Brain perfusion SPECT analysis
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Chapter 6. The Number of Optic Neuritis Attacks is a Potential Confounder When Comparing Patients with NMO vs. Controls by Voxel-based Neuroimaging Analysis

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ABSTRACT

Background: Voxel-based morphometric (VBM) studies in neuromyelitis optica (NMO) have shown limited reproducibility. A previous study suggests that the number of optic neuritis (ON) attacks may be a confounding factor when comparing NMO patients with controls if it is not taken into account during VBM analysis.

Purpose: To investigate the potential confounding effect of the number of ON attacks, for both tissue volumes and perfusion by voxel-based statistical analysis.

Material and Methods: Volumetric magnetic resonance imaging (MRI) and perfusion SPECT were obtained from 15 controls and two patient subgroups: subgroup I was composed of nine patients with one or two ON attacks; and subgroup II of six patients with three or four ON attacks. We performed non-parametric voxel-based comparison of tissue volumes and perfusion between controls versus the two patient subgroups and for the whole patient group.

Results: Subgroup I presented no volume reductions, contrary to subgroup II that showed unequivocal reduction. We also found hypoperfusion in different brain regions in different subgroups. The results were quite different for the whole patient group.

Conclusion: These findings highlight the confounding effect of the number of ON attacks, providing a new methodological insight that could explain the limited reproducibility of previous VBM studies in NMO.
INTRODUCTION

Neuromyelitis optica (NMO) is a demyelinating disease, clinically characterized by optic neuritis (ON) and transverse myelitis attacks with a relapsing course in most patients (1, 2). In the past it was considered an uncommon and severe variant of multiple sclerosis (Devic disease). The finding that most NMO patients have autoantibodies (NMO-IgG) against the water channel aquaporin-4 changed the comprehension of NMO pathogenesis (1–3).

Recent voxel-based morphometry (VBM) studies have shown limited reproducibility in NMO (4–10). This needs to be addressed in order to make VBM more reliable as potential imaging biomarker in this complex disease.

In a previous study we found that the number of ON attacks negatively correlates with regional white matter (WMV) and gray matter volumes (GMV) in patients with NMO; while negatively correlating with cerebral perfusion in some regions and positively in other regions (11). This suggests that the number of ON attacks per patient may be a confounding factor when comparing patients with controls if this is not taken into account during the analysis, especially if the patient sample includes patients with both smaller and larger number of ON attacks. This might also explain a limitation of our previous work that did not substantiate differences between patients and controls for regional WMV/GMV after multiple comparisons correction (11). Other clinical variables seemingly do not have a similar effect as the number of ON attacks in NMO (5, 7, 11).

Our previous study focused on investigating how regional changes of brain volumes and perfusion are associated to the ON attack-related pathological process in NMO. Here our aim was to investigate a methodological aspect of VBM in NMO that had not been explicitly addressed before, which refers to the potential confounding effect of the number of ON attacks suffered by the patients. For this purpose, we performed group-wise comparison of regional WMV/GMV and perfusion differences between patients and controls, by assessing
these differences in two subgroups of patients grouped according to the number of ON attacks and for the whole patient group.

METHODS

Subjects

The enrollment, diagnostic, and exclusion criteria as well as the demographic and clinical characteristics of the sample of 15 NMO patients have been described previously in detail (11). Briefly, all patients fulfilled revised diagnostic criteria for NMO (12) and were in stable phase (no acute relapse at least 4 months prior to the study).

The sample of patients was divided into two subgroups using the median of the variable number of ON attacks (n= 2). Thus, subgroup I consisted of nine patients with one or two ON attacks (8 women, 1 man; mean age, 41.7 ± 8.4 years; median age, 41 years; age range, 25–50 years); and subgroup II consisted of six patients with three or four ON attacks (5 women, 1 man; mean age, 39.2 ± 13.8 years; median age, 42 years; age range, 17–56 years). Disease duration was similar in both subgroups (subgroup I: mean duration, 7.9 ± 4.2 years; median duration, 8 years; range, 2–14 years; subgroup II: mean duration, 10.1 ± 5.4 years; median duration, 8.5 years; range, 5–17 years).

The control group has also been also described previously (11). Briefly, it was composed of 15 healthy volunteers (13 women, 2 men; mean age, 46.6 ± 12.5 years; median age, 49 years; age range, 20–58 years). Although the mean age of the control group was higher than the mean ages of both NMO subgroups, the difference was not statistically significant (P= 0.1712, Kruskal-Wallis test).

All subjects gave written informed consent. The study was approved by the Ethics Committee of the Center for Neurological Restoration of Havana.
Image data and analysis

Acquisitions and preprocessing of volumetric magnetic resonance imaging (MRI) (T1-weighted [T1W]) and 99mTc-ECD SPECT imaging, corrected for partial volume effect, from patients and controls have been described previously in detail (11). MRI was performed with a 1.5 Tesla Symphony scanner (Siemens, Erlangen, Germany); while SPECT was carried out using a double-head gamma camera (DST Xli, Sopha Medical Vision, Buc, France) equipped with ultrahigh-resolution fan-beam collimators.

No T1-visible lesions could be identified on brain MRI for any of the patients. However, five patients had minor non-specific T2/FLAIR-visible lesions. As described in our previous study, before the preprocessing step, a lesion mask from manually segmented lesions visible on T2-weighted (T2W) images was created for these five patients (11). The co-registered lesion mask was used to zero out respective areas in the patients’ volumetric T1W images, in order to reduce the influence of these lesions during segmentation and normalization procedures. These lesion areas were not incorporated into statistical analyses since they were few and small. Although these predominated in patient subgroup II, they were localized in different brain regions in each patient, which precluded potential bias towards patients with lesions for VBM analyses, considering also that we controlled for total intracranial volume.

For this study, non-parametric voxel-based comparison between the control group and each patient subgroup was performed for each image modality using the SnPM5 toolbox (http://www.sph.umich.edu/ni-stat/SnPM).

Suprathreshold cluster size tests (pseudo T-tests) were performed based upon 10,000 random permutations to compare patient subgroup I with the control group; while 54,264 random permutations (maximum of permutations) were used for patient subgroup II.
Multiple comparisons corrected P values were obtained for each suprathreshold cluster in the observed statistical image by comparing their size to the permutation distribution (13, 14). These tests were performed to map WMV/GMV and perfusion values in each patient subgroup, which were either incremented or decremented compared to the control group. The theta parameter was set to 0.5, which corresponds to equally weighted statistics (equally sensitive to high intensity peaks and large clusters) (14).

Age and sex were included as standard nuisance covariates for perfusion, and total intracranial volume as an additional nuisance for the WMV/GMV analysis. A variance smoothing of 12mm was used. We collected suprathreshold clusters using a primary threshold T = 2.3.

To allow comparison with previous VBM studies, we also used statistical parametric mapping (SPM8) to perform a group wise-comparison for the whole patient group and for each imaging modality. Unlike our previous study, where false positives were controlled, here statistical maps were thresholded at an uncorrected P = 0.005 and minimum cluster size = 30 voxels. These values are comparable to what was used in three previous VBM studies (1, 5, 9). The SnPM analysis described above was also applied to the whole patient group.

Anatomical regions were determined with a brain atlas that comprises deep WM (DWM), superficially located WM (SWM), cortical GM, subcortical GM, and other brain labels (15). The most significant voxels were reported in MNI coordinates, with P values corrected for multiple comparisons by family-wise error (FWE).
RESULTS

No significant differences were found between patient subgroup I and the control group for WMV and GMV. On the contrary, significant regional decrements of WMV and GMV were found in patient subgroup II (Fig. 1 a and Supplemental Table 1). WMV decrement was observed in one extensive cluster that comprised DWM and SWM bilateral regions. The most significant voxel was found at the left angular gyrus (SWM) (MNI: x, y, z = -36, -58, 36; PFWE = 0.047, pseudo-T = 3.93); while GMV decrement was found in one cluster that comprised frontal and anterior cingulate bilateral regions but mostly in the right side. The most significant voxel was found at the right anterior cingulate (MNI: x, y, z = 2, 33, -12; PFWE = 0.05, pseudo-T = 4.52, Brodmann area (BA) 11).

On the other hand, no significant differences were found between the whole group and the control group for GMV (uncorrected p = 0.005). Unlike GMV, significant differences were found for WMV (Fig. 1 b). WMV reductions were observed in few and small regions comprising the fornix, posterior thalamic radiation and supramarginal region (SWM) bilaterally. Significant reductions were also found in small SWM regions in the frontal, temporal and occipital lobes on the right side. The most significant voxel was found at the right cerebral peduncle (MNI: x, y, z = 20, -20, -11; PFWE = 0.89, T = 3.19). Similar results were found for SnPM analysis.
Fig. 1. NMO patient samples versus control group for brain tissue volumes. (a) Pseudo T-maps showing reductions of white matter volume (in blue) and gray matter volume (in red) in patient subgroup II as compared with the control group. In patient subgroup I no significant differences were found for tissue volumes. Corrected P values in the observed pseudo T-maps were obtained by comparing their size to the permutation distribution, using statistical non-parametric mapping. (b) White matter volume reduction in whole sample of patients compared with the control group. Unlike patient subgroup II, it can see in Fig. 1b that in the whole patient group few and small regions show white matter volume reduction. The results are overlaid on selected slices of MNI 152 T1 template.
Supplemental table 1. Brain regions with white and grey matter volume decrements in the patient subgroup II compared to healthy controls.

<table>
<thead>
<tr>
<th>P</th>
<th>No. of voxels</th>
<th>Brain regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.018</td>
<td>60537</td>
<td><strong>Bilateral DWM:</strong> superior longitudinal fasciculus; superior and inferior fronto-occipital fasciculus; posterior thalamic radiation; anterior, posterior and superior corona radiata; cingulum (cingulated gyrus); cingulum (hippocampus); fornix(cres) stria terminalis; sagittal stratum; external capsule; anterior limb, and retro lenticular part of internal capsule; genu, body and splenium of corpus callosum; tapatum; and cerebral peduncle. <strong>Right DWM:</strong> posterior limb of internal capsule. <strong>Bilateral SWM:</strong> superior parietal; postcentral; angular; supramarginal; cingulum; precuneus; cuneus; lingual, fusiform, superior, middle and inferior occipital; superior, middle and inferior temporal; precentral; superior, middle and inferior frontal; and lateral fronto-orbital.</td>
</tr>
<tr>
<td>0.039</td>
<td>9170</td>
<td><strong>Bilateral GM:</strong> cingulate; superior frontal and rectus gyri. <strong>Right GM:</strong> middle and inferior frontal gyri; lateral and middle fronto-orbital gyri; and insular.</td>
</tr>
</tbody>
</table>

P, P value corrected for multiple comparisons at cluster level; DWM, deep white matter; SWM, superficially located white matter; and GM, grey matter.
Unlike WMV and GMV, patient subgroup I showed a significant hypoperfusion in one extensive cluster, mainly involving an extensive WM area (Fig. 2 a and Supplemental Table 2). Although the cluster was mainly located on WM, it also comprised adjacent GM areas, including the mesial region of the temporal lobe and the thalamus on the right hemisphere. The most significant voxel was found at the right sagittal stratum (MNI: x, y, z= 41, -39, -5; $P_{FWE} = 0.004$, pseudo-$T = 5.80$).

Unlike patient subgroup I, patient subgroup II showed significant hypoperfusion in one cluster, mainly located in the occipital cortex bilaterally, including the primary visual area; and infero-mesial temporal region and the cerebellum on the right side (Fig. 2 b and Supplemental Table 3). The most significant voxel was found at the right cuneus (MNI: x, y, z= 2, -82, 9; $P_{FWE} = 0.001$, pseudo-$T = 6.66$, BA 17).

Significant differences were also found between the whole patient group and the control group at uncorrected $p= 0.005$ (Fig. 2 c). Perfusion reductions were mainly in an extensive WM area and occipital cortex bilaterally. The most significant voxel was found at the right fusiform region (SWM) (MNI: x, y, z= 23, -76, -2; $P_{FWE} = 0.004$, $T = 6.83$). It can be seen in Fig.2 c that when analyzing the entire sample, the two effects showing in Fig. 2 a and b appear together on the same map of hypoperfusion. Similar results were found for SnPM analysis.
Fig. 2. NMO patient samples versus control group for brain perfusion. Pseudo T-maps showing perfusion reduction in patient subgroup I (a) and in patient subgroup II (b). Corrected P values in the observed pseudo T-maps were obtained by comparing their size to the permutation distribution, using statistical non-parametric mapping. (c) Perfusion reduction in the whole patient group. Fig. 2c shows that the two effects in Fig. 2a and b appear together when analyzing the whole patient group. The results are overlaid on selected slices of MNI 152 T1 template.
Supplemental table 2. Brain regions with perfusion decrement in the patient subgroup I compared to healthy controls.

<table>
<thead>
<tr>
<th>P</th>
<th>No. of voxels</th>
<th>Brain regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^{-4}$</td>
<td>56595</td>
<td><strong>Bilateral DWM</strong>: superior longitudinal fasciculus; superior fronto-occipital fasciculus; posterior thalamic radiation; anterior, posterior and superior corona radiata; cingulum (cingulated gyrus); fornix (cres) stria terminalis; sagittal stratum; external capsule; anterior limb, posterior limb and retrolenticular part of internal capsule; genu, body and splenium of corpus callosum; tapatum; cerebral peduncle; substantia nigra and midbrain. <strong>Bilateral SWM</strong>: superior parietal; postcentral; angular; supramarginal; cingulum; precuneus; cuneus; lingual; superior and middle occipital; superior and middle temporal; precentral; superior, middle and inferior frontal; and lateral fronto-orbital. <strong>Right SWM</strong>: fusiform; inferior occipital and inferior temporal. <strong>Left SWM</strong>: rectus. <strong>Bilateral GM</strong>: cingulate gyrus. <strong>Right GM</strong>: lingual, fusiform and parahippocampal gyri; superior and middle temporal gyri; insular; hippocampus; and thalamus.</td>
</tr>
</tbody>
</table>

P, P value corrected for multiple comparisons at cluster level; DWM, deep white matter; SWM, superficially located white matter; and GM, grey matter.
Supplemental table 3. Brain regions with perfusion decrement in the patient subgroup II compared to healthy controls.

<table>
<thead>
<tr>
<th>P</th>
<th>No. of voxels</th>
<th>Brain regions</th>
</tr>
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<tbody>
<tr>
<td>0.01</td>
<td>13938</td>
<td><strong>Bilateral GM:</strong> superior and middle occipital gyri; precuneus, cuneus and lingual gyri; <strong>Right GM:</strong> cingulate, fusiform, parahippocampal, inferior temporal and inferior occipital gyri; and cerebellum. <strong>Bilateral SWM:</strong> superior and middle occipital; cuneus and lingual. <strong>Right SWM:</strong> fusiform; inferior temporal; and inferior occipital. <strong>Right DWM:</strong> posterior thalamic radiation and sagittal stratum.</td>
</tr>
</tbody>
</table>

P, P value corrected for multiple comparisons at cluster level; GM, grey matter; SWM, superficially located white matter; and DWM, deep white matter.

**DISCUSSION**

In this study we demonstrated: (i) that the patient subgroup with one or two ON attacks shows no decrease of regional WMV/GMV as compared with controls; (ii) that in contrast, the patient subgroup with more than two ON attacks showed unequivocal reductions, especially extensive in WMV; (iii) that in the whole group of patients few and small regions showed WMV reduction; and (iv) that hypoperfusion was present in different brain regions in different subgroups, while both appear when analyzing the whole group. This shows that the number of attacks ON attacks per patient in each group significantly influences the results, which highlights its confounder effect when comparing patients with controls, for both tissue volumes and perfusion by voxel-based statistical analysis. This provides a new methodological insight that could explain the limited reproducibility of previous VBM studies in NMO and could be useful for future studies.
The non-parametric voxel-based method used to substantiate our main findings is robust in the presence of outliers and increased noise, and for detecting small differences in small samples (13, 14). This approach appropriately solves the multiple comparisons problem by a non-parametric randomization and permutation test.

In previous VBM studies, where the number of ON attacks per patient was not controlled, few and small brain regions showed WMV reduction in patients as compared with controls (4, 5, 7), or showed no reduction (8). Moreover, studies with positive findings showed quite different patterns of regional WMV reduction (4–7). Nevertheless, some common regions could be identified, such as the posterior thalamic radiation in two studies (4, 5), and parietal WM in two other studies (6, 7). We obtained comparable results in the whole patient group as shown in Fig. 1b (e.g. posterior thalamic radiation and parietal WM), using uncorrected P values similar to those used in previous studies (4, 5).

On the other hand, except one, all of the studies mentioned above also analyzed regional GMV (4–6, 8). One of these studies showed no reduction of GMV (6), which is similar to our findings in the whole patient group, even using uncorrected P values. Two other studies analyzed only GMV, with positive findings (9, 10). Like our preceding comments on WMV results, studies with positive findings showed different pattern of regional GMV reduction (4,5,8–10), in four of them without correction for multiple comparisons (4,5,8,9). However, GMV reduction of the prefrontal cortex was a common finding in these studies. This was also detected in the present study but only in the patient subgroup with the larger number of ON attacks (Fig. 1a).

Previous VBM studies in NMO showed limited reproducibility. Although we cannot exclude other explanations, the results presented here suggest that one important reason for this could be that the number of ON attacks per patient was not controlled during analyses.
On the other hand, a supplementary analysis performed in our previous study has found decreased perfusion in the NMO patient group as compared to controls, including brain regions that showed both positive and negative correlation with the number of ON attacks, but by using a lenient control for false positives (11). The results of the present study clearly show that patients in initial stages of the ON attack-related process have extensive WM hypoperfusion (Fig. 2a). In contrast, in more advanced stages (more attacks) patients have nearly no WM hypoperfusion, but show bilateral hypoperfusion in the occipital cortex (Fig. 2b). Fig. 2c illustrates that by analyzing the patient group as a whole, the two effects appear together on the hypoperfusion map, which is confusing, because it was not considered that, with increasing ON attacks, perfusion changes are opposite in different brain regions. Thus, our results indicate that the number of ON attacks per patient should be taken into account for analysis in future studies of cerebral perfusion in NMO.

The sample of patients is the same of our previous article, because of the long time needed to have a larger well characterized sample in our setting. NMO has a very low prevalence/incidence in Cuba (16). Havana has an incidence of one case per year approximately, often difficult to differentiate from MS at the onset. Even so, this sample served for the aim of the present study that was focused on a new and distinct issue. It is also justified because our findings with the whole patient sample, using uncorrected P values, were comparable to those obtained in other similar and larger patient samples in recently published VBM studies in NMO (4, 5, 7). Furthermore, this suggests that sample size is relatively less important than the effect of the number of ON attacks.

Although the results of the present study could be inferred from our previous correlation analysis in NMO patients, the confounding effect of the number of ON attacks is now clearly
shown by using group-wise comparison of controls and patient subgroups. This gives a new methodological insight for VBM in NMO that was not verified in our preceding study.

Finally, other clinical variables seemingly do not have a similar effect as ON attacks in NMO. Several of the studies mentioned before did not find correlation between WMV and the Expanded Disability Status Scale (5, 7), NMO-IgG status (5, 11), the number of myelitis attacks and/or total attacks (11). One study found an association between disease duration and GMV (4); although other studies found no association with GMV (11) and WMV (5, 7, 11). Another study found more extensive WMV reduction in patients with cognitive impairment as compared to patients without impairment (6); while still another study failed to find such differences for global WMV (17).

Other recent studies have also failed to find associations between brain tissue volumes and other clinical characteristics in NMO (18, 19). The reason for this apparent peculiarity of ON attacks remains unclear. However, it is possible that ON attacks (and related symptoms) are only part (clinically detectable) of a more complex pathological process of this not fully understood disease. More studies are needed to clarify this issue further.

Our analysis has limitations. First, the patient grouping was arbitrary and the applied threshold has influence on the outcome. However, this grouping allowed comparison of controls with patient samples where the distribution of number of ON attacks per patient was different in each sample. Yet, more studies in other and larger populations of NMO patients are necessary to clarify our findings further, including the use of other thresholds for grouping patients according to the number of ON attacks. Second, our results should be taken with caution since we cannot exclude imprecisions related to the spatial segmentation step, particularly for WMV images using a 1.5 T scanner. This could be improved by a 3.0 T machine (20–22). Automated spatial segmentation procedures may perform better in this case, especially close to tissue borders. Nevertheless, for this particular problem a 1.5 T machine
also seems adequate. One of the studies discussed above was carried out by 3.0 T scanner and showed WMV reduction in few and small regions (4) similar to two other studies using a 1.5T machine (5, 7).

In conclusion, this study supports that the number of ON attacks per patient is a potential confounder when comparing NMO patients with controls, for both tissue volumes and perfusion by voxel-based statistical analysis, and calls for attention in future studies.
REFERENCES


