 Association of OPN with lesion volumes in brain WM but not in the cervical spinal cord

The LST segmented median total lesion volume in the cerebral white matter was 2774 mm^3 (701.75-7713.25). NFL concentrations in CSF and paired serum before MRI, and in serum at the time of MRI did not show significant association with the LST segmented total lesion volume. Higher baseline CSF OPN levels were associated with increased total brain WM lesion volume over a mean of 10 years ($p=0.009$, $t= 2.877$).

The median segmented lesion volume in the cervical spine was 300 mm^3 (88-652), and 57% of patients had multiple lesions. Neither OPN, nor NFL showed any association with the number of lesions, the total lesion volume, or the number of lesions larger than 300 mm^3.

3.4. Higher OPN but not NFL in the CSF predicted microstructural changes in the NAWM

Gender, age, and total cerebral lesion volume were included as nuisance variables. CSF OPN levels were significantly associated with microstructural alterations in the NAWM 10.3±2.8 years later: higher levels were associated with reduced FA, increased MD, and reduced RD affecting left superior and inferior longitudinal fasciculi, external capsule, forceps minor (genu of corpus callosum), anterior corona radiata, and to a smaller extent the right inferior longitudinal fasciculus and corona radiata (**Figure 1)**. OPN levels in the sera showed no association with the microstructure of the WM skeleton.

Baseline serum/CSF NfL levels were not correlated with alterations in the NAWM found at 10.3±2.8 years later, neither with the serum at the time of the MRI.

3.5 Trend of association between CSF OPN and single-voxel spectroscopy

OPN measured from CSF obtained 10.3±2.8 years earlier was associated with tCr (t=2.578, p=0.018) and Ins (t=2.796, p=0.0129) concentrations, but none survived multiple comparisons correction. tCho was strongly associated with DMT applied (**Figure 2**).

OPN and NFL levels in the sera obtained 10.3±2.8 years earlier or at the time of MRI, as well as CSF NFL showed no association with metabolic changes in the NAWM voxel.

3.6 OPN and NFL are associated with functional connectivity state after 10 years

Permutation-based non-parametric tests showed no significant association between ALFF and soluble biomarkers, irrespective of sample type and time of collection.

However functional connectivity between the 11 default mode network nodes showed several associations with OPN and NFL after correcting for age, gender, anxiety, depression, disease type and current DMT.

Baseline CSF NfL concentration was associated with functional connectivity strength at year 10.3±2.8 of follow-up regarding the following connections: PHC-pIPL (t=-3.611, p=0.002), aMPFC-pIPL (t=-2.483, p=0.025), vMPFC-HF (t=2.413, p=0.028), and TempP-TPJ (t=-2.138, p=0.048) (**Figure 3A, Table 3**). NFL in the paired serum was associated with functional

connectivity between vMPFC-pIPL (t=-3.831, p=0.004). Serum NFL collected at the time of MRI showed no significant associations.

OPN measured from CSF 10.3±2.8 years before MRI showed association with TempP-vMPFC functional connectivity (t=2.435, p=0.026), while serum OPN levels measured in paired serum showed numerous associations with functional connectivity strengths between the nodes of DMN (**Figure 3B, Table 3**). Serum OPN levels at the time of the MRI showed no significant associations.

Functional connectivity between vMPFC-pIPL and vMPFC-HF were related to both OPN and NFL concentrations measured 10.3±2.8 years before MRI (**Table 3**).

3.7 MRI and biomarker outcomes are associated with EDSS and EDSS changes

Baseline EDSS was significantly associated to lesion load (volume) assessed on FLAIR images (p=0.05) and cervical spine lesion volume assessed on PSIR images (p=0.016), controlling for age, gender and total intracranial volume. EDSS change showed no association with either volumetric measures or lesion load.

EDSS baseline also showed significant association with the following connection strengths aMPFC-TempP (p=0.025), PCC-TempP (p=0.046), and vMPFC-PHC (p=0.003), adjusted for age, gender, current DMTs, anxiety, and depression. EDSS change was not associated with functional connectivities.

Neither EDSS baseline, nor EDSS change showed any significant associations with white matter microstructure after correcting for age and gender. However, EDSS baseline showed nearly significant associations with mean diffusivity (MD) in the left forceps major (p-values between 0.052-0.06).

Neither EDSS at the time of MRI nor EDSS change showed significant correlation with baseline NfL in the serum and in the CSF, and with baseline OPN in the serum and in the CSF, respectively.

4. DISCUSSION

OPN is regarded as a proinflammatory biomarker produced by both peripheral immune cells and CNS resident cells (Rittling and Singh, 2015)·(Niino and Kikuchi, 2011)·(Hammond et al., 2019; Neumann et al., 2014a). NFL is primarily a marker of axonal destruction in both neuroinflammatory and neurodegenerative conditions, and its CSF level reflects acute neuronal and axonal damage in MS (Khalil et al., 2018). Our study controlled for age, sex, MS subtype, depression, anxiety and treatments examined the so far unaddressed questions of OPN and NFL

association with (i) microstructural and (ii) metabolic changes in the NAWM, and (iv) functional connectivity in 10 years.

Our results indicate that OPN and NFL may be related to different aspects of developing MS pathology. OPN measured from CSF was associated with extensive changes in NAWM microstructure, and changes in functional connectivity at year 10.3±2.8 of follow-up. NFL on the other hand showed no such association with NAWM microstructural changes, albeit showed a strong association with the functional connectivity of two main DMN nodes, the MPFC and the pIPL. Thus, while CNS inflammation in MS contributes to lesion evolution and extensive abnormalities in the NAWM in a decade associated with changes in functional connectivity, the inflammation-related axonal damage affects global functional changes on the long term.

The association of OPN levels in the CSF with increased lesion volume in 10 years is not surprising, as OPN produced by both infiltrating peripheral lymphocytes and CNS resident cells may contribute to lesion evolution. Elevation of CSF and peripheral blood OPN levels have been detected in RRMS and SPMS, and higher OPN levels in CSF were measured in patients with active MS compared to patients with stable disease (Agah et al., 2018). Interferon beta, a platform therapy in MS regulates OPN expression (Chen et al., 2009). We also found that high levels of OPN in the CSF were associated with reduced FA, increased MD and reduced RD at different CNS sites in 10 years indicating that inflammation also affects microstructural changes in the NAWM on the long term. Correlation of OPN levels with NAWM changes was also supported by the observed trend of association between OPN levels in the CSF and metabolic changes (tCr and Ins) in an NAWM voxel. Currently applied DMT was a highly significant predictor in the statistical models used to track metabolic changes. In a clinical trial of patients with progressive MS, natalizumab that prevents the migration of inflammatory lymphocytes reduced the level of OPN (Christensen et al., 2014). Besides peripheral lymphocytes, OPN expressed by microglia and reactive astrocytes in the NAWM distant from MS lesions (Chabas et al., 2001; Neumann et al., 2014b) may contribute to such microstructural abnormalities. In the insulted brain, OPN recruits microglia, macrophages and astrocytes, modulating inflammatory responses and attenuating secondary neurodegeneration (Rabenstein et al., 2016; Riew et al., 2019). Expression of the OPN gene *SPP1* is especially high in activated microglia (Hammond et al., 2019). OPN promotes progression and relapse in MS by enhancing the survival of activated T cells (Hur et al., 2007).

NFL concentration in the CSF was associated with reduced functional connectivity between PHC-pIPL and aMPFC-pIPL, while increased functional connectivity between the vMPFC and hippocampus. Serum OPN levels 10.3±2.8 years before MRI also correlated positively with the

vMPFC-hippocampus functional connectivity strength. Two more general observations can be made based on Table 3. First, neither NFL, nor OPN measured from serum at the day of MRI showed any significant association with the examined functional connectivity strengths. This may indicate that such changes need time to develop, and although effect of inflammation on network functioning is not immediate, it has long-term consequences. And second, while NFL concentration showed mainly negative associations with functional connectivity strength between DMN nodes (except for vMPFC-HF), OPN concentrations showed mainly positive associations (except for vMPFC-pIPL). The negative associations related to NFL may reflect the general loss of axons and connection in the brain, while the positive associations of OPN may be related to reorganization due to the inflammatory damage.

Associations between the functional connectivity of DMN and the biomarker concentrations measured from samples collected approximately a decade ago point towards the anterior DMN, especially the MPFC. The functional connectivity of the MPFC showed characteristic associations with OPN or NFL concentrations. Except for the MPFC-pIPL connections, all other MPFC related connections showed a positive association with the measured biomarker concentrations. This indicates that 10 years after the sample collection, patients with higher OPN or NFL concentrations showed stronger functional connectivities between MPFC and other regions (hippocampus, posterior cingulum, inferior parietal lobule, and retrosplenial cortex). This finding can be partially explained by plasticity, which allows an adaptive and effective reorganization to limit impairment. The recruitment of MPFC is a form of adaptive brain plasticity in MS to compensate for relative deficits in information processing. Indeed, MS patients had significantly larger activation in the MPFC compared to healthy controls in Stroop fMRI test, although performance was not different (Parry et al., 2003). The neurobiological background behind this adaptive change is probably driven by the ‘unmasking’ of latent pathways (Parry et al., 2003). We may speculate that higher OPN and NFL concentrations predict worse outcome and enhanced progression on the long run causing higher information processing deficiency, resulting in enhanced activation and more effective functional connectivity of the MPFC to alleviate the deficit. OPN may be associated with WM damage (Selvaraju et al., 2004), which is supported by our data: CSF OPN levels were related to a wide-spread alterations in the NAWM of the left superior and inferior longitudinal fasciculi, external capsule, forceps minor (genu of corpus callosum) and anterior corona radiata, indicating myelin loss.

Although we found correlation between NFL levels in blood and CSF similarly to recent studies (Disanto et al., 2017b; Sejbaek et al., 2019), OPN levels in the two compartments did

not correlate. While NFL in the serum reflects production in the CNS, concentration of OPN in the serum indicates both peripheral and possibly CNS sources. Since we did not find association between serum OPN and microstructural abnormalities, our data may suggest that OPN produced within the CNS/CSF by infiltrating lymphocytes and resident cells may be important in the development of such alterations.

In conclusion, our data indicate that both OPN and NFL levels in the CSF predict changes in functional connectivity in 10 years, while OPN also predict microstructural abnormalities in the NAWM. These data add additional layers to the predictive role of NFL in MS outcome measures and strengthen the early introduction of DMTs in order to prevent the long-term effect of inflammation.

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6. DECLARATION OF CONFLICTING INTERESTS

The Author(s) declare(s) that there is no conflict of interest.

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Figure legends

Figure 1. Tract-based spatial statistics analysis of the white matter in patients with multiple sclerosis

Upper table (A): Areas with significant associations (corrected $p<0.05$) between CSF NFL levels and fractional anisotropy (FA) and mean diffusivity (MD) values measured ~10 years later are shown in yellow and blue, respectively. Overlapping areas (FA+MD) are depicted in green. A lesion mask (red) is overlaid on top of all other layers to mask associations within

multiple sclerosis (MS) lesions. Lower table (B): Areas with significant associations (corrected $p<0.05$) between CSF NFL levels and radial diffusivity (RD) values measured ~10 years later are shown in blue. Overlapping areas (FA+MD+RD) are depicted in green. A lesion mask (red) is overlaid on top of all other layers to mask associations within MS lesions.

Figure 2. Effect of therapy on total Choline levels measured in multiple sclerosis patients
Single-voxel MR spectroscopy in multiple sclerosis (MS) patients measured from centrum semiovale normal-appearing white matter. Horizontal line: median; box: interquartile range (25–75%), whiskers are set to minimum and maximum. Outliers are marked with ◦. Kruskal-Wallis Test ($p=0.004$). Post-hoc pairwise comparisons revealed a significant difference between patients without disease modifying therapy and patients treated with fingolimod, $p=0.001$ (Adjusted $p=0.027$, after Bonferroni correction). tCho: total choline

Figure 3. Association between NFL and OPN concentration and functional connectivity strengths within the default mode network
A, Associations between NFL measured from CSF sample collected ~10 years before MRI and functional connectivity between the nodes of DMN are depicted. B, Associations between OPN measured from serum sample collected ~10 years before MRI and functional connectivity between the nodes of DMN are depicted. Only the significant associations are shown, line thickness corresponds to the 1-"p-value". Statistical data are summarized in Table 3.