# Regional Selectivity of Neuromelanin Changes in the Substantia Nigra in Atypical Parkinsonism

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# Regional selectivity of neuromelanin changes in the substantia nigra in atypical Parkinsonism

**Running head: Patterns of nigral degeneration in parkinsonism**

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## Abstract

**Background**. Neurodegeneration in the substantia nigra *pars compacta* (SNc) in parkinsonian syndromes may affect the nigral territories differently.

**Objective**. To investigate the regional selectivity of neurodegenerative changes in the SNc in patients with Parkinson's disease (PD) and atypical parkinsonism using neuromelanin-sensitive MRI.

**Methods**. Twenty-two healthy controls (HC), 38 patients with PD, 22 with progressive supranuclear palsy (PSP), 20 with multiple system atrophy (MSA, 13 with the parkinsonian variant, 7 with the cerebellar variant), 7 with dementia with Lewy body (DLB) and 4 with corticobasal syndrome (CBS) were analyzed. Volume and signal-to-noise ratio (SNR) values of the SNc were derived from neuromelanin-sensitive MRI in the whole SNc. Analysis of signal changes was performed in the sensorimotor, associative and limbic territories of the SNc.

**Results**. SNc volume and corrected volume were significantly reduced in PD, PSP, and MSA versus HC. PSP had lower volume, corrected volume, SNR and CNR than HC, PD and MSA. PSP patients had greater SNR reduction in the associative region than HC, PD and MSA patients. PD patients had reduced SNR in the sensorimotor territory, unlike PSP patients. MSA patients did not differ from PD patients.

**Conclusions**. This study provides the first MRI comparison of the topography of neuromelanin changes in parkinsonism. The spatial pattern of changes differed between PSP and synucleinopathies. These nigral topographical differences are consistent with the topography of the extra-nigral involvement in parkinsonian syndromes.

**Abbreviations:** CBS: corticobasal syndrome; DLB, dementia with Lewy body; HC: healthy controls; MRI: magnetic resonance imaging; MSAc: multiple system atrophy of the cerebellar type; MSAp: multiple system atrophy of the parkinsonian type; PD: Parkinson's disease; PSP: progressive supranuclear palsy; ROI: region of interest; SN: substantia nigra; SNc: substantia nigra pars compacta, SNR: signal-to-noise, CNR: contrast-to-noise.

# Introduction

Parkinsonism is clinically defined by the association of bradykinesia, plastic rigidity and asymmetrical resting tremor. Parkinson's disease (PD) is the most common neurodegenerative cause of parkinsonism. Atypical parkinsonism includes tauopathies (progressive supranuclear palsy - PSP and corticobasal degeneration) and synucleinopathies (multiple system atrophy - MSA, with its cerebellar - MSAc - and parkinsonian - MSAp - subtypes, and dementia with Lewy body - DLB).[1–3]

The hallmark of neurodegenerative parkinsonism is the neurodegeneration of dopaminergic neurons in the substantia nigra *pars compacta* (SNc).[1–4] Degeneration of dopaminergic neurons has been studied in PD and atypical parkinsonism using neuromelanin-sensitive MRI. Neuromelanin is a pigment contained in the dopaminergic neurons that has paramagnetic T1-shortening effects when bound to metals. Volume and signal intensity using neuromelanin-sensitive MRI have been used as surrogate markers for degeneration of dopaminergic neurons in the SNc.[5,6] Reduced SNc neuromelanin volume and signal were reported in PD,[6–11] PSP, MSA and corticobasal syndrome (CBS) with high diagnostic accuracy versus healthy controls (HC).[12–15] Although all studies reported significant changes in PD and atypical parkinsonism, the differences reported between the different types of parkinsonian syndromes were sometimes discordant. Some studies reported greater reductions in SNc neuromelanin volume[15] in PSP vs. PD, whereas one study reported no significant change in the SNc signal in PSP vs PD and MSA[13] or no significant difference in SNc volume[12] or signal between PD, PSP and MSA.[16] Similar changes were reported between MSA and PD[13,17] while others have reported lower changes in MSAp vs PD.[18]

Disagreement between studies may be due to differences in patient characteristics and methodology. Previous studies have also suggested a differential topography of the SNc involvement between diseases. Histological studies have demonstrated a regional selectivity of the dopaminergic neuronal loss in PD. Indeed, the greatest depletion of dopaminergic neurons was shown to occur first in the posterolateral part of the SNc, in the so-called nigrosome 1, before spreading to other nigrosomes and the matrix along rostral, medial, and dorsal axes of progression.[19] This pattern has been recently confirmed using neuromelanin-sensitive MRI.[10] Histological studies have shown that this pattern was similar to MSA but differed from PSP and from aging where the lateral ventral tier was relatively spared.[4]

Here, we investigated the topography of neurodegenerative SNc involvement in patients with PD and atypical parkinsonism in comparison with HC using neuromelanin-sensitive MRI. We assessed changes in signal intensity and volume, identified the different spatial patterns of neurodegeneration in the SNc, and quantified degenerative changes in the motor, associative and limbic territories of the SNc.

# Methods

### Population

Participants were prospectively enrolled between April 2017 and June 2020 in the movement disorders clinic of the Pitié-Salpêtrière University Hospital, Paris. The study population included parkinsonian patients with a diagnosis of PD,[20] PSP,[21] MSA,[22] DLB,[23] and CBS[24] established by an expert in movement disorders according to the international diagnostic criteria. Moreover, the clinical diagnosis had to be in agreement with the MRI pattern: i)for PSP, midbrain atrophy with hummingbird sign,[25] midbrain to pons sagittal area ratio<0.21[26] or MRPI>13.6,[27] ii)for MSAp, posterior putamen atrophy with flattening of lateral border, increased diffusivity and iron load,[28] iii)for MSAc, pons and cerebellar atrophy with a hot cross bun sign,[29] iv)for CBS, asymmetrical parietocentral atrophy, v)no such changes and a third ventricle/internal skull diameter ratio<5.88 in PD.[30]

We recruited age and sex-matched HC with patients, without any history of neurological or psychiatric disease. Subjects were excluded if they had additional neurological disorder including stroke or brain tumor on MRI examinations or when the MRI pattern was inconsistent with the clinical diagnosis (e.g. MRI typical of MSA in a patient with a clinical diagnosis of PSP or vice versa). The local institutional review board approved the study (Parkatypique: CPP Ile-de-France VI08012015). We obtained written informed consent from all participants.

The clinical examination included the Unified Parkinson's Disease Rating Scale part III scores (UPDRS III) performed in a variable ON state during the routine clinical examination with a delay between the MRI and the clinical evaluation ranging from 0 to 94 days (mean: 26±33days).

### Image acquisition

Participants were scanned in clinical conditions for diagnostic purposes at the hospital's Neuroradiology department using a 3T SKYRA scanner (Siemens, Erlangen, Germany), with a 64-channel head coil. The MRI protocol included 1)high-resolution three-dimensional T1-weighted gradient-recalled echo sequence (Magnetization-prepared rapid acquisition with gradient-recalled echo, MPRAGE) with 1-mm isovoxel size and 2)two-dimensional turbo spin echo neuromelanin-sensitive $T_1$-weighted imaging (Repetition time (TR)/echo time (TE)/flip angle=890 ms/13 ms/180°, 5 averages; voxel size, 0.4×0.4×3 $mm^3$).

Overall, 13 scans were not analyzed: 6 due to motion artefacts, 5 because of presence of exclusion criteria (3 MSA and 1 PSP patients with normal MRI, 1 PSP patient with MRI typical of MSA), 2 PD patients due to misregistration issues.

## Analyses in native space based on manual segmentations

### Data processing and segmentation

All analyses were performed using MATLAB (MathWorks Inc, MA, USA, vR2017b), Statistical Parametric Mapping (SPM12, UK), FreeSurfer (MGH, USA, v5.3.0), and FSL (FMRIB, UK, v5.0). Using FreeSurfer viewer, similar to a previous study,[9] SN contours of the left and right SN were manually delineated on neuromelanin-sensitive images by one examiner (E.A.) as the border of hyperintense area dorsal to the cerebral peduncle and ventral to the red nucleus. Contours were continuous as they did not include non-contiguous voxels. The examiner was blind to the clinical status. A background region including the tegmentum and superior cerebral peduncles was also traced.

### Volume and signal analyses

Volumes of each neuromelanin-based ROIs were calculated as the product between the voxel size and the number of voxels in the ROIs of the three lowest contiguous image slices where the SN was visible. Total intracranial volume (TIV) was used to correct for variations in individual head size. White matter, grey matter and cerebrospinal fluid volumes were summed

up to provide an estimate of TIV using SPM12. Hence, we calculated corrected volume by dividing SN volumes by TIV to normalize for respective head sizes of the subjects. For each slice, signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) were calculated by normalizing the mean signal in SN relative to the background signal using these formulas:

$SNR=Mean\_over\_slices\{(Sig_{SN}/Sig_{BND})*100)\}$

$CNR=Mean\_over\_slices\{(Sig_{SN}-Sig_{BND})/STD_{BND}\}$

where $Sig_{SN}$ is the signal intensity in SN ROI, $Sig_{BND}$ the signal intensity in background ROI and $STD_{BND}$ the standard deviation in background ROI.

For SN volumes and signal intensity, we used the mean values of the left and right SN.

Intra-examiner reproducibility for the segmentations was evaluated using DICE similarity coefficient on a sample of 51 subjects.

## Analyses in template space

To enable anatomical alignment of all SN segmentations, we used a template of the average brain which was calculated in a previous study to be equally representative of HC and patients with prodromal and clinical parkinsonism.[10]

For each subject, the neuromelanin-sensitive image was aligned to the brain template using NiftyReg.[31,32] First, the neuromelanin-sensitive image was rigidly aligned to the corresponding T1-weighted image. Second, the T1-weighted image was aligned to the template using a concatenation of one affine and on nonlinear transformation. The resulting T1-to-template transformation was applied to the neuromelanine-sensitive image with nearest-neighbor interpolation.[10,33] (Supplementary Fig.S1)

An analysis was then performed based on the calculation of the neuromelanin SNR in the SNc and its subregions in template space. An SNc mask and a background mask which were previously calculated in template space based on neuromelanin-sensitive MRI of sixty-one HC were used.[10,33] The SNc mask was manually segmented into three regions based on the functional subdivision of the SN in a posterolateral sensorimotor, anteromedial associative, and posteromedial limbic regions.[33,34] These masks were applied to each subject's neuromelanin-sensitive image previously aligned to the brain template. Then, signal values in the whole SNc, in each SNc subregion and in the background masks were extracted and mean SNR values of both sides of SNc were calculated for each subject (Fig.S2). The codes used for

the analyses are available at *https://github.com/emmabiondetti/substantia-nigra-neuromelanin*.

Of note, analyses in native space and template space studied the SNR in two different and complementary ways: in the remaining less affected SNc and in the entire SNc using the mask of healthy subjects, respectively.

## Statistical analyses

Statistical analyses were performed using R (R Core Team 2019 *v3.6.1*). Clinical and demographic data were compared between groups using the Kruskal-Wallis test, followed by pairwise Dunn's tests with Bonferroni correction, or the Fisher's exact test.

Sex and age were included as covariates of no interest as both sex and age influence the phenotypical expression of PD,[35] healthy aging[36,37], and sex[37] influences neuromelanin accumulation. MSA patients were analyzed together and DLB and CBS patients were excluded from the main analyses given the low number of subjects in each group. Exploratory pairwise analyses were subsequently performed to compare HC to DLB, CBS, MSAp and MSAc patients on the one hand, and MSAp to MSAc patients on the other hand.

Between-group differences in volume, corrected volume, SNR and CNR were evaluated by fitting general linear models (one model per type of measurement) including Group (HC and parkinsonian groups) as the only between-group factor, with age and sex for covariate adjustment. Based on the fitted GLMs, the group difference was tested by type II analysis of variance (ANOVA) F-test. If a significant difference was found, post hoc pairwise comparisons were conducted by using Tukey's method in the emmeans package.

For measurements in template space, the data were first analyzed using the same procedure as before to test for between-group differences in SNR values in the whole SNc. Then, a second analysis was performed to investigate the spatial pattern of dopaminergic neuron loss in the SN in parkinsonian syndromes. SNR values in the sensorimotor, associative and limbic territories were compared through linear mixed-effect models (LMMs, one model per parameter of interest). In these models, Group, Region, and their interaction terms were regarded as fixed effects, while the subject identifier was assigned as a random effect (intercept) to account for the repeated measurements acquired in the different ROIs for the same subject. Age and sex were also included for covariate adjustments. All LMMs were fitted using restricted maximum-

likelihood estimation (REML) from the function lmer in the lme4 package. Significance for the main effects and the two-way interaction of Group and Region was assessed based on Type II Wald chi-square tests using the function Anova in the car package. Post hoc pairwise comparisons were performed on a significant interaction or main factor effect with the emmeans package to further determine where the differences occurred across the study groups and the ROIs. All p-values from the post hoc tests were obtained using Kenward-Roger's approximation for degrees of freedom (df), and after adjustment for multiple testing by Tukey's method.

For each model, the assumptions of normality and constant variance of residuals were checked afterwards. The level of statistical significance was set at $p<0.05$ for all tests.

Correlations between SNR values and clinical variables were also investigated (see supplementary material).

## Results

### Subject characteristics

We included 22 HC, 38 PD, 22 PSP, 13 MSAp, 7 MSAc, 7 DLB and 4 CBS (Table 1). MRI scans were obtained in average within 22.5±26.9 days after the clinical examination. There were no between-group differences in sex proportion ($p=0.40$). Age was significantly different between groups ($p=0.001$), PSP patients being older than MSAc ($p=0.02$). UPDRS III scores ($p=0.11$) and disease duration ($p=0.21$) did not differ significantly between patient groups.

### Group differences in neuromelanin volume and signal based on manual segmentations

On neuromelanin-sensitive imaging, the signal intensity of the SNc was visually decreased in parkinsonian patients in comparison with HC (Fig.S3).

There was a high intra-rater reproducibility for manual segmentations (DICE=0.81).

There was a significant difference in SNc volume ($F=52.49$, $df=3$, $p<0.001$), corrected volume ($F=44.18$, $df=3$, $p<0.001$), SNR ($F=7.50$, $df=3$, $p<0.001$) and CNR ($F=6.70$, $df=3$, $p<0.001$) when comparing all groups. Post hoc comparisons showed that HC had higher SN volume and corrected volume than PD, PSP, MSA ($p\leq0.001$) and higher SNR and CNR values than PSP ($p<0.01$). PSP patients had lower volume and corrected volume than PD ($p<0.01$ and $p<0.001$, respectively) and MSA ($p<0.05$), lower SNR values than PD ($p<0.001$) and MSA ($p<0.05$),

and lower CNR values than PD ($p<0.001$) with a trend for MSA ($p=0.05$) (Table 2, Fig. 1). There were no differences between PD and MSA.

The exploratory pairwise comparisons showed higher SN volume and corrected volume in HC versus DLB, CBS ($p<0.01$ for volume and $p<0.05$ for corrected volume, respectively), MSAp and MSAc ($p<0.0001$). There was no SNR or CNR difference. MSAp had higher SN volume than MSAc ($p<0.05$), without any difference in corrected volume, SNR or CNR.

**Spatial distribution of neuromelanin changes**

Analyses performed in the mask of the whole SNc in template space confirmed that SNR values were significantly different between groups (Type II ANOVA, $F=5.20$, $df=3$, $p=0.002$). Post hoc comparisons indicated lower values in PD ($p=0.016$), PSP ($p=0.002$) and MSA ($p=0.02$) subjects versus HC without differences between patient groups (Fig. 2, Supplementary Table).

When comparing the three SN territories between groups, there was a significant effect of the group ($\chi^2=16.01$, $df= 3$, $p=0.001$) and region ($\chi^2=51.90$, $df= 2$, $p<0.0001$) factors with a significant Group by Region interaction ($\chi^2=47.02$, $df= 6$, $p<0.0001$). Post hoc comparisons showed that SNR values in the associative territory were lower in PSP ($p<0.0001$), PD ($p=0.04$) and MSA ($p=0.03$) subjects than in HC, and lower in PSP versus PD ($p<0.0001$) and MSA ($p=0.01$) subjects. In the sensorimotor territory, SNR values were lower in PD than in HC ($p=0.03$), and also lower in MSA patients than in HC but the difference did not reach significance ($p=0.08$). There were no significant differences in the limbic territory or between PD and MSA (Fig. 2 and 3, Supplementary Table).

Exploratory pairwise comparisons showed that SNR values in the whole SN were lower in DLB ($p=0.01$) and MSAp ($p=0.001$) in comparison with HC, with an involvement of the associative ($p=0.002$) and sensorimotor ($p=0.02$) territories in DLB, and all territories in MSAp (associative: $p=0.004$, limbic: $p=0.04$, sensorimotor: $p=0.006$). No difference was seen between HC and CBS, HC and MSAc, or MSAp and MSAc subjects.

When comparing SNR values between the three territories within each group, in HC, PD and MSA patients, the associative territory had significantly higher SNR values than the limbic and sensorimotor territories (Fig.S4). In PD but not in HC and MSA, the sensorimotor territory had also significantly lower SNR value than the limbic territory. In contrast, in PSP patients, this pattern was reversed, the associative territory having lower SNR values than the limbic and sensorimotor territories.

For all models, a visual inspection of the residual distributions did not show any important deviation from the normality and variance-variance assumptions.

## Discussion

This study provides the first MRI comparison of the topography of neuromelanin changes in parkinsonism. We confirm that neuromelanin volume and signal were reduced in parkinsonian disorders. The spatial pattern of changes differed between PSP and synucleinopathies. Compared to other groups, PSP had greater changes in the associative region. SNR in the sensorimotor territory was preserved in PSP, but reduced in PD and there was a trend in MSA. There was no significant difference between MSA and PD. Exploratory analyses showed reduced SN volume in DLB and CBS groups and no differences between MSAp and MSAc.

### SN anatomy

The anatomy of the SN is complex, involving several distinct compartments with different afferent and efferent projections. Human histological studies have parcellated the SN into three oblique bands including the dorsal tier of pars compacta, which corresponded to the upper and posterior part of the SNc, the ventral tier of pars compacta, corresponding to the lower and anterior part of the SNc, and the *pars reticulata*, located anteriorly to the SNc.[4] These SN regions have distinct connections to the striosome and matrix compartments of the striatum [38] and to the sensorimotor (lateral SN), associative (central SN), and limbic (medial SN) striatal regions.[39] Using diffusion-based tractography in humans, these regions were shown to be connected to the sensorimotor cortex (dorsolateral SNc), the frontal cortex and insula (ventral SNc), and limbic areas including the lateral and medial orbitofrontal cortex, hippocampus and amygdala (dorsomedial SNc).[34] A better description of the topographical differences in the involvement of SN in parkinsonian syndromes could thus allow a better understanding of their pathophysiology.

### Regional selectivity of neuromelanin changes

In PD, degeneration predominated in the posterolateral part of the caudal SN as shown in histological [4,19,40,41] and MRI studies. [10,33] This region corresponded to the lateral ventral tier of the SN [4] and to the sensorimotor territory of the SNc.[10] It was shown to project to the sensorimotor striatum in primates,[42] corresponding to the posterior sensorimotor territory of the putamen, the area of greatest dopaminergic denervation in PD patients.[10,43] Changes in this nigral region correlated with the severity of motor symptoms in PD as demonstrated using

neuromelanin imaging [9,10,15,16] or diffusion imaging.[44,45] In our study, the associative territory was also involved in PD patients. Histological studies showed that in PD the regional neuronal loss started in the ventrolateral sensorimotor territory and then spread to the ventromedial associative territory.[4] A recent study using neuromelanin imaging reported similar spatial and temporal gradient in the SNc involving first the sensorimotor, then the associative and limbic regions.[33]

In MSA patients, neuromelanin signal was decreased in the associative territory with a trend in the sensorimotor territory compared to HC. There was no significant difference between MSA and PD. Histology studies reported variable results. In MSAp, formerly known as striatonigral degeneration, the pattern of cell loss has been variously described as affecting the lateral and medial,[46] lateral and caudal,[47] lateral and midportion,[48] dorsolateral,[49] and ventral and middle parts of the SNc.[50] Lateral predominance therefore seemed to be reported by many authors. The absence of difference between MSA and PD is also in agreement with previous histological studies, in which the distribution of pigmented neuron loss in MSA estimated by neuronal counting was similar to that of PD,[4] and with neuromelanin-based MRI studies, which found no difference between MSA and PD.[13,17] In MSA, although a predominant involvement of the sensorimotor posterolateral putamen was reported in histological,[49,51] MRI,[52–54] $^{18}$F-fluorodeoxyglucose [55] and $^{18}$F-dopa positron emission tomography studies,[56] and presynaptic dopamine transporter single photon emission tomography studies,[57] hypometabolism [56] and reduction in dopaminergic function [57] is also observed in the anterior striatum in line with the involvement of the associative SN observed in our study.

PSP patients had lower SNc volume and SNR than other groups using manual ROI, with greater involvement of the associative territory. These findings are consistent with those from a previous study using free-water imaging that showed higher free water values in PSP compared with other groups. However, while both anterior and posterior SN were affected in that study, only the anterior portion, partly including the associative territory, was significantly involved here.[58]Our results were also in agreement with a histological study that did not find elective involvement of the lateral ventral region of the SN, the region most affected in PD, but greater cell loss in the ventromedial SN.[4] In monkeys, using retrograde transport of horseradish peroxidase, the dorsolateral frontal cortex was connected to the more anterior portions in the medial one-half and dorsal-most part of the SNc.[59] In humans, the ventral SNc was preferentially connected with the prefrontal cortex, anterior cingulate cortex and anterior insula.[34] In PSP patients, these frontal regions are particularly affected as shown using positron

emission tomography with $^{18}$F-fluorodeoxyglucose [55] and flortaucipir tau tracer as well as MRI structural and diffusion imaging.[60] In many neurodegenerative disorders such as PD [61,62] and Alzheimer's disease,[63,64] neuropathological changes follow a stereotyped regional pattern of progression over time. Changes are greater in brain regions that are densely connected, probably due to transneuronal spread of the disease process.[65,66] Imaging studies also provided support to this mechanism of transmission.[67] In PSP, early histological lesions were most prominent in the midbrain and deep brain nuclei and spread to cortical regions in advanced stages of the disease.[68] Our results are in agreement with the hypothesis that the greatest changes in neuromelanin signal should be observed in the SN region connected to the frontal cortical areas most affected in PSP.

In DLB patients, exploratory analyses showed reduced neuromelanin signal in the whole SNc and in the associative and sensorimotor territories. Histological [69,70] and MRI studies [6,16] showed a decrease in dopaminergic neurons density and neuromelanin signal in DLB. Previous histological studies reported an overlap in the pattern of neuropathological changes between PD and DLB.[69,70]

In CBS patients, there was a decrease in SN volume in line with previous findings,[12] but not in signal using the template-based approach probably likely due to a lack of statistical power.

**Limitations**

There were some limitations to this work. Some patient groups included only a few subjects which may have masked differences due to a lack of statistical power. There was no histological confirmation of the diagnosis of parkinsonism, this latter relying on international clinical criteria. However, we took care to ensure the diagnoses by adding an imaging inclusion criterion characteristic of each pathology, which limited the risk of subjects being misclassified. PSP patients were older than MSAc subjects. To take into account the potential effect of age in the analyses, age was used as a covariate. Assessment of UPDRS III scores was performed in a variable ON state during the routine clinical examination, with a variable delay between MRI and clinical examination, and a number of values were missing, which limited correlation analyses. Further, we used the average values on both sides of the SNc because information on which side was clinically more or less affected was missing for many participants. Therefore, the possible asymmetry of the SNc degeneration was not investigated. We used manual segmentation to delineate the SN, which allows careful quality control of images, with a good reproducibility in previous studies.[9,10] However, automated or semi-

automated segmentation could be faster and more reproducible.[7172,73] Regarding the registration procedure, some degree of misalignment may occur between neuromelanin-sensitive images and the template. However, we took several measures to mitigate this effect.

## Conclusion

Spatial patterns of SNc degeneration differed between PD and PSP but not between synucleinopathies (PD and MSA). In future studies, multimodal neuroimaging analysis of the involvement of the SNc territories, the striatum and the cortex would allow to better define the networks affected in parkinsonian syndromes. Further, longitudinal studies in larger populations are needed to confirm these findings and could allow determining the progression of neuromelanin changes over time.

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**Authors' roles**

LC: designed and conceptualized the study, collected and analyzed the data, drafted and revised the manuscript for intellectual content
EA: collected and analyzed the data, drafted the manuscript
RG: analyzed the data and revised the manuscript
EB: analyzed the data and revised the manuscript
AF: performed data collection and revised the manuscript
RV: analyzed the data and revised the manuscript
NP: collected and analyzed the data and revised the manuscript
GD: performed data collection and revised the manuscript
FL: analyzed the data and revised the manuscript
FC: performed data collection and revised the manuscript
JCC: performed data collection and revised the manuscript
MV: performed data collection and revised the manuscript
BD: performed data collection and revised the manuscript
DG: performed data collection and revised the manuscript
SL: designed and conceptualized the study, collected and analyzed the data, drafted and revised the manuscript for intellectual content

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NP: nothing to report related to the present work

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