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NEUROLOGY AND PRECLINICAL NEUROLOGICAL STUDIES - ORIGINAL ARTICLE

# Levodopa-induced dyskinesias in Parkinson's disease increase cerebrospinal fluid nitric oxide metabolites' levels

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## Abstract

Levodopa-induced dyskinesia (LID) is a common complication of Parkinson's disease (PD) therapy. Nitric oxide in the central nervous system may have a role in its pathophysiology. The present work investigates plasma and CSF levels of nitric oxide metabolites nitrite and nitrate in patients with PD, LID, and healthy control. We measured plasma and CSF nitrite and nitrate levels in patients with PD with and without LID and in healthy controls. The levels of plasma and CSF nitrite and nitrate were measured by ozone-based chemiluminescence. Sixty-seven participants were enrolled. CSF nitrite levels in patients with PD and LID were higher than in patients with PD without LID and healthy controls. CSF/plasma ratio of nitrite was higher in patients with PD and LID than in patients with PD without LID. The CSF/plasma ratio of nitrite in patients with PD and LID was higher than 1, indicating an intrathecal production of NO in patients with this motor complication. There was an increase in nitrate levels of CSF and CSF/plasma ratio of nitrate in patients with PD and LID compared to the healthy controls. Sex, age at evaluation, disease duration, and levodopa equivalent daily doses, as well as processing and storage time, did not critically influence these results. The present study demonstrated an increase in nitrite and nitrate levels in the central nervous system of patients with PD and LID. This finding strengthens the role of NO on LID pathophysiology.

**Keywords** Nitric oxide · Parkinson's disease · Dyskinesia · Levodopa

## Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disease, occurring in approximately 7 million individuals worldwide (Dorsey and Bloem 2018). Levodopa is the most efficacious drug to treat the symptoms of PD. However, 33–51.2% of patients develop levodopa-induced dyskinesias (LID) after 5 years of levodopa therapy (López et al. 2010).

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There is evidence implicating the role of nitric oxide (NO) in the central nervous system on motor control and on the pathophysiology of LID (Bel et al. 2005; Del-Bel et al. 2015). Pharmacological inhibition of NO synthesis in the brain attenuated LID in parkinsonian rodents (Padovan-Neto et al. 2009, 2015). The transcription factor ΔFosB is a trigger for the appearance of LID (Andersson et al. 1999). Its expression in the dopamine-depleted striatum occurs in NO synthase (NOS) interneurons (Pavón et al. 2006; Padovan-Neto et al. 2015). The basal ganglia nitrergic system has been described in healthy human brains (Santos-Lobato et al. 2016) and the brains of patients with PD (Böckelmann et al. 1994; Mufson and Brandabur 1994; Eve et al. 1998). Nitrergic activity in PD has also been explored in cerebrospinal fluid (CSF) of patients with the measurement of their metabolites, nitrite and nitrate (Kuiper et al. 1994; Ikeda et al. 1995; Qureshi et al. 1995; Molina et al. 1996; Shukla et al. 2006; Boll et al. 2008).

Nitrite ($NO_2^-$) and nitrate ($NO_3^-$) are the primary metabolites produced by the oxidation of NO. Nitrite derives directly from NO and has a high biological activity, with

a half-life of 11–13 min in whole blood (Tsikas 2005). NO synthesis is the main source of nitrite, and it seems to be a specific indicator of the L-arginine/NO pathway activity (Tsikas 2007). Nitrate is derived from the oxidation of nitrite (Tsikas 2007). Nitrate levels are usually much higher than nitrite in circulation, as hemoglobin converts almost totally nitrite to nitrate, the major metabolic pathway for endogenously formed NO (Wennmalm et al. 1993). Also, different factors can influence nitrate levels (dietary nitrate intake, bacterial nitrate synthesis, and renal function), and its half-life is longer than nitrite. Thus, nitrate is not a direct and reliable indicator of NO generation, but it can reflect its synthesis to some extent (Hendgen-Cotta et al. 2008).

No previous study assessed the association of NO synthesis with LID in patients with PD. Therefore, to explore the possible role of NO on the pathophysiology of LID in PD, we evaluated a possible association between plasma and CSF levels of nitrite and nitrate in patients with PD and LID.

## Materials and methods

### Study design and participants

To analyze nitrite and nitrate levels in blood plasma and CSF of patients with PD with and without LID and healthy controls, we conducted an observational cross-sectional study. The methods were previously described (Marchioni et al. 2020). In brief, participants were recruited in the Movement Disorders Unit of Ribeirão Preto Medical School, Brazil. All patients with PD met the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria for PD. For analysis, we divided participants into three groups: healthy controls (HC), patients with PD without LID (PD-ND), and patients with PD with LID (PD-D). Healthy controls were enrolled from a volunteer cohort (individuals followed up at our hospital due to other medical reasons, not neurological diseases). Patients with PD and healthy controls were included using a 1:1 ratio matching sex and age within four years.

We excluded participants if they had acute infections, severe chronic systemic diseases, autoimmune or other neurological diseases, high nitrite/nitrate dietary intake (as strict vegetarian or vegan diet), or drugs containing nitrite/nitrate. Additionally, patients with PD were excluded if they had PD-associated dementia or mild-to-severe psychosis according to the International Parkinson and Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (score > 1 on item 1.2).

### Evaluations

All patients with PD were examined by the same movement disorder specialist (B.L.S-L.) using a standardized assessment including the MDS-UPDRS (Goetz et al. 2008a) and the Unified Dyskinesia Rating Scale (UDysRS) (Goetz et al. 2008b) in the ON-stage for patients with LID. LID was determined by a score ≥ 1 on item 4.1 of the MDS-UPDRS Part IV (time spent with dyskinesias) and confirmed if the patient presented abnormal movements in ON-stage. We performed the clinical evaluations on the same day, and period blood and CSF samples were collected.

### Collection, processing, and storage of biological samples

Peripheral blood and CSF were collected on the same day and period, between 08:00 and 10:00 AM, without fasting. We instructed patients to take their morning dose of levodopa and samples were collected in ON-stage. Both peripheral blood and CSF samples were placed on ice immediately after collection. We collected peripheral blood into Vacutainer tubes (BD Diagnostics, Plymouth, UK) with anticoagulant (EDTA) by venipuncture, and whole blood was centrifuged 4 °C at 1600 g for 15 min. Coded aliquots of 1 mL containing supernatant plasma were stored in cryotubes at − 80 °C until use.

We performed lumbar puncture in a lateral recumbent position for CSF collection, using a Quincke needle at the L3/L4 or L4/L5 level. We separated 1–2 mL of CSF for routine analysis (cell counts, glucose, and proteins). All remaining CSF (10–12 mL) was collected in polypropylene tubes and gently mixed to avoid gradient effects, centrifuged at 4 °C at 4000 g for 10 min to remove cells, aliquoted into 1-mL cryotubes, coded, and after stored at − 80 °C until use without preservatives added. CSF samples contaminated with blood (CSF red cells > 500 per $mm^3$) were excluded.

### Measurement of nitrite

Nitrite levels in plasma and CSF were measured by chemiluminescence as previously described (MacArthur et al. 2007). Briefly, to measure nitrite levels, 50 µl of plasma or CSF were injected into a solution of acidified tri-iodide, purging with nitrogen in line with ozone-based chemiluminescence using a Sievers® Nitric Oxide Analyzer 280 (GE Analytical Instruments, Boulder, USA). A standard curve to measurement was obtained by injections of sodium nitrite solution (0.01–1.00 µmol/L). Nitrite levels were reported in µmol/L.

### Measurement of nitrate

Nitrate levels in plasma and CSF were also measured by chemiluminescence. Fifty microliters of plasma or CSF were deproteinated by precipitation before measurement using 100 µL 100% ethanol at 4 °C, followed by agitation and resting for 30 min in freezer − 20 °C, and centrifuged

at 25 °C at 4000 g for 10 min. For measurement, 5 µL of prepared samples were injected into the purge vessel with a reducing agent [0.8% vanadium (III) chloride in 1 N hydrochloric acid at 95 °C], converting nitrate to NO, after being measured by ozone-based chemiluminescence NO analyzer. As a result, the essay converts all nitrite and nitrate to NO. Therefore, the preliminary result of this method is the sum of nitrite and nitrate levels on plasma and CSF. To obtain the nitrate levels, we subtracted from the sum of nitrite and nitrate of each sample the nitrite level, which was measured according to the methodology described above. Nitrate levels were reported in µmol/L.

### Statistical analysis

We applied logarithm transformation of the nitrite and nitrate levels and their CSF/plasma ratio to comply with the normal distribution assumption. We performed the *t* test or the one-way analysis of variance (ANOVA) (followed by the Tukey post hoc test for multiple comparisons) to compare two or more independent groups of continuous variables or the Pearson's correlation test. To confirm the results from ANOVA, we performed the analysis of covariance (ANCOVA) adjusting for confounding variables (sex, age at evaluation, disease duration, and levodopa equivalent daily dose—LEDD). We used the Chi-square test to compare categorical variables. All analyses were performed using SPSS for Windows version 23.0 (SPSS Inc., Chicago, USA), and graphical representations were generated using the R software version 4.0.4 and the R package *ggplot2*.

## Results

### Clinical and epidemiological data

We recruited a total of 76 participants for this study. Sixty-seven participants fulfilled inclusion and exclusion criteria. We did not perform CSF analysis in four patients with PD due to technical problems in the lumbar puncture. Data are summarized in Suppl Table 1 and 2.

There was no difference between groups in age at the time of evaluation, but males predominated in the groups of patients with and without LID. Patients in the PD-D group had earlier disease onset, longer disease duration, more extended levodopa therapy, and used higher doses of antiparkinsonian drugs than patients in the PD-ND group. However, the motor symptoms severity and disease staging were similar.

### Influence of collection and processing factors on plasma and CSF nitrite and nitrate levels

The median time between sample collection and freezing was 240 min (interquartile range 180–305). The median storage time of plasma and CSF for measurement of nitrate was 21 months (interquartile range 18–25) and 49 months for nitrite (interquartile range 46–53). These two intervals have not critically influenced nitrite (Fig. 1a–d) and nitrate levels (Fig. 1e–h), except for a weak correlation between plasma nitrate and time between blood collection and freezing ($r = 0.33$, $p = 0.02$; Fig. 1e). No participants were excluded due to CSF contamination with blood.

### Plasma and CSF nitrite levels

Plasma and CSF nitrite levels are shown in Fig. 2. CSF nitrite levels were higher in the PD-D group than in the PD-ND and HC groups [$F(2,47) = 5.89$, $p = 0.005$—PD-D group versus PD-ND group, $p = 0.05$; PD-D group versus HC group, $p = 0.005$] (Fig. 2b). Plasma nitrite levels were higher in the PD-ND group than in the HC group [$F(2,47) = 3.25$, $p = 0.04$—PD-ND group versus HC group, $p = 0.03$] (Fig. 2a). CSF/plasma ratio of nitrite was higher in the PD-D group than in the PD-ND group [$F(2,46) = 4.32$, $p = 0.019$—PD-D group versus PD-ND group, $p = 0.01$] (Fig. 2c). After adjusting for sex, age at evaluation, disease duration, and LEDD, CSF nitrite levels, and CSF/plasma ratio of nitrite were higher in the PD-D group than in the other groups (CSF nitrite levels adjusted $p = 0.022$; CSF/plasma ratio of nitrite: adjusted $p = 0.016$).

### Plasma and CSF nitrate levels

Plasma and CSF nitrate levels are shown in Fig. 2 and Suppl Table 2. CSF nitrate levels were higher in the PD-D group than in the HC group, but not higher than the PD-ND group [$F(2,47) = 5.26$, $p = 0.009$—PD-D group versus HC group, $p = 0.008$; PD-D group versus PD-ND group, $p = 0.07$] (Fig. 2e). Regarding plasma nitrate, there was no difference between groups [$F(2,47) = 1.74$, $p = 0.18$] (Fig. 2d). CSF/plasma ratio of nitrate was higher in the PD-D group than in the HC group [$F(2,45) = 7.65$, $p = 0.001$—PD-D group versus HC group, $p = 0.001$) (Fig. 2f]. After adjusting for sex, age at evaluation, disease duration, and LEDD, CSF nitrate levels, and CSF/plasma ratio of nitrate were higher in the PD-D group than in the HC group (CSF nitrate levels adjusted $p = 0.002$; CSF/plasma ratio of nitrite adjusted $p = 0.001$).

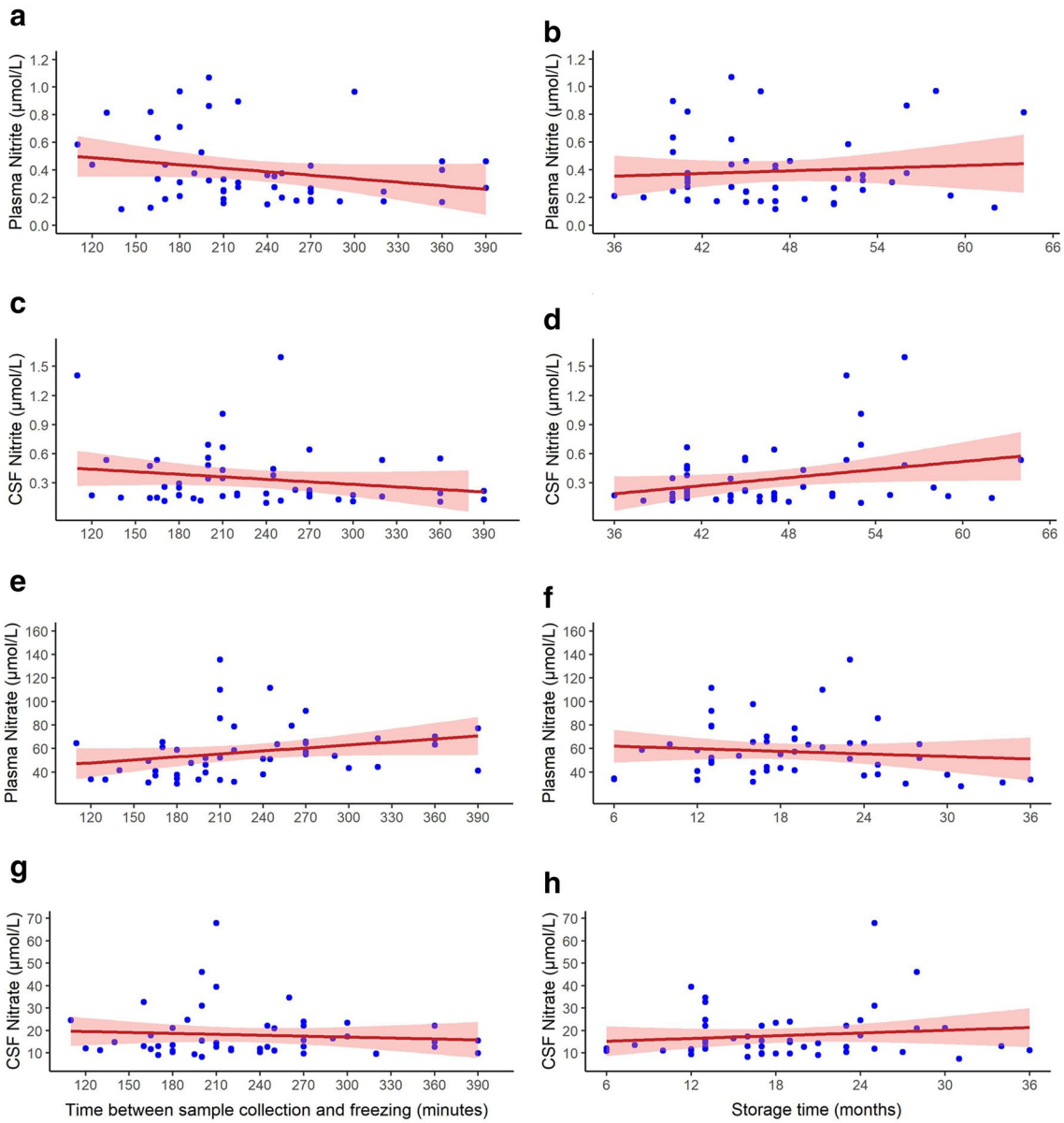


**Fig. 1** Correlations between processing time and storage time with plasma and CSF nitrite and nitrate levels. **A** Plasma nitrite and time between sample collection and freezing; **B** plasma nitrite and storage time; **C** CSF nitrite and time between sample collection and freezing; **D** CSF nitrite and storage time; **E** plasma nitrate and time between sample collection and freezing; **F** plasma nitrate and storage time; **G** CSF nitrate and time between sample collection and freezing; **H** CSF nitrate and storage time

## Associations between plasma and CSF nitrite and nitrate levels and clinical variables

Regarding only the PD-D group, there was a moderate negative correlation between plasma nitrite and historical subscore and total score of UDysRS (historical subscore $r = -0.57$, $p = 0.02$; total score $r = -0.59$, $p = 0.01$). Regarding only patients with PD, there was no correlation between nitrite and nitrate levels with age at PD onset, age at evaluation, disease duration, levodopa therapy duration, doses of antiparkinsonian drugs, MDS-UPDRS (total scores and subscores), and Hoehn and Yahr stage.

## Discussion

Our findings suggest that nitrite and nitrate levels are increased in the CSF of patients with PD and LID. Nitrite levels were increased in the CSF of the PD-D group compared to the PD-ND and HC groups. Furthermore, the CSF/

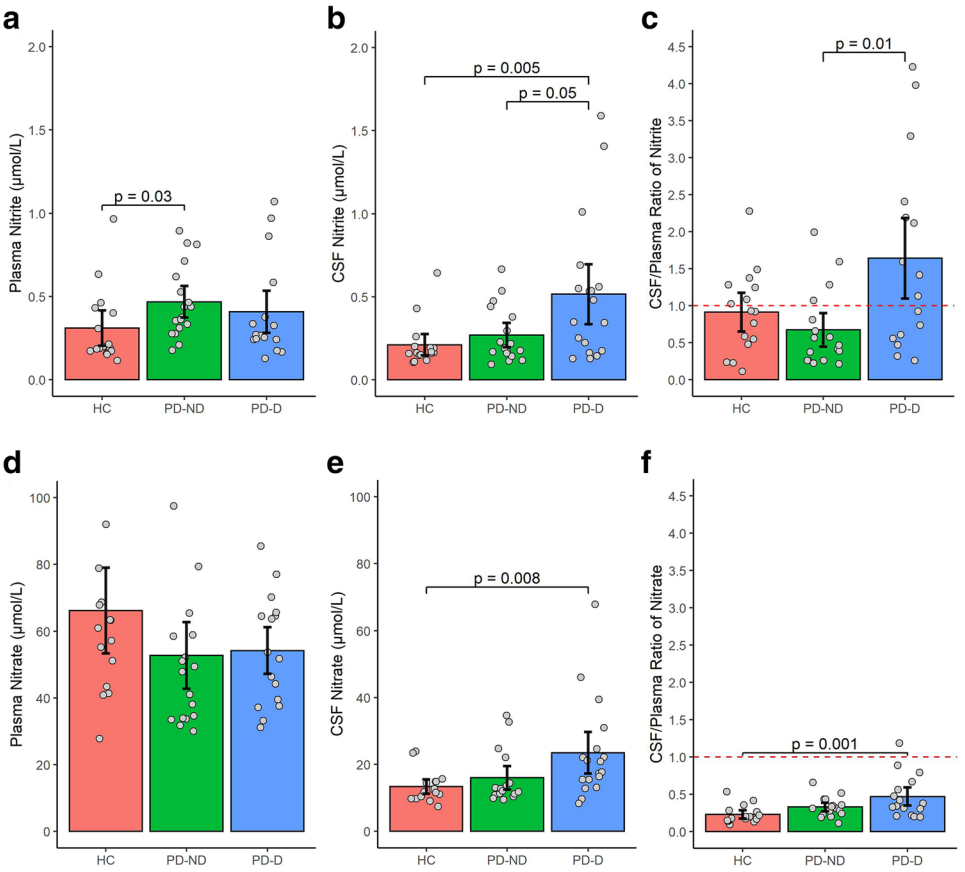


**Fig. 2** Plasma and CSF nitrite and nitrate levels and CSF/plasma ratio of nitrite and nitrate levels in patients with Parkinson's disease and healthy controls. **A** Plasma nitrite; **B** CSF nitrite; **C** CSF/plasma ratio of nitrite; **D** plasma nitrate; **E** CSF nitrate; **F** CSF/plasma ratio of nitrate. Black error bars indicate 95% confidence interval, grey circles indicate individual data values, and red dot lines indicate a CSF/plasma ratio of 1.0. To compare the nitrite and nitrate levels and CSF/plasma ratio between groups, we performed the one-way analysis of variance with the Tukey post hoc test for multiple comparisons (*p* values shown in the figure). Abbreviations: HC, healthy controls; PD-D, Patients with PD with LID; PD-ND, Patients with PD without LID

plasma ratio of nitrite in the PD-D group was higher than the PD-ND group, with a ratio over 1. Nitrate levels in CSF and CSF/plasma ratio of nitrate were also increased in the PD-D group compared to the HC group, but not higher than the PD-ND group. Processing and storage time did not influence nitrite and nitrate levels critically.

Higher CSF nitrite and nitrate levels in the PD-D group may suggest an increased synthesis of NO in these patients' brains, supporting the previous evidence of the role of NO in the pathophysiology of LID (Del-Bel et al. 2015). Preclinical results demonstrated an increase in the NO production in the brain of Parkinsonian rodents presenting LID (Pavón et al. 2006; Padovan-Neto et al. 2015). Considering that inducible nitric oxide synthase (iNOS) isoform, present in astrocytes and microglia, generates large amounts of NO in the central nervous system, the intrathecal production of NO and its metabolites may be influenced by neuroinflammation (Del-Bel et al. 2016).

The CSF/plasma ratio of nitrite showed increased values in the PD-D group, with a median ratio of 1.27, indicating that nitrite was generated at higher rates in the nervous system than in peripheral tissues. Multiple sclerosis and neuromyelitis optica are two inflammatory neurologic diseases in which clinical exacerbations are associated with a CSF/plasma ratio of nitrite higher than one (Danilov et al. 2003; Haghikia et al. 2015). A CSF/plasma ratio of nitrite higher than one may be a specific hallmark of neuroinflammation, also present in LID.

Regarding the source of CSF nitrite, there is evidence showing CSF nitrite and nitrate levels are independent of other body fluids, as the blood–brain barrier impedes the movement of these metabolites across compartments due to being charged moieties (Kuiper et al. 1994; Rejdak et al. 2003). Moreover, the fast conversion of nitrite to nitrate in circulation and the high renal excretion of NO metabolites would favor a movement from CSF into plasma to be rapidly excreted in the urine (Rejdak et al. 2003). Therefore, an inverse direction of nitrite from plasma into CSF would not be probable, supporting the NO pathway in the central nervous system as the source of CSF nitrite.

Our results agree with the previous studies that reported higher CSF nitrite levels (Qureshi et al. 1995) and nitrate levels (Boll et al. 2008) in patients with PD than controls (Table 1) (Molina et al. 1994; Cristalli et al. 2012; Kouti et al. 2013; de Farias et al. 2016; Medeiros et al. 2016; Çubukçu et al. 2016). However, some studies did not show the same findings (Ikeda et al. 1995; Molina et al. 1996; Shukla et al. 2006) and even described reduced CSF nitrite levels in PD (Kuiper et al. 1994). Likewise, the previous studies did not support the correlation between plasma

**Table 1** Studies with analysis of plasma and CSF nitric oxide metabolites in people with Parkinson's disease

| Author, Year | NO metabolite analyzed | N PD/Controls | Age at evaluation in PD | Disease duration (years) | Hoehn & Yahr stage | Method of detection | NO metabolites levels in PD (µmol/L) | Results in PD compared to controls |
|---|---|---|---|---|---|---|---|---|
| Plasma | | | | | | | | |
| Molina et al. (1994) | Nitrate | 68/68 | 65.8 | 5.3 | 2.5 | Griess | Nitrate 44.5 | Nitrate = |
| Molina et al. (1996) | Nitrate | 31/38 | 64 | 7 | 3.2 | Griess | Nitrate 34.1 | Nitrate = |
| Cristalli et al. (2012) | NOx | 87/134 | 70 | 6.6 | Not described | Not described | NOx 27 | NOx = |
| Kouti et al. (2013) | Nitrate | 58/15 | 64.4 | 5.4 | Not described | Griess | Nitrate 7.49 | Nitrate = |
| Medeiros et al. ( 2016) | NOx | 40/46 | 65.9 | 8.5 | 2.5 | Modified Griess | NOx 112.8 | NOx ↓ |
| de Farias et al. (2016) | NOx | 56/56 | 70.3 | 6.5 | Not described | Cadmium-based Semi-automated | NOx 5.57 | NOx = |
| Çubukçu et al. (2016) | Nitrite | 32/32 | 66.7 | 4.9 | 2.06 | Modified Griess | Nitrite 95.4 | Nitrite ↓ |
| Santos-Lobato et al. (this study) | **Nitrite and Nitrate** | **47/20** | **60** | **10.4** | **2.31** | **Chemiluminescence** | **Nitrite 0.43/ Nitrate 52.6** | **Nitrite = / Nitrate =** |
| HC Group | | 20 | 63.9 | Not applicable | Not applicable | | Nitrite 0.3 / Nitrate 66.14 | |
| PD-ND Group | | 23 | 61.6 | 8.43 | 2.21 | | Nitrite 0.46/ Nitrate 51.22 | |
| PD-D Group | | 24 | 58.9 | 12.45 | 2.41 | | Nitrite 0.4 / Nitrate 54.15 | |
| CSF | | | | | | | | |
| Kuiper et al. (1994) | Nitrite and Nitrate | 86/20 | 66 | Not described | Not described | Griess | Nitrite 0.5/ Nitrate 5.4 | Nitrite = / Nitrate ↓ |
| Ikeda et al. (1995) | NOx | 11/17 | 66 | Not described | Not described | Griess | NOx 5.8 | NOx = |
| Qureshi et al. (1995) | Nitrite | 16/14 | 70 | 5 | 2.25 | HPLC + Electrochemical Detection | Nitrite 0.02 | Nitrite ↑ |
| Molina et al. (1996) | Nitrate | 31/38 | 64 | 7 | 3.2 | Griess | Nitrate 7.5 | Nitrate = |
| Shukla et al. (2006) | Nitrite | 21/20 | 57 | 2.7 | 2.28 | Griess | Nitrite 5.8 | NOx = |
| Boll et al. (2008) | NOx | 22 | Not described | Not described | Not described | HPLC + Ultraviolet Detection | NOx 80 | NOx ↑ |
| Santos-Lobato et al. (this study) | **Nitrite and Nitrate** | **43/20** | **60** | **10.4** | **2.31** | **Chemiluminescence** | **Nitrite 0.4/ Nitrate 20.1** | **Nitrite ↑/ Nitrate ↑** |
| HC Group | | 20 | 63.9 | Not applicable | Not applicable | | Nitrite 0.21/ Nitrate 13.3 | |

**Table 1** (continued)

| Author, Year | NO metabolite analyzed | N PD/Controls | Age at evaluation in PD | Disease duration (years) | Hoehn & Yahr stage | Method of detection | NO metabolites levels in PD (μmol/L) | Results in PD compared to controls |
|---|---|---|---|---|---|---|---|---|
| PD-ND Group | | 20 | 61.6 | 8.43 | 2.21 | | Nitrite 0.27/ Nitrate 15.95 | |
| PD-D Group | | 23 | 58.9 | 12.45 | 2.41 | | Nitrite 0.51/ Nitrate 24.33 | |

The data in bold refer to the results of this study

*HPLC* high-performance liquid chromatography, *N* sample size, *NO* nitric oxide, *NOx* sum of nitrite and nitrate levels, *PD* Parkinson's disease, *HC* healthy controls, *PD-D* PD with LID, *PD-ND* PD without LID

↓ indicates NO metabolite levels are lower in patients with Parkinson's disease than in healthy controls; ↑ indicates NO metabolite levels are higher in patients with Parkinson's disease than in healthy controls; = indicates that there were no differences in NO metabolites levels between patients with Parkinson's disease and healthy controls. Except for the present study, other studies did not distinguish between patients with and without levodopa-induced dyskinesias. Age at evaluation, disease duration, Hoehn & Yahr stage, and NO metabolites levels are represented by mean

nitrite and the severity of LID shown in our findings. A recent systematic review with meta-analysis reviewed six articles that measured blood nitrite in PD. Patients with PD had higher blood nitrite levels than controls, with a high level of heterogeneity among studies (Wei et al. 2018), probably related to the different methods for measurement of nitrite and nitrate.

This study used an ozone-based chemiluminescence technique to measure nitrite and nitrate, considered as a direct method to detect smaller quantities of NO in real time with high accuracy (MacArthur et al. 2007). None of the previous studies involving nitrite and nitrate measurement in patients with PD employed this technique. Griess reaction, an indirect method with a low cost to measure NO by detecting nitrite in samples, was used in many previous studies (Kuiper et al. 1994; Ikeda et al. 1995; Qureshi et al. 1995; Molina et al. 1996; Shukla et al. 2006). However, the Griess reaction has some technical issues (external nitrite contamination, sample nitrite degradation, and reagent problems) (MacArthur et al. 2007), explaining different results from similar works.

As study strengths, we had efficient CSF collection and processing protocols without external factor influences. According to the Movement Disorders Society, we used one of the main instruments to evaluate LID (UDysRS) (Goetz et al. 2013), and our dyskinetic patients had average scores in UDysRS. The three groups (HC, PD-ND, and PD-D) were age-matched. As limitations, we had a small number of participants. Still, patients with PD and healthy controls were not sex-matched, and the difference in clinical severity between the two PD groups could affect statistical analyses. The inclusion of a drug-näive group of patients with PD could help to explore CSF nitrite and nitrate levels in untreated patients.

## Conclusions

The present study demonstrated that the central nervous system generates more NO in patients with PD and LID. This finding strengthens the role of NO on LID pathophysiology.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00702-021-02447-4.

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**Author contributions** All authors contributed to the study conception and design. In detail: conceptualization: BLS-L, MB, EAD-B, and VT; methodology: BLS-L, MB, EAD-B, and VT; clinical data collection: BLS-L, ÂVP, and VT; nitrite and nitrate analysis: LCP, MEB, and EC-C; formal analysis and investigation: BLS-L, L CP, EAD-B, and VT; writing—original draft preparation: BLS-L; writing—review and editing: B LS-L, LCP, EAD-B, and VT; funding acquisition: EAD-B and VT; supervision: EC-C, EAD-B, and VT.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** Dr. TUMAS received honoraria from Teva Brasil, UCB Biopharma and Ipsen, and travel support for medical conferences from Roche.

**Ethical approval** The study was approved by institutional review board of the Ribeirão Preto Medical School (Number 3.036.243), and each participant provided written informed consent to participate.

**Consent to participate** Authors declare that informed consents for participation were obtained from all participants. Moreover, informed consents for lumbar puncture examination were also obtained from all participants.

**Consent for publication** Authors declare that consent for publication of blinded data was a part of informed consents obtained from participants.

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