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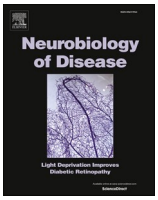

# Differential profiles of serum cytokines in Parkinson's disease according to disease duration

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ABSTRACT

*Objective:* Neurodegeneration and neuroinflammation are two intertwined mechanisms contributing to the pathophysiology of Parkinson's disease. Whether circulating biomarkers reflecting those two processes differ according to disease duration remains to be established. The present study was conducted to characterize the biomarkers individuals with PD with short (≤5 years) or long disease duration (>5 years).

*Methods:* We consecutively enrolled 104 patients with Parkinson's disease and evaluated them using validated clinical scales (MDS-UPDRS, Hoehn and Yahr staging, MMSE). Serum samples were assayed for the following biomarkers: neurofilament light chain (NfL), brain-derived neurotrophic factor (BDNF), interleukin (IL-) 1β, 4, 5, 6, 10, 17, interferon-γ, and tumor necrosis factor α.

*Results:* Mean age of participants was 66.0 ± 9.6 years and 45 (34%) were women. The average disease duration was 8 ± 5 years (range 1 to 19 years). Patients with short disease duration (≤ 5 years) showed a pro-inflammatory profile, with significantly higher levels of pro-inflammatory IL-1β and lower concentrations of IL-5, IL-10 and IL-17 ($p < 0.05$). NfL serum levels showed a positive correlation with disease duration and age (respectively rho = 0.248, $p = 0.014$ and rho = 0.559, $p < 0.001$) while an opposite pattern was detected for BDNF (respectively rho −0,187, $p = 0.034$ and rho = −0.245, p = 0.014).

*Conclusions:* Our findings suggest that a pro-inflammatory status may be observed in PD patients in the early phases of the disease, independently from age.

## 1. Introduction

The main mechanism underlying Parkinson's disease (PD) is commonly identified with the degeneration of the nigrostriatal pathway. However, it is not clear which is the *primum movens* of the degenerative process. Several evidence support the role of neuroinflammation as a contributor to the development and progression of PD (Harms et al., 2021; Hirsch and Hunot, 2009). Indeed, the inflammatory processes seem to be involved in the protein misfolding cascade. On the other hand, abnormal forms of α-synuclein (α-syn) deposition leads to oxidative stress and inflammation, in a vicious circle (Calabresi et al., 2023; Hirsch and Standaert, 2021). In fact, preclinical evidence demonstrated that abnormal α-syn trigger innate immunity responses via leucine-rich-repeat and pyrin-domain-containing3 (NLRP3) inflammasome and toll-like receptors (TLRs) (Panicker et al., 2019; Mazzotta et al., 2023), leading to the release of pro-inflammatory cytokines. This promotes α-syn internalization, transport, and degradation via lysosomal pathway by neurons and glial cells. However, when these mechanisms reach saturation, the partial protein degradation leads to further intracellular accumulation and neuroinflammation (Lindström et al., 2017). Chronic neuroinflammation could, therefore, be seen both as an expression of the neurodegenerative process and as a process inducing neurodegeneration itself. Indeed, it has been established that chronic neuroinflammation can lead to the alteration of the blood–brain barrier, allowing

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chemokines and cells from the peripheral immune system to enter the central nervous system (CNS) and activate resident glial cells, T lymphocytes, and mast cells (Jung et al., 2022). The activation of inflammation in the CNS would accelerate the neurodegenerative process, causing oxidative stress, and inflicting damage to the dopaminergic neurons (Kempuraj et al., 2017; Chen et al., 2023).

However, several questions remain open. When does the neuroinflammatory process start? Is it more prominent in the earlier or later stages of the disease? Which could be the optimal time-window to eventually intervene with anti-inflammatory approaches?

Inflammatory markers, such as soluble cytokines and chemokines measured in cerebrospinal fluid (CSF) or in blood samples, have been found to be increased in PD patients as compared to healthy controls (Brodacki et al., 2008), however their relationship with disease stages has not been defined yet.

In the present study, we measured the levels of a selected panels of pro- and anti-inflammatory cytokines in patients with PD of different disease duration, to test the hypothesis of a major role of neuroinflammation in the earliest phases of the disease. We also evaluated the profiles of biomarkers of neurodegeneration in the serum, such as neurofilament light chain (NfL), and that of the brain-derived neurotrophic factor (BDNF) in a cohort of people with PD.

## 2. Materials and methods

### 2.1. Patients' enrolment and clinical measures

PD patients were consecutively recruited at the Movement Disorders outpatient service, at Gemelli Hospital, Rome, Italy, between February 2022 and September 2022.

Inclusion criteria were the diagnosis of a clinically defined idiopathic PD according to the Movement Disorders Society (MDS) criteria (Postuma et al., 2015) and age between 18 and 80 years old. Exclusion criteria included ongoing inflammatory or autoimmune diseases, pregnancy or the presence of a known pathogenetic variant in genes associate with PD. The study was approved by the local Ethical Committees (ID 4687) and was carried out according to the Declaration of Helsinki. All patients included signed a written informed consent.

All patients underwent a clinical evaluation in the best ON-medication state by a neurologist expert in movement disorders. Motor impairment and disease stage were assessed by means of MDS Unified Parkinson's Disease Rating Scale (MDS -UPDRS parts I-IV) and Hoehn & Yahr staging scale, respectively (Goetz et al., 2008; Martinez-Martin, 2010). Non-motor symptoms burden was evaluated with the Non-motor Symptoms Questionnaire (NMSQuest) Global cognitive abilities were evaluated by Mini Mental Status Evaluation Scale (MMSE) (Hoops et al., 2009; Chaudhuri et al., 2006).

### 2.2. Biochemical measures

Blood samples were collected in the morning by venipuncture of the median cubital vein after overnight fasting, using commercial collection tubes (BD Vacutainer®). Serum separation was obtained after 30 min of clotting at room temperature and subsequent centrifugation at 1000 xg for 15 min at 4 °C. The upper clear fraction (serum) was collected in 0.5 mL aliquots. The aliquots were stored at −80 °C until analysis.

The patient's inflammatory profile was assessed by measuring Th1, Th2 and Th17 cytokines in the serum. In particular, the levels of Interleukin (IL)-1β, Interferon-γ (IFN- γ) and Tumor Necrosis Factor α (TNF-α) for Th1 inflammatory response, IL-4, IL-5, IL-6 as representative of Th2 response, IL-10 for both Th1 and Th2 inflammatory response, and IL-17 for the Th17 one were determined using custom-designed multiplex assays (Calvani et al., 2020; Chen et al., 2020). Furthermore, NfL was assayed as biomarker of neurodegeneration and BDNF as growth factor. All determinations were carried out in serum using commercially available kits on an ELLA™ automated immunoassay system (Bio-Techne, San Jose, CA, USA) according to the manufacturer's instructions.

### 2.3. Data collection

Data collection was realized by a neurologist expert in movement disorders. The following data were recorded for all study participants: age, sex, family history of movement disorders, clinical characteristics (age at onset, premorbid symptoms, concomitant neurological medications, presence of fluctuations and wearing-off and comorbid conditions), morphological and/or functional imaging performed (brain MRI or CT, 123I-FP-CIT-SPECT). Ongoing dopaminergic treatments were recorded, and L-dopa Equivalent Daily Dose (LEDD) was calculated.

### 2.4. Statistical analysis

Continuous clinical and demographic features of the cohort considered are summarized as mean ± standard deviation (SD), and categorical features as count and percent. Distribution was checked for normality with Levene's test. The correlation of biomarker values with clinical scores and demographic data was performed by means of Spearman's correlation coefficients. A cut-off of 5 years was set to analyze data in patients with a short disease course vs. patients with long disease course as this threshold is classically reported to be the length of the "levodopa honeymoon", before motor fluctuations appear (Alonso-Canovas et al., 2023). To study the different biomarkers expression in patients with long and short disease duration, a logistic regression model with the binomial variable "long" and "short" disease duration as dependent variable and age as covariate was used. *P*-values smaller than 0.05 have been considered statistically significant. All the *p*-values were corrected for multiple testing according to Benjamini and Hochberg. The statistical analysis was conducted using the program "Statistical Package for Social Science (SPSS)", version 26 for MAC.

## 3. Results

### 3.1. Clinical-demographic characteristics

Clinical-demographical characteristics are summarized in Table 1. 104 PD patients were enrolled in the study, 59 male (57%) and 45 female (43%), with a mean age of 66 ± 9.55 years. The mean disease duration was 8 ± 5 years, with a range from 1 to 19 years. At the time of clinical observation, patients presented a mild-moderate parkinsonism with mean MDS-UPDRS III score of 22.2 ± 13. Considering MDS-UPDRS III subscores, the mean score assigned to the area of speech was 0.9 ± 0.7, to facial expression 1.2 ± 0.6, to tremor 3.3 ± 2.5, to rigidity 4.7 ± 3.1, to bradykinesia 8.4 ± 4.9, to gait and posture 2.9 ± 5.8 and to global spontaneity of movement (body bradykinesia) 1.2 ± 0.6.

**Table 1**
Clinical-demographical characteristics of PD patients. LEDD = levodopa equivalent daily dose; UPDRS = Unified Parkinson's disease rating scale; y = years; mg = milligrams.

| | Patients ($n$ = 104) |
|---|---|
| Sex (m/f) | 59 (57%) / 45 (43%) |
| Age | 66 ± 9.55y |
| Family history of movement disorders | 34 (33%) |
| Age at onset of symptoms | 59 ± 10y |
| Laterality of onset (right / left / bilateral) | 50 (53%) / 41 (43%) / 4 (4%) |
| Motor fluctuations (y/n) | 42/62 |
| Disease duration | 8 ± 5y |
| LEDD | 581 ± 403 mg |
| MDS-UPDRS I | 1.9 ± 1.5 |
| MDS-UPDRS II | 8.8 ± 5.7 |
| MDS-UPDRS III | 22.2 ± 13 |
| MDS-UPDRS IV | 0.3 ± 1.5 |
| NMSQuest | 3.6 ± 2.4 |

As expected, a positive correlation between disease duration and MDS-UPDRS II (rho = 0.569, $p < 0.001$) and IV (rho = 0.284, $p = 0.004$) scores was found. Also, longer disease duration correlated with higher UPDRS III (rho = 0.436, $p < 0.001$).

Regarding non-motor symptoms, 35 patients (34%) had anosmia, 55 (53%) suffered from stypsis, 32 (31%) from depression and 44 (42%) had RBD. The mean NMSQuest score was 3.6 ± 2.4. NMSQuest positively correlated with UPDRS III score and with LEDD (respectively, rho = 0.253, $p = 0.013$; rho = 0.272, $p = 0.008$).

Among patients under levodopa, motor fluctuation occurred in 42 (45%). Fluctuations started at a mean age of 62 ± 9.3 years old, and after a mean time from the diagnosis of 7 ± 4.1 years and a mean duration of levodopa therapy of 5 ± 4.3 years. The age of onset of symptoms was significantly earlier in patients with fluctuations than in patients without (average 55 ± 10 years vs 62 ± 10 years, $p = 0.01$). Moreover, patients with fluctuations had higher MDS-UPDRS III and a higher LEDD intake compared with those without (UPDRS III: 25 ± 13 vs 20 ± 9, $p = 0.028$, LEDD: 837 ± 411 vs 475 ± 269, $p < 0.001$).

### 3.2. Biomarkers profile

The mean concentration of mediators measured in the serum of all participants are reported in Table 2. Biomarkers showed an equal distribution between males and females. No significant correlation was found between biomarkers levels and LEDD.

#### 3.2.1. Inflammatory biomarkers

Serum concentrations of IL-1β, IL-5 and IL-6 positively correlated with age (IL-1β: rho = 0.042, $p = 0.042$; IL-5: rho = 0.239, $p = 0.018$; IL-6: rho = 0.288, $p = 0.004$, respectively). Also, significant correlations between inflammatory biomarkers levels and clinical scores were observed. In particular, higher IL-1β levels correlated with higher MDS-UPDRS I (rho = 0.308, $p = 0.039$), as well as IFN-γ levels positively correlated with a NMSQuest scores (rho = 0.211, $p = 0.047$).

#### 3.2.2. Differences in patients with short and long disease course

A cut-off of 5 years was set to analyze data in patients with a short disease course vs. patients with long disease course. 35 patients (34%) had a short disease course (< 5 years old) while 69 (66%) showed a longer disease duration. Clinical-demographical characteristics are reported in Table 3. Patients with a shorter and longer disease duration showed a different inflammation profile (Table 4, Fig. 1), independently from age. In particular, patients with a shorter disease duration showed higher levels of pro-inflammatory cytokines (IL-1β, $p = 0.048$) and lower levels of those driving a Th2 and Th17 anti-inflammatory response (IL-5, IL-10, IL-17, $p = 0.049$, $p = 0.023$ and $p = 0.018$ respectively). Moreover, those with a longer disease duration showed higher levels of NfL ($p < 0.001$).

#### 3.2.3. Neurodegeneration biomarkers and BDNF

BDNF concentrations were negatively correlated with age (rho = −0.245, $p = 0.014$, Fig. 2A), while higher NfL levels correlated with advancing age (rho = 0.559, $p < 0.001$, Fig. 2B). BDNF showed an opposite trend (rho = −0.187, $p = 0.034$, Fig. 2C), while higher levels of NfL correlated with longer disease duration (rho = 0.248, $p = 0.014$,

**Table 2**
Mean ± SD concentration of selected biomarkers.

| Biomarker | mean ± SD |
|---|---|
| NfL | 21.87 ± 9.04 pg/ml |
| BDNF | 39,960 ± 7551 pg/ml |
| IL-1β | 0.60 ± 0.53 pg/ml |
| TNF-α | 12.13 ± 2.29 pg/ml |
| IFN-γ | 0.78 ± 0.32 pg/ml |
| IL-4 | 0.16 ± 0.11 pg/ml |
| IL-5 | 0.38 ± 0.25 pg/ml |
| IL-6 | 3.59 ± 1.91 pg/ml |
| IL-10 | 2.51 ± 1.14 pg/ml |
| IL-17 | 1 ± 0.98 pg/ml |

**Table 3**
Clinical-demographical differences between short and long disease duration groups.

| | Short disease duration ($n = 35$) | Long disease duration ($n = 69$) | *P* value |
|---|---|---|---|
| Sex (m/f) | 19/16 | 40/29 | $p =$ 0.88 |
| Age | 64 ± 10y | 68 ± 9.5y | $p =$ 0.37 |
| Family history of movement disorders | 10 (29%) | 24 (35%) | $p =$ 0.67 |
| Age at onset of symptoms | ***63 ± 9y*** | ***58 ± 9y*** | ***p = 0.002*** |
| Laterality of onset (right / left / bilateral) | 17 (49%) / 16 (45%) / 2 (6%) | 33 (53%) / 25 (44%) / 2 (3%) | $p =$ 0.75 |
| Motor fluctuations (y/n) | ***3/32*** | ***39/30*** | ***p < 0.001*** |
| LEDD | ***312 ± 192 mg*** | ***752 ± 410 mg*** | ***p < 0.001*** |
| MDS-UPDRS I | 2 ± 1 | 2 ± 1.4 | $p =$ 0.96 |
| MDS-UPDRS II | 6 ± 4 | 10 ± 5.9 | $p =$ 0.06 |
| MDS-UPDRS III | ***16 ± 8*** | ***26 ± 14.3*** | ***p = 0.021*** |
| MDS-UPDRS IV | 0 | 1 ± 1.9 | $p =$ 0.12 |
| NMSQuest | 3 ± 2 | 4 ± 2.5 | $p =$ 0.45 |

**Table 4**
Biomarkers levels differences between short and long disease duration groups.

| Biomarker | Short disease duration ($n = 35$) | Long disease duration ($n = 69$) | P value |
|---|---|---|---|
| NfL (pg/ml) | ***19.16 ± 7.8*** | ***24.21 ± 10.1*** | ***<0.001*** |
| BDNF (pg/ml) | 40,238 ± 7061 | 38,694 ± 7656 | 0.094 |
| IL-1β (pg/ml) | ***0.69 ± 0.5*** | ***0.55 ± 0.48*** | ***0.048*** |
| IFN-γ (pg/ml) | 0.78 ± 0.31 | 0.78 ± 0.32 | 0.28 |
| TNF-α (pg/ml) | 12.13 ± 2.3 | 12.19 ± 2.21 | 0.281 |
| IL-4 (pg/ml) | 0.14 ± 0.08 | 0.16 ± 0.11 | 0.249 |
| IL-5 (pg/ml) | ***0.29 ± 0.16*** | ***0.41 ± 0.28*** | ***0.049*** |
| IL-6 (pg/ml) | 3.49 ± 2.21 | 3.49 ± 1.63 | 0.34 |
| IL-10 (pg/ml) | ***2.43 ± 1.22*** | ***2.57 ± 1.05*** | ***0.023*** |
| IL-17 (pg/ml) | ***0.62 ± 0.37*** | ***1.12 ± 0.99*** | ***0.018*** |

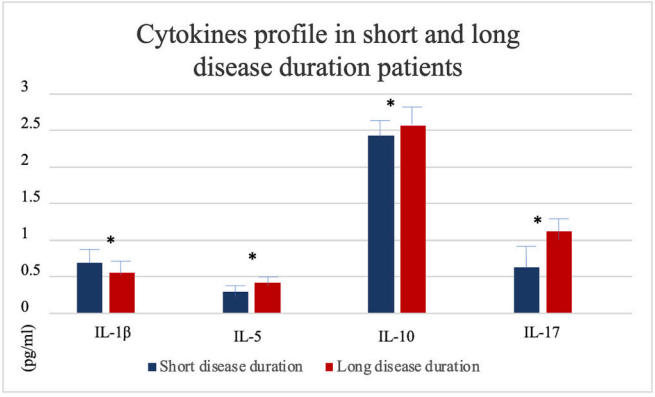


**Fig. 1.** Selected biomarkers levels differences between short and long disease duration groups.

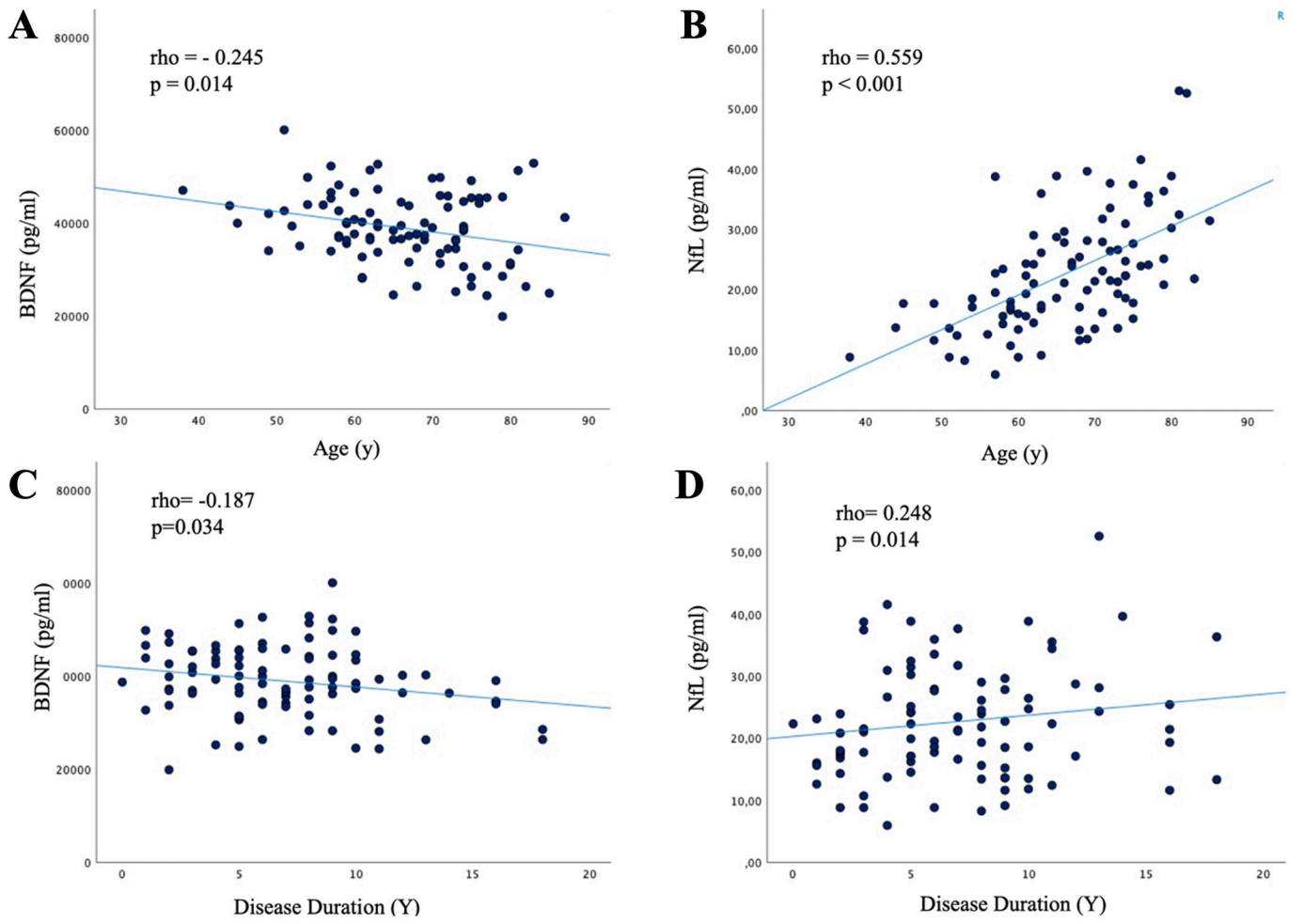


**Fig. 2.** A: Inverse correlation between BDNF levels and age (rho = − 0.245, $p = 0.014$). B: Positive correlation between NfL level and age (rho = 0.559, $p < 0.001$). C: Negative correlation between BDNF levels and disease duration (rho = −0.187, $p = 0.034$). D: Positive correlation between NfL levels and disease duration (rho = 0.248, $p = 0.014$).

Fig. 2D). Tremor dominant patients showed higher levels of BDNF ($p = 0.045$). NfL levels also positively correlated with MDS-UPDRS I (rho = 0.332, $p = 0.027$) and MDS-UPDRS II (rho = 0.377, $p = 0.015$) scores.

## 4. Discussion

In this cross-sectional study, we investigated whether the levels of peripheral inflammatory biomarkers differ in patients at different stages of the disease, independently from age. Also, to enlighten the potential role of neuroinflammation in different PD characteristics, we investigated correlations between these selected biomarkers and clinical variables.

From the clinical point of view, as expected, longer disease duration was associated with a more severe impairment as assessed through motor (MDS-UPDRS III) and non-motor (NMSQuest) clinical scales and to higher LEDD. MDS-UPDRS III positively correlated with NMSQuest and LEDD, indicating that the progression of motor and non-motor symptoms proceeded at the same pace and was associated with a gradual increase in the need for dopamine-replacement therapy. Fluctuations occurred more frequently in patients with earlier disease onset and were related to more critical clinical conditions and to a higher Levodopa intake.

About neuroinflammation, we found a different profile between patients with longer and shorter disease duration. In fact, lower levels of IL-5, IL-10 and IL-17 and higher level of IL-1β were found in patients with a more recent disease onset. IL-1β is a potent proinflammatory mediator involved in many reactions, including the recruitment of innate immune cells and modulation of adaptive immunity. IL-1β maturation and activation is driven by the inflammasomes, multiprotein complexes that activate caspase-1 (Muthuraman et al., 2012). On the other hand, IL-5, IL-10, and IL-17 have different immunomodulatory roles. IL-5 is one of the main cytokines involved in the Th2-response, and IL-10 has a prominent role in inhibiting cytokine production by Th1 cells, thus exerting an anti-inflammatory effect (Swain et al., 1988; Kokubo et al., 2022; Saraiva et al., 2019). IL-17 is the signature cytokine of a subset of CD4+ helper T cells known as Th17 cells, which promote inflammatory response along with tissue repair, usually in a chronic inflammation context (Chen et al., 2020). This cytokine seems to play a role in several neurodegenerative diseases such as Alzheimer's disease, PD, Amyotrophic lateral sclerosis, multiple sclerosis, contributing to the maintenance of a chronic inflammatory environment activating glial cells (Di Filippo et al., 2021; Gelders et al., 2018; Chen et al., 2018). In this context, our finding strongly emphasizes the role of inflammation in the first phase of the disease, followed by a shift toward a Th2 and Th17 driven response in the later stages (Chen et al., 2020). This is in line with previous findings showing that patients with shorter disease duration had lower levels of IL-5, IL-10 and IL-17 (Lindestam Arlehamn et al., 2020). Age also seems to shape the profile of peripheral cytokines as younger patients showed higher levels of IL-1β and lower of IL-10, independently from disease duration. These data, along with previous preclinical evidence (Panicker et al., 2019; Lindström et al., 2017; Chen et al., 2023), support a major role of neuroinflammation in the earlier

stages of the disease and in younger patients, defining a population more susceptible to therapeutic interventions both pharmacological and non-pharmacological, aiming at blunting neuroinflammation. Indeed, drugs modulating the inflammatory processes are under investigation (NCT04015076 (Gordon et al., 2018), NCT05790382 (Reilly et al., 2013), NCT05083260 (Reading et al., 2021)) (Hirsch and Hunot, 2009; Balzano et al., 2022). To define the patients' population which could benefit the most from this approach would help designing clinical trials envisaging a more tailored therapeutic approach.

With respect to biomarkers profiles, BDNF levels decreased with advancing age and disease duration; these findings are in line with the current knowledge of BDNF as a relevant role in neuronal survival (Kowiański et al., 2018). Decrease of circulating levels of BDNF in people with PD may be explained by decline of dopaminergic neurons expressing BDNF. Indeed, the tremor-dominant group, which is typically associated with a more benign course, had higher BDNF levels compared with the akinetic-rigid group. Greater neuroprotective mechanisms may be in place in these patients.

As for NfL, higher levels of NfL were related to longer disease duration and severity of motor impairment. This result may be consistent with more white matter axonal degeneration in advanced disease and adds to the hypothesis of NfL as a biomarker of disease progression (Lerche et al., 2020; Parnetti et al., 2019). While the evaluation of NfL in CSF has been widely recognized as a biomarker of PD, our data indicated that also serum NfL may be a reliable biomarker accurately reflecting the disease course, more accessible in the clinical practice, also for longitudinal evaluations.

One limitation of the study is the relatively limited sample size and the lack of longitudinal measures. As widely known, PD has a high interpersonal variability, with very different disease course and progression, and people have a variable lifestyle, including aspects such as physical activity which have a known influence on inflammatory and neurotrophic factors. We tried to mitigate parkinsonism heterogeneity including only patients with a clinically defined diagnosis of PD, excluding those presenting red flags which have higher risk of developing atypical parkinsonism. We also excluded patients undergoing regular intense physical activity. However, our findings should be confirmed by longitudinal studies with larger sample size. Lastly, the choice to assay biomarkers in serum has the advantage of being more accessible but exposes to a higher risk of obtaining less reliable data, as they can be influenced by peripheral conditions.

Our study has also several strengths that deserve discussion. First, the clinical assessment was carried out by an established group of clinicians. Also, a comprehensive number of mediators were analyzed in a fairly large number of participants. Our results on inflammatory mediators and their association with disease duration allowed us to gather indications on the profile of inflammatory profile of study participants for which we could observe specific cytokine patterns and association with disease duration. Indeed, we do not have any reference to the minimum cytokine variations corresponding to a biological meaning, nor our experimental setting could allow establish the biological meaning of the difference identified in cytokine variations, even considering the lack of a control population, Nonetheless, we could verify that the changes observed may be biologically meaningful as the decline of the pro-inflammatory cytokine IL-1b in participants with long disease duration is paralleled with an increase of the anti-inflammatory IL-10. Furthermore, an interesting and possibly supporting finding is that the concentration of α-Klotho, a protein regulating aging-related processes (neuroinflammation, oxidative stress) seems to follow the same trend of IL-1b along the disease course (Sancesario et al., 2021; Grillo et al., 2022). Moreover, our findings are supported by findings in several preclinical studies and, to the best of our knowledge, is the first report on such a wide biomarkers panel in patients with PD at different stages, filling a gap of knowledge and providing an insight on the different preponderance of the inflammatory processes along the disease course.

## 5. Conclusion

The present study shows that patients with PD have a different inflammatory profile according to disease duration. A shorter disease duration is associated with a more pro-inflammatory cytokine expression, which later in the disease course seems to switch toward a Th2- and Th17- response. These findings are important for clarifying the role of neuroinflammation in PD pathogenesis, matching previous preclinical evidence. Moreover, peripheral inflammatory biomarkers profile could be useful in identifying patients targetable by immunomodulating drugs.

Our data also confirm an association of serum NfL and PD and indicate that NfL levels reflect disease duration and severity at all stages. These observations also suggest that the monitoring of serum NfL levels in at-risk populations, such as REM sleep behaviour disorder patients and LRRK2 or GBA carriers, might provide a strategy to identify individuals for preventive treatments and immunotherapy approaches. Even if preliminary, our data represent promising basis for future studies, in which a neuroinflammatory profiles could be explored in a deeper manner and targeted approaches could be tested.

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## Author roles

(1) Research Project: A. Conception, B. Organization, C. Execution;
(2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique;
(3) Manuscript Preparation: A. Writing of the First Draft, B. Review and Critique.

GDL: 1A,1B,1C, 2A, 2B, 3A
AP: 1A, 1C, 2C, 3B
EM: 3B
MP: 1A, 3B
FB: 1A, 2C, 3B
CP: 3B
ARB: 3B
PC: 1A, 2C, 3B

## Ethical compliance statement

The authors confirm that the approval of an institutional review board was not required for this work. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines.

## Financial disclosures for previous 12 months

None relevant for this work.

## CRediT authorship contribution statement

**Giulia Di Lazzaro:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Picca:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Sofia Boldrini:** Investigation, Data curation. **Francesco Bove:** Writing – review & editing, Investigation. **Emanuele Marzetti:** Writing – review & editing. **Martina Petracca:** Writing – review & editing, Investigation. **Carla Piano:** Writing – review & editing. **Anna Rita Bentivoglio:** Writing – review & editing, Supervision, Resources. **Paolo Calabresi:** Writing – review & editing, Supervision, Resources, Conceptualization.

## Declaration of Competing Interest

The authors report no sources of funding and no conflicts of interest.

## Data availability

The data that has been used is confidential.

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