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Plasma Cystatin C correlates with plasma NfL levels and predicts disease progression in Parkinson's disease

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ABSTRACT
Introduction: Previous studies reported increased plasma levels of Cystatin C (Cys-C) in Parkinson's disease (PD) and claimed for a possible association with disease severity and progression. The aim of this study was to evaluate plasma Cys-C in PD and healthy controls (HC) and test its association with markers of peripheral inflammation, neurodegeneration and clinical progression in a longitudinal study.
Methods: Plasma Cys-C, high-sensitive C-reactive protein (hsCRP), interleukin 6 (IL-6) and Neurofilament Light Chain (NfL) were assessed at the baseline in 71 consecutive non-demented PD and 69 HC. PD patients underwent an extensive motor and cognitive assessment at baseline and after 2 years of follow-up. The association of Cys-C with disease severity was evaluated in a multilinear model adjusted for the effect of age, sex, disease duration and peripheral inflammation.
Results: Cys-C levels appeared to be higher in PD compared to controls and correlated with the plasma neuronal marker NfL ($r = 0.204$, $p = 0.046$). In longitudinal analyses, PD patients with higher Cys-C levels exhibited faster motor progression at two years of follow-up independently from the peripheral inflammatory profile.
Conclusions: Cys-C was associated with higher NfL levels and a remarkably faster motor progression in PD independently from peripheral inflammation. Further studies are needed in order to understand the mechanisms underpinning the association of Cys-C with higher neuronal damage markers in neurodegenerative diseases.

INTRODUCTION

Parkinson's disease (PD) represent a neurodegenerative condition characterized by an heterogeneous clinical presentation and prognosis [1–4]. Recently, faster PD progression have been associated with increased level of CSF and blood neurofilament light chain (NfL) as proxy of neuronal damage [5–9]. Still, the biological factors underpinning the wide clinical heterogeneity and individual vulnerability to neurodegeneration are theme of debate. Several preclinical and clinical studies claimed for an important role of inflammation as modulator of neurodegeneration in alpha-synucleinopathies. Specifically, high levels of peripheral pro-inflammatory mediators and lower levels of anti-inflammatory cytokines have been associated with early DLB stages [10] and faster progression in PD [11].
Cystatin-C (Cys-C) is a protein made up of 120 amino acids known to be a cysteine proteases inhibitor and a reliable biomarker of kidney disfunction [12] with anti-amyloidogenic properties [13,14]. Cys-C has been also reported to regulate several biological processes, such as matrix proteases activity, inflammation and autophagy [15,16]. Recently, a preclinical study suggested that Cys-C can increase *in vitro* the neurotoxicity of glial-mediated inflammatory response in rat dopaminergic cells [17]. In addition, few cross-sectional studies indeed claimed for an association between elevated blood levels of Cys-C with disease severity measures in PD [18,19].
In this work, we evaluate the relationship between Cys-C, neuronal and inflammatory peripheral markers in PD and we test Cys-C as potential predictor of clinical progression in a longitudinal study.

METHODS

Patients selection

Consecutive patients with a clinical diagnosis of PD [20] were evaluated at the outpatient Movement disorder Clinic, Neurology Unit at the University of Brescia, Italy from October 2016 to March 2018 for baseline enrolment. Healthy controls (HC) were selected among patients' caregivers at the university of Brescia and from the Biobank (BioBIM®) of the IRCCS San Raffaele Roma, Italy. This study was approved by the local ethics committee (NP 1471, DMA) and was in conformity with the Helsinki Declaration. Informed consent was obtained from all participants. Levodopa equivalent daily dose (LEDD) was calculated according to standard conversion [21] and the diagnosis of PD was supported by levodopa/dopaminergic response and at least two years of clinical follow-up. All patients underwent routine blood analyses, magnetic resonance imaging to exclude prominent cortical or subcortical infarcts or brain/iron accumulation or atypical parkinsonian disorders and [^{123}I]FP-CIT SPECT imaging to support

the diagnosis [22]. The following exclusion criteria were applied: (1) atypical parkinsonism, at baseline or during follow-up; (2) prominent cortical or subcortical infarcts in structural imaging; (3) other neurologic disorders or medical conditions potentially associated with cognitive deficits; (4) bipolar disorder, schizophrenia, history of drug or alcohol abuse or impulse control disorder; (5) negative nigrostriatal dopaminergic imaging; (6) recent traumatic events and acute or chronic fever/inflammation (potentially influencing plasma biomarkers levels); (7) acute and chronic kidney disease as well as chronic inflammatory diseases or immunomodulator treatments potentially influencing levels of Cys-C, high-sensitive C-reactive protein (hsCRP) and interleukin 6 (IL-6).

Clinical and neuropsychological assessment

At baseline, standardized neurological examination was performed, including the Movement Disorder Society - Unified Parkinson Disease Rating Scale (MDS-UPDRS) [23] and Hoehn and Yahr stage (H&Y) assessment [24]. A comprehensive, standardized cognitive and behavioural assessment was applied, including Mini-Mental State Examination (MMSE) and ten tests covering five cognitive domains as previously described [25]. All patients included in the analyses underwent a complete motor and cognitive assessment follow-up at 2 years as previously described [8].

Biochemical analyses

hsCRP, IL-6 and Cys-C blood measurement were performed at the Laboratory of Biochemical Analyses, University of Brescia, Brescia, Italy using Commercially available assays according to manufacturer's instruction. At the time of assessment, approximately 10 ml of venous blood was collected in glass tubes containing lithium heparine (LH) from each subject. Participants were required to fast for at least 2 h prior to collection. The blood samples were centrifuged at 2000 x g at 4 °C for 8 min within 2 h of collection. Plasma supernatant was collected, divided into aliquots, and frozen at − 80 °C until further use. Plasma Cys-C concentration was assessed using Tina-quant Cystatin C Gen.2 (Roche Diagnostics, Mannheim, Germany). hsCRP concentration was measured with Cardiac C-reactive protein (Latex) high sensitive – Cobas c 701 System (Roche Diagnostics, Mannheim, Germany). IL-6 concentration was measured with Elecsys® IL-6 immunoassay – Cobas e801 System (Roche Diagnostics, Mannheim, Germany).
NfL blood measurement were performed at the Maurice Wohl Clinical Neuroscience Institute, London, UK. Plasma NfL concentration was measured using the Simoa platform (NF-light; Quanterix, Billerica, MA). Samples were randomized, blinded and measured in duplicate using a batch of reagents from the same lot. The intra-assay and inter-assay coefficients of variation were 8.1 and 11.2%, respectively. The lower limit of quantification was 0.136 pg/mL and the lower limit of quantification (LLOQ) was 0.696 pg/mL when compensated for a 4-fold sample dilution. Outliers with plasma biomarkers value above 3 interquartile range from the 75° percentile were excluded from the study analysis [26].

Statistical analyses

Differences in demographic features between PD and HC were assessed with Mann-Whitney *U* test and chi square test for continuous and dichotomic variables, respectively. Comparisons of Cys-C, hsCRP, IL-6 and NfL levels between PD and HC were evaluated using age- and sex adjusted one-way ANCOVA.
Partial correlation analyses adjusted for the effect of age, sex and disease duration was applied in order to test significant relationships between plasma biomarkers, NfL (as neuronal marker) and clinical variables. Each patient was then stratified according to the cut-off of Cys-C corresponding to the mean plus one standard deviation in “high” (H-Cys-C) and “normal” Cys-C (N-Cys-C) subgroups. The ability of Cys-C to predict motor and cognitive impairment was assessed using a 2-way repeated measures mixed ANCOVA adjusted for age, sex, disease duration, IL-6 (as marker of peripheral inflammation) and baseline clinical measures (namely MDS-UPDRS-III for motor and MMSE for cognitive progression). The ability of Cys-C to predict clinical progression was additionally tested in a multiple linear regression model with ΔMDS-UPDRS-III ($MDS\text{-}UPDRS\text{-}III_{2\text{-}years} - MDS\text{-}UPDRS\text{-}III_{baseline}$) as dependent variable and taking in consideration the effect of age, sex and disease duration. SPSS 26 (IBM, Armonk, NY) was used for statistical analyses.

RESULTS

Recruitment, clinical and demographic features

Seventy-nine patients with parkinsonism were screened for enrollment, and 71 patients with a confirmed diagnosis of PD after two years of follow-up were included in the final analyses. Eight patients were excluded because an atypical parkinsonism ascertained at baseline (n = 2 progressive supranuclear palsy and n = 2 multiple system atrophy), severe vascular chronic encephalopathy by brain MRI (n = 2), or lost at follow-up for death (n = 2). Sixty-nine healthy controls were included. PD patients were younger compared to HC, but showed similar sex distribution (Table 1).

Plasma inflammatory biomarkers

Between-groups comparisons of plasma biomarkers are shown in Table 1. In age- and sex-adjusted analyses PD exhibited higher levels of Cys-C and IL-6 than HC, while there were no significant between-group differences in hsCRP levels (Fig. 1). In the whole cohort (including PD and HC) Cys-C, IL-6 and hsCRP correlated with age (Cys-C: $\rho = 0.328$; $p = 0.001$; IL-6: $\rho = 0.423$; $p < 0.001$; hsCRP: $\rho = 0.328$; $p < 0.001$). hsCRP and IL-6 were slightly higher in males (hsCRP: males 2.12 ± 2.03 mg/L, females: 1.78 ± 2.33 mg/L, $p = 0.048$; IL-6: males 3.51 ± 1.67 pg/mL, females 2.70 ± 1.22 pg/mL; $p = 0.020$), while there were no significant associations of Cys-C with sex. Age-adjusted analyses in PD revealed a positive correlation between Cys-C and hsCRP ($r = 0.300$; $p = 0.017$) and between Cys-C and IL-6 ($r = 0.242$; $p = 0.052$).

Plasma inflammatory biomarkers and NfL levels

In age- and sex-adjusted analyses, NfL levels appeared to be slightly increased in PD compared to controls (Table 1).

In the whole cohort and PD patients, Cys-C exhibited a positive correlation with NfL ($r = 0.270$; $p = 0.022$ for all and $r = 0.310$; $p = 0.014$ for PD) (Fig. 2), whereas no significant correlation between NfL and hsCRP ($r = -0.014$; $p = 0.890$) or IL-6 ($r = -0.026$; $p = 0.799$) were observed.

Cys-C and clinical measures of disease severity at baseline

In PD, Cys-C showed a positive correlation with H&Y stage (Spearman $\rho = 0.267$; $p = 0.026$) and a negative correlation with MMSE (Spearman $\rho = -0.293$; $p = 0.026$), not confirmed after age and sex adjustment. Conversely, IL-6 correlated with MDS-UPDRS-III ($r = 0.298$; $p = 0.005$) and H&Y stage ($r = 0.245$, $p = 0.022$) in adjusted analyses, whereas hsCRP was not associated with clinical measures of disease severity.

Longitudinal progression of patients according to Cys-C stratification

In PD, patients with higher Cystatin levels (H-Cys-C) were older and showed higher NfL levels than patients with normal levels (N-Cys-C) (Suppl. Table 1). In the 2-years longitudinal follow-up, H-Cys-C showed faster MDS-UPDRS-III progression (N-Cys-C: 0.8 ± 9.6 vs H-Cys-C: 9.7 ± 9.6 points, Fig. 3 and Suppl. Table 1) using repeated-measures mixed ANCOVA adjusted for the effect of age, sex, disease duration, baseline MDS-UPDRS-III and IL-6 [group*time interaction: $F = 6.410$; $p = 0.014$; partial $\eta^2 = 0.101$]. Within-group post-hoc analysis revealed a significant longitudinal increase of MDS-UPDRS-III in the H-Cys-C group only ($p = 0.002$; Bonferroni corrected). Moreover, Cys-C was confirmed as predictor of changes within MDS-UPDRS-III progression taking in consideration age, sex, disease duration and IL-6 as independent variables in a multiple linear regression model ($\beta = 0.381$; $p = 0.017$) (Table 2). The ANCOVA and linear regression analyses did not reveal any association between Cys-C and cognitive progression over time.

DISCUSSION

This is to the best of our knowledge the first longitudinal study to evaluate plasma Cys-C as biomarker of disease progression in PD. Findings showed that PD patients with higher Cys-C levels exhibited faster motor progression over a 2-years follow-up, as measured by MDS-UPDRS-III changes, after adjusting for age, sex, disease duration and peripheral inflammation. In addition, the present work provides the first evidence of a significant correlation between plasma levels of Cys-C and NfL, a well-known marker of axonal damage in neurodegenerative diseases [7,8,27,28].

According to previous cross-sectional reports we found increased plasma levels of Cys-C in PD compared to HC [18,19,29–31](Suppl. Table 2). Cys-C is a cysteine proteases inhibitor ubiquitously expressed and secreted into all body fluids [15,32]. It has been proposed as a regulator of several cellular functions, such as apoptosis, autophagy and inflammatory response [16]. In line with this, several preclinical investigations have suggested that Cys-C

could be also an important player in neurodegenerative processes. There are indeed evidences suggesting that Cys-C may exert a neuroprotective role through inhibition of cathepsins-mediated proteolysis [33,34]. Other previous *in vitro* and *in vivo* data from Alzheimer's disease (AD) models have shown that soluble Cys-C has also anti-amyloidogenic properties [14,35]. Co-incubation of Cys-C with monomeric $A\beta_{42}$ attenuated the formation of Aβ oligomers and protofibrils *in vitro* [35,36], while increased expression of Cys-C reduced parenchymal Aβ load in mouse models of AD [14,37]. However, investigations assessing blood Cys-C in AD gave conflicting results, with reports of serum/plasma levels unchanged [38], increased [39] and decreased in AD [40] or decreased levels associated with increased risk of future AD [41]. Importantly, Cys-C colocalizes with amyloid plaques in brain parenchyma and vascular walls of AD and cerebral amyloid angiopathy patients [42], while CSF Cys-C levels were decreased in AD patients compared to HC [43,44].

In Parkinson's disease, the preclinical studies addressing the role of Cys-C gave conflicting results. On one hand, Cys-C was able to rescue dopaminergic cell loss in 6-hydroxydopamine models [45] whereas the injection of Cys-C promoted angiogenesis and autophagy, thus reducing apoptosis in several brain regions in α-synuclein transgenic rats [46]. On the other, a recent work proposed Cys-C as a mediator of neurodegeneration, able to induct microglial activation and consequent neurotoxic factors release in rat dopaminergic cell cultures [17]. The present study added an important piece to this complex picture, revealing a strong correlation between plasma levels of Cys-C and NfL, a highly reliable biomarker of neuronal damage [8,27,47]. This association is of particular relevance because constitutes the first evidence of a direct link between neurodegeneration and Cystatin C, independently from peripheral inflammation. These findings fit with the ability of Cys-C to predict motor progression in PD observed after 2-years follow-up. These results are clinically significant, as the mean increase of MDS-UPDRS-III between baseline and 2-years timepoint was 8.9 point higher in H-Cys-C than N-cys-C subgroup. It should be also underlined that these findings were obtained after adjusting for the effects of age, sex, disease duration, baseline MDS-UPDRS-III and IL-6 and then strengthened by a multivariable linear regression model (Table 2).

Several limitations should be acknowledged. First, a 2-year follow-up period did not permit to properly evaluate the association between plasma biomarkers and cognitive progression, as the changes in general cognition is quite low in this timeframe [4]. Thus, these results cannot be considered as definitive and they definitively need further validations in longer on-going larger longitudinal studies. Second, the relatively small sample size required the use of non-parametric statistical analysis thus potentially increasing the risk of effect underestimation. Third, the patients included were well into the disease course and replication in de novo – PD cohorts are thus warranted in order to confirm the value of Cys-C in this specific population suitable of neuroprotective intervention trials. The major strengths of the study were the exclusion of patients with inflammatory conditions and kidney disfunction, the evaluation of inflammatory/neuronal markers and conservative statistical models adopted both in cross-sectional and longitudinal analyses, which gives reliability to the results. Notably, plasma Cys-C can be easily and quickly measured by most clinical laboratories, differently from the majority of the putative markers currently emerging in PD research (e.g. NfL). Moreover, the availability of well-defined reference values in the healthy subjects and the low cost of measurement would facilitate the use of Cys-C in PD clinical practice.

In summary, the present study provides the first evidence of a strong association between plasma levels of Cys-C and NfL, a reliable marker of neuronal damage. Moreover, higher baseline levels of Cys-C predicted faster motor progression in PD patients. Despite the limitations associated with an observational single-center study, our results significantly advance the evidences suggesting a role of Cys-C as modulator of neurodegenerative processes and potential marker of progression in alpha-synucleinopathies.

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STATEMENT OF ETHICS

This study protocol was reviewed and approved by Brescia Hospital Ethic Committee, approval number NP 1471, DMA. Written informed consent was obtained from all participants.

CONFLICT OF INTEREST STATEMENT

All the authors report no disclosures related to this manuscript. Other disclosures are listed below.
Alberto Imarisio has no financial conflicts to disclose.

Andrea Pilotto served in the advisory board of Z-cube (technology division of Zambon pharmaceuticals); he received honoraria from Z-cube s.r.l., Abbvie, Bial, Biomarin, Zambon, Nutricia and Chiesi pharmaceuticals. He received research support from Vitaflo Germany and Zambon Italy.
Emirena Garrafa has no financial conflicts to disclose.
Francesca Conforti has no financial conflicts to disclose.
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Cristina Gussago has no financial conflicts to disclose
Marina Pizzi has no financial conflicts to disclose.
Fiorella Guadagni has no financial conflicts to disclose.
Henrik Zetterberg has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).
Nicholas Ashton has no financial conflicts to disclose.
Abdul Hye has no financial conflicts to disclose.
Alessandro Padovani is consultant and served on the scientific advisory board of GE Healthcare, Eli-Lilly and Actelion Ltd Pharmaceuticals, received speaker honoraria from Nutricia, PIAM, Lansgstone Technology, GE Healthcare, Lilly, UCB Pharma and Chiesi Pharmaceuticals. He is founded by Grant of Ministry of University (MURST).

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AUTHOR CONTRIBUTIONS
Alberto Imarisio: study concept and design, acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.
Andrea Pilotto: study concept and design, acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.
Emirena Garrafa: acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.
Francesca Conforti: acquisition of data, drafting/revising the manuscript for content.
Stefano Masciocchi: acquisition of data, drafting/revising the manuscript for content
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Nicholas Ashton: acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.
Abdul Hye: acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.
Alessandro Padovani: acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

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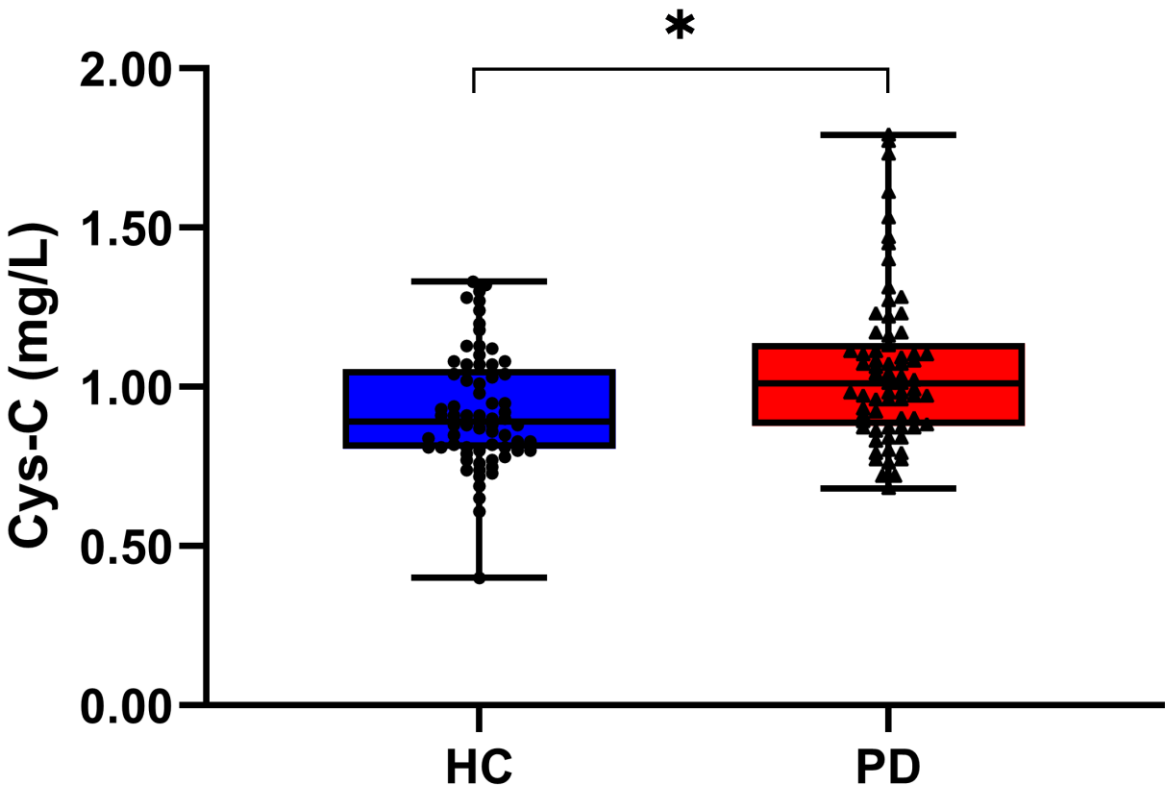
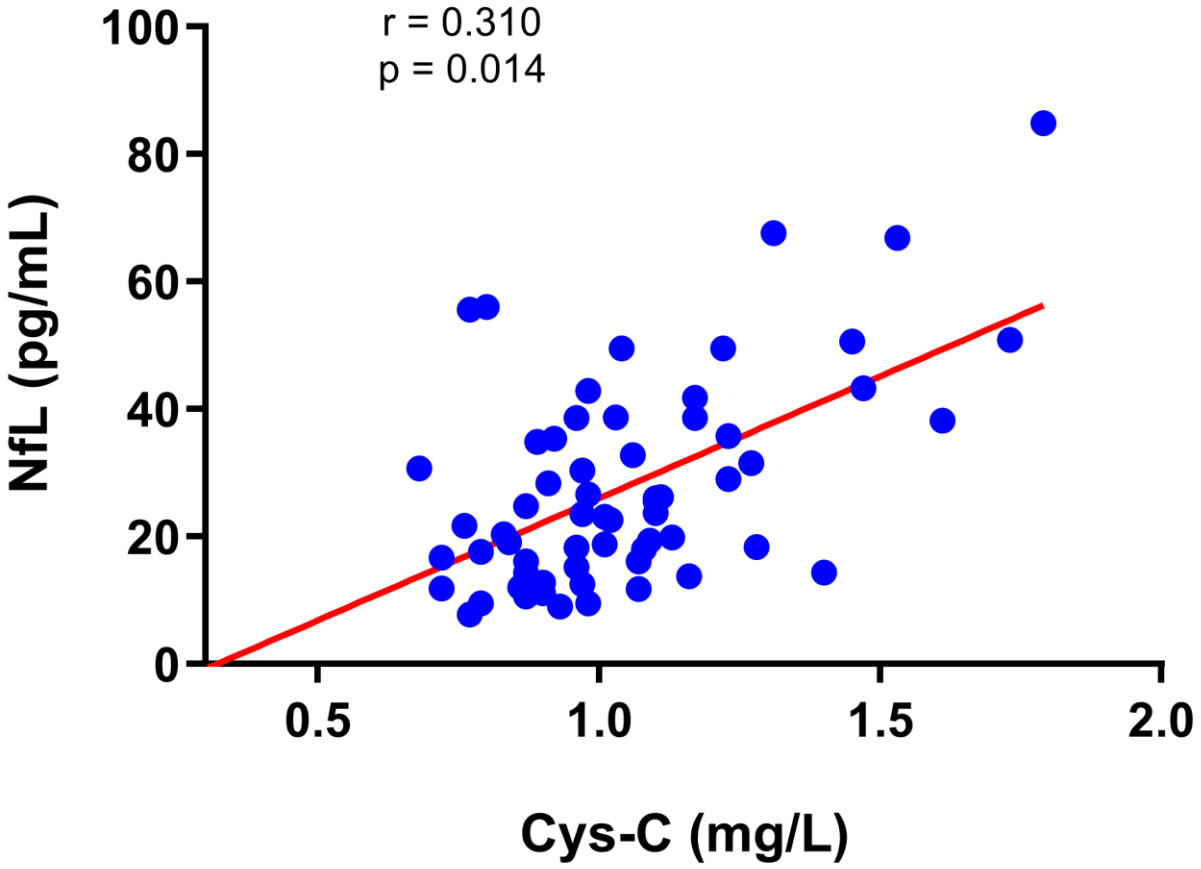
FIGURE LEGENDS

Fig. 1. Box and whiskers plot showing plasma Cys-C levels in HC and PD patients. Data are shown as medians with 25◦–75◦ percentiles (boxes), min-max values (whiskers), and individual values (dots). **Abbreviations:** HC, healthy controls; PD, Parkinson's disease patients; $*p < 0.001$.

Fig. 2. Age-adjusted correlation between plasma Cys-C and NfL in PD cohort. **Abbreviations:** Cys-C, cystatin C; NfL, neurofilament light chain.

Fig. 3. Motor progression between H-Cys-C and N-Cys-C subgroups. Dots represent the mean of each group, while bars represent the standard error of the mean. **Abbreviations:** H-Cys-C and N-Cys-C: patients with high and normal levels of plasma Cys-C, respectively; MDS-UPDRS-III, Movement Disorder Society Unified Parkinson's Disease Rating Scale, part III.





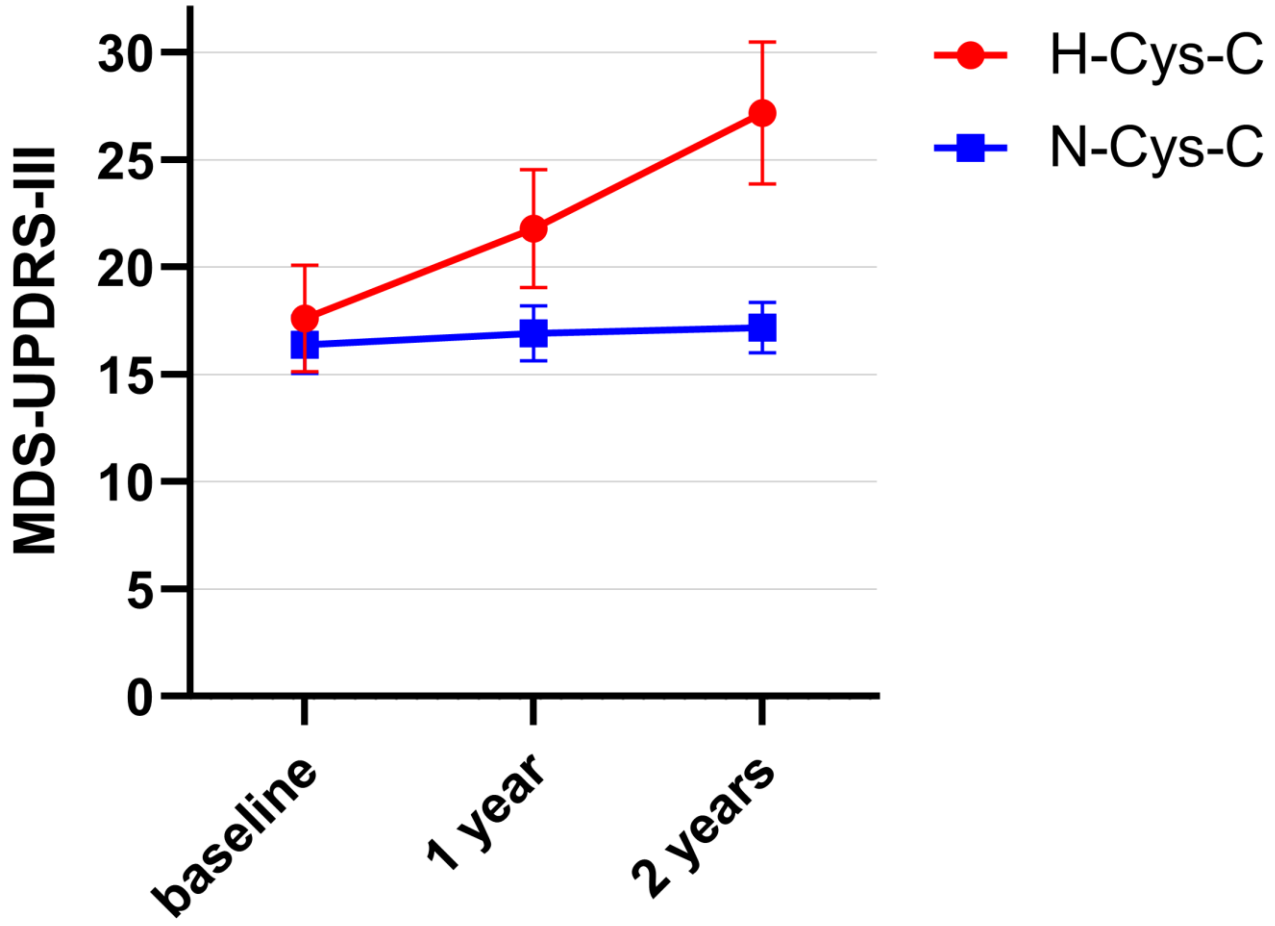


Table 1. Demographic and clinical features of participants at baseline and after 2-years follow-up.

	HC (n = 69)	PD (n = 71)	p-value
Age (years)	71.7 ± 7.1	65.1 ± 10.5	**0.003**[α]
Male Sex, n (%)	41 (59.4)	46 (64.8)	0.595[ζ]
Disease duration, years	-	5.2 ± 7.0	-
MDS-UPDRS-III	-	16.6 ± 10.1	-
LEDD, mg/day	-	374.2 ± 344.6	-
MMSE, total score	-	27.6 ± 2.5	-
Cys-C (mg/L)	0.92 ± 0.18	1.05 ± 0.24	**< 0.001**[γ]
hsCRP (mg/L)	2.15 ± 2.04	1.51 ± 1.62	0.061[γ]
IL-6 (pg/mL)	2.04 ± 1.91	3.27 ± 1.57	**< 0.001**[γ]
NfL (pg/mL)	25.7 ± 10.6	27.7 ± 16.3	**0.046**[γ]
2 years follow-up			
MDS-UPDRS-III	-	18.2 ± 9.6	-
ΔMDS-UPDRS-III	-	1.6 ± 3.5	-
LEDD, mg/day	-	560.8 ± 387.5	-
ΔLEDD, mg/day	-	186.6 ± 195.5	-
MMSE	-	25.1 ± 4.0	-
ΔMMSE	-	-2.3 ± 3.1	-

Table 2. Multivariable linear regression model for motor progression in PD cohort defined by changes in MDS-UPDRS-III score including demographic variables, plasma Cys-C and IL-6 levels. In the first line "B" refers to unstandardized coefficients, while Beta refers to the standardised coefficients.

Model for MDS-UPDRS-III progression

Independent variables	B	Standard error	Beta	t	p-value
Constant	-6,889	8,730		-0,789	0,433
age	-0,116	0,137	-0,129	-0,846	0,401
sex	1,889	2,468	0,097	0,765	0,447
Disease duration	-0,223	0,176	-0,157	-1,263	0,212
Cys-C	14,517	5,918	**0,381**	2,453	**0,017**
IL-6	-0,031	0,084	-0,046	-0,367	0,715

Abbreviations: Cys C, Cystatin C; IL-6, interleukin 6.