

Phosphorylation of RPS6 at Ser235 and Il-6 Release Emerge as Components of a Peripheral Inflammatory Signature in Parkinson's Disease Manifestation

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Abstract

Background

Parkinson's disease (PD) is a neurodegenerative disorder traditionally viewed as a central nervous system disease, but increasing evidence indicates that peripheral immune alterations contribute to disease pathophysiology. Moreover, the molecular features of peripheral blood mononuclear cells (PBMCs) in idiopathic and genetic forms of PD remain incompletely understood.

Methods

We performed mass spectrometry-based proteomic and phosphoproteomic profiling of PBMCs obtained from healthy controls, patients with idiopathic PD (iPD), and carriers of the LRRK2 G2019S mutation with (L2PD) or without (L2NMC) motor manifestations. Key findings were validated using biochemical and functional approaches in patient-derived PBMC cultures, including assessment of interleukin-6 (IL-6) secretion, and by evaluating downstream signaling effects in primary mouse astrocytes exposed to PBMC-conditioned media.

Results

Distinct proteomic and phosphoproteomic patterns were observed across PD groups, with consistent enrichment of ribosome-related pathways in manifesting disease. Phosphorylation of ribosomal protein S6 at Ser235 (pSer235-RPS6) was increased in iPD and LRRK2-associated PD but not in non-manifesting mutation carriers. pSer235-RPS6 levels correlated with clinical severity across multiple Unified Parkinson's Disease Rating Scale domains. PBMCs derived from patients with iPD released markedly higher levels of IL-6 compared with other groups. Moreover, conditioned media from iPD PBMC cultures induced RPS6 phosphorylation in primary astrocytes, supporting a functional link between peripheral immune signaling and inflammatory glial responses.

Conclusions

These findings define a disease-associated peripheral immune signaling state in Parkinson's disease, characterized by coordinated activation of translational and inflammatory pathways. Phosphorylation of ribosomal protein S6 at Ser235 and increased IL-6 release distinguish manifest from non-manifest disease across idiopathic and LRRK2-associated PD, correlate with clinical severity, and occur independently of direct LRRK2 kinase activity. Together, these results support a functional link between peripheral immune dysregulation and central nervous system-relevant signaling, highlighting peripheral translational control as a biologically meaningful and accessible marker of disease manifestation with translational relevance for patient stratification and monitoring of phenoconversion.

BACKGROUND

Parkinson's disease (PD) is a progressive neurodegenerative disorder traditionally defined by dopaminergic neuronal loss and motor impairment, affecting 1% of the population over 60 years, with no cure yet [1]. PD is also increasingly recognized as a systemic condition involving peripheral immune dysfunction. Altered cytokine profiles, chronic low-grade inflammation, and immune cell activation are consistently observed in patients with PD and correlate with both

motor and non-motor symptoms [2–5]. These findings suggest that peripheral immune signaling is not merely a consequence of neurodegeneration but may actively participate in disease manifestation and progression.

Peripheral blood mononuclear cells (PBMCs) provide an accessible and biologically relevant window into these systemic alterations. Beyond their role as immune effectors, PBMCs integrate metabolic, inflammatory, and translational signals that reflect cellular activation states. While transcriptional and cytokine changes in PBMCs have been described in PD [2, 3, 6], the post-translational signaling mechanisms that define disease-associated immune states, particularly those linked to protein synthesis and translational control, remain poorly characterized.

Emerging evidence implicates dysregulation of the translational machinery as a convergent feature of neurodegenerative disorders. Ribosomal proteins and their phosphorylation states are central regulators of selective mRNA translation, coupling extracellular inflammatory cues to intracellular responses through pathways such as PI3K–Akt–mTOR [7, 8]. In PD, alterations in translational control have been reported in vulnerable brain regions [8–10]. However, whether similar ribosome-centered signaling changes occur in peripheral immune cells, and whether they associate with clinical disease manifestation, is largely unknown.

Genetic forms of PD offer a unique opportunity to dissect disease-associated signaling states independently of overt neurodegeneration. Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene represent the most common monogenic cause of PD yet display incomplete and age-dependent penetrance [11, 12]. Many LRRK2 mutation carriers remain asymptomatic for decades, indicating that additional molecular events are required for the transition from a non-manifesting to a clinically manifest disease state, a process known as phenoconversion [12].

Importantly, LRRK2 is highly expressed in immune cells [13] and has been linked to inflammatory and translational pathways [14, 15], making LRRK2 carriers an informative model in which to study peripheral signaling changes associated with disease manifestation.

Notably, increased LRRK2 activity has also been reported in idiopathic PD [16], suggesting convergence between genetic and sporadic forms at the level of downstream signaling rather than mutation-specific effects. However, the few established LRRK2 substrates do not fully explain the spectrum of immune and inflammatory alterations observed in PD [17, 18], raising the possibility that disease manifestation is defined by broader signaling states that extend beyond direct LRRK2 kinase activity.

Here, we combined unbiased proteomic and phosphoproteomic profiling of PBMCs from healthy controls, patients with idiopathic PD, and LRRK2 G2019S mutation carriers with or without motor manifestations, with functional and clinical validation approaches. We hypothesized that PD manifestation is associated with a distinct peripheral signaling state characterized by altered translational and inflammatory pathways. Our data identify phosphorylation of ribosomal protein S6 at Ser235 and interleukin-6 (IL-6) release as components of a peripheral inflammatory and translational signature that distinguishes manifest from non-manifest disease and links peripheral immune activation to central nervous system–relevant signaling.

METHODS

Participants

Blood samples from 120 patients with idiopathic PD (iPD), G2019S carriers (L2PD and L2NMCs), and healthy controls (CT) were collected at the Hospital Clínic, Barcelona's Movement Disorders Unit, IDIVAL/Hospital Marqués de Valdecilla, Santander and Biodonostia Health and Research Institute/Hospital Universitario de Donostia. Patients

with PD were clinically identified according to the UK PD Society Brain Bank guidelines and having more than one affected relative with PD was not an exclusion condition. PBMC sample pools from N = 120 participants were included in the investigation; these subjects included CT (n = 36), patients with G2019S L2PD (n = 29), G2019S L2NMCs (n = 18) and iPD patients (n = 37) (Table 1). Every subject was a Caucasian person of Spanish ancestry. Patients with G2019S L2PD were matched by age and sex to healthy CTs, based on availability. All participants provided written informed consent, and the study was approved by the Hospital Clínic de Barcelona local ethics committee.

Table 1

Participant clinical features and demographics. Data are expressed as the mean $\pm$ standard deviation (SD), with the number of available subjects/totals in parentheses. AAO = age at onset; C = controls; iPD = idiopathic PD; L2NMC = LRRK2 non-manifesting carriers; L2PD = LRRK2-associated PD patients; MoCA = Montreal cognitive assessment; PD = Parkinson's disease; UPDRS I-IV = Unified Parkinson's Disease Rating Scale; – = not applicable;

Cohort	N (males/females)	Age at sampling (years)	PD AAO (years)	UPDRS- I	UPDRS- II	UPDRS- III	UPDRS- IV	MoCA
Entire cohort	120							
Controls	36 (12/24)	57,80		0,21	0,16	0,50	0,16	19,04
L2NMC G2019S	18 (12/6)	52,22		1,13	2,01	0,99	0,49	27,46
L2PD G2019S	29 (17/12)	62,29	53,76	2,55	9,71	13,64	2,68	25,81
iPD	37 (21/16)	66,94	61,11	3,91	11,57	22,37	3,87	25,07
Barcelona	61							
Controls	21 (7/14)	64,90		0,62	0,48	1,50	0,48	27,86
L2NMC G2019S	7 (4/3)	42,71		0,71	2,57	0,17	0,71	28,71
L2PD G2019S	14 (6/8)	63,71	53,09	2,69	10,08	8,75	3,08	26,82
iPD	19 (14/5)	68,26	64,00	2,95	6,95	15,00	1,17	27,16
Santander	42							
Controls	7 (1/6)	54,00		0,00	0,00	0,00	0,00	0,00
L2NMC G2019S	11 (8/3)	61,73		1,55	1,45	1,82	0,27	26,20
L2PD G2019S	15 (11/4)	60,87	54,43	2,40	9,33	18,53	2,27	24,80
iPD	9 (5/4)	67,22	61,78	2,67	9,67	17,78	1,89	23,56
Donostia	17							
Controls	8 (4/4)	54,5		0	0	0	0	29,25
L2NMC G2019S	0	-						
L2PD G2019S	0	-						
iPD	9(2/7)	65,33	57,56	6,11	18,11	34,33	8,55	24,5

PBMC isolation

Peripheral vein puncture was used to obtain 8 mL of blood while fasting. Sodium-citrate collector tubes (BD Vacutainer CPT, Franklin Lakes, New Jersey, United States, #EAN30382903627821) were used to isolate PBMCs using a density gradient. After being flash-frozen in liquid nitrogen, dry pellets were kept at -80°C until needed, not more than one year. Blood collection and flash-freezing of dry pellets took an average of 4 hours. For target validation, PBMCs were cryo-preserved in DMEM culture media with 10% DMSO and stored at -80°C (decreasing 1 degree per minute) overnight before transferring them to liquid nitrogen (-196°) for long-term preservation.

Proteomics

Protein extraction, quantitation, digestion, phosphopeptide enrichment, peptide purification and liquid chromatography mass spectrometry analysis was performed as previously described [17].

MS data and search results files are available in the Proteome Xchange Consortium via the PRIDE partner repository [19] (<https://www.ebi.ac.uk/pride/>) with the dataset identifiers PXD050865 for the proteome and PXD050944 phospho-proteome analyses. DOI: 10.1093/nar/gkab1038

Identification of Differential Proteins and Phospho-Proteins

To account for the multiple testing problem in our phosphoproteomic and proteomic datasets, we estimated the effective number of tests (ENT) using the eigenvalues of the correlation matrix, as previously described in [20]. This method evaluates the overall correlation structure among variables by computing the variance of the eigenvalues derived from the correlation matrix. The performed analysis took into consideration the ENTs to minimize the impact of dependent variable error. Based on the ENTs, we considered significant pairwise comparisons with a significance threshold of $p\text{-value} < 0,05$ and fold-change (as calculated by $2^{(\text{mean difference})}$) with a difference of at least 0.3 in both senses (*i.e.*, $\log_2(1,3) \leq \log_2\text{FC} \leq \log_2(0,7)$). Since in our datasets the number of markers (e.g., phosphosites or proteins) exceeds the number of samples, we followed the approach of Patterson et al., [21] to compute the correlation matrix as $X = (M \times M^T)/n$, where M is the data matrix and n , the number of samples. We then performed eigenvalue decomposition of this matrix. Following the criterion used by Yin et al., [22], we considered as independent tests only the principal components with eigenvalues greater than 1. This estimated ENT was subsequently used to adjust statistical thresholds in downstream analyses, including count plots, UpSet plots, and volcano plots.

R (v.4.2.1) package EnhancedVolcano was used for multivariate data analysis and visualization.

Gene site enrichment analysis (GSEA) of the differential protein sets in the different experimental groups was computed using R package gProfileR (v. 0.7.0). The differential proteins with nominal p-value below the established threshold by the ENT in each comparison with positive and negative fold change were tested. The background was set to the input set of proteins detected by mass spectrometry. External gene names of the differential proteins were used as a query. Organism was set as a human. Electronic annotations were excluded, the p-value correction method was set to “fdr” and results with $\text{FDR} < 5\%$ were considered. ggplot2 R package was used for other statistical results representation, such as the UpSet plot.

PBMCs culture and LRRK2 inhibition with MLI-2

Cryo-preserved PBMCs from patients were cultured in RPMI media and treated for 1 hour at 37°C with 100 nM MLI-2 (Abcam #254528) or an equal volume of vehicle (diluted DMSO) as a control. Cells were spun down, and pellets were lysed in lysis buffer supplemented with protease and phosphatase inhibitors as described above. 50 µL of lysis buffer was used per 1x10⁶ cells. Samples were then centrifuged at 14.000 g for 10 minutes to separate the insoluble fraction and the pellet was discarded. Supernatant was stored at -80°C until use. As positive control of LRRK2 kinase inhibition by MLI-2 we assessed phospho-protein levels of its downstream target pThr73-Rab10 [23].

PBMC culture media were randomly selected from available cryo-preserved samples for the secretome experiments.

Western Blots

PBMC pellets were lysed using cell lysis buffer (Cell Signaling Technology, #cst9803S) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄ and 1 µg/ml leupeptin and supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) and additional protease (Roche cOmplete #11697498001) and phosphatase (Roche PhosSTOP #4906845001) inhibitors. Protein analysis was performed by Western immunoblotting as previously described [24], loading 20 µg of protein per lane. We used the following primary antibodies: rabbit monoclonal pThr73-Rab10 (Abcam #230261, MJF-R21), mouse monoclonal total RAB10 (NanoTools #0680 – 100, 605B11 clone), and mouse monoclonal β-actin (Sigma #A3854). We also tested the rabbit monoclonal pSer235/236-RPS6 antibody (Cell Signaling Technology, #4858) and the mouse monoclonal total S6 (Cell Signaling Technology, #2317S). We used the following HRP-conjugated secondary antibodies: goat polyclonal Mouse IgG (Thermo Fisher Sci. #31460) and goat polyclonal Rabbit IgG (Thermo Fisher Sci. #31430). Proteins were detected with Supersignal™ West Pico Plus chemiluminescent substrate (Thermo Fisher Sci. #34580). Chemiluminescent images were acquired using a Chemidoc (BioRad) imager. Samples were run in 17-well acrylamide gels, and samples from each condition were randomly distributed in the different gels. In the first experiment to compare between blots, for each membrane and protein of interest, values were relativized for the mean value of all control samples included in the gel. For re-incubation with other primary antibodies, membranes were incubated for 15 minutes with the Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Sci.). Signal quantification was done using the ImageJ software, and relative quantification of phospho-proteins was performed to corresponding total protein levels of the loading control. All uncropped and quantified WB membranes are collected in **Supplementary Fig. 1**.

Interleukin-6 determination by enzyme-linked immunosorbent assay (ELISA).

Cryo-preserved media from PBMCs cultures were randomly selected per each group based on protein content integrity, and 50 µL were assayed for IL-6 detection. The Human IL-6 ELISA Kit-Quantikine (R&D, #D6050B) was used. IL-6 ELISA was carried out in accordance with the manufacturer's instructions. Every sample from a particular individual was examined on the same assay plate. The IL-6 hs-ELISA showed intra-assay variability of 2,3% and inter-assay variability of 4,3%. According to the manufacturer, the assay's sensitivity was 0,626 pg/ml, and its lower limit of detection was 3,1 pg/ml. Each sample was analysed in triplicate wells (three technical replicates) within the same ELISA plate to minimize intra-assay variability.

Primary culture of mouse astrocytes and treatment with PBMCs media

C57Bl/6 mice were housed under controlled conditions (22°C, 40–60% humidity in a 12-h light/dark cycle) with water and food available *ad libitum*.

Hippocampus from mouse pups (P2) were dissociated in 0,025% trypsin, mechanically minced, centrifuged and plated at a density of 370 cells/mm² on plates coated with poly-D-lysine. DMEM supplemented with 10% FBS, 20 U/mL penicillin, and 20 µg/mL streptomycin (all from Thermo Fisher Scientific), was used to maintain the astrocytes. Once a week, the medium was changed. Cell cultures were kept at 37°C in an atmosphere with 5% CO₂. Complete media was changed at day 10 and replaced by the PBMC derived- RPMI media for 24 hours. Three independent cultures were tested with two technical replicates each.

Immunocytochemistry

Mouse primary astrocytes were fixed with 4% paraformaldehyde in 1X PBS and stained.

In short, samples were blocked with Superblock-PBS (Thermo Fisher Scientific, #37515)

after being permeabilized with Superblock-PBS and 0,25% Triton X-100 (Sigma Aldrich).

Primary antibodies such as rabbit monoclonal pSer235/236-RPS6 (1:500, Cell

Signaling Technology, #4858), mouse polyclonal GFAP (1:1000, Synaptic Systems,

#135303). Thermo Fisher Scientific provided the secondary antibodies that were used: donkey anti-rabbit Alexa Fluor 555 (1:500, #A31572) and Donkey anti-mouse 647 (1:500, #A31571). Nuclei were stained with Hoechst 33342 (1:5,000, Thermo Fisher Scientific, #H3570). Thermo Fisher Scientific's ProLong® Gold Antifade Mountant was used to mount the coverslips.

Statistical analysis

For statistical analysis, we used the software GraphPad Prism 6.0. The normal distribution was investigated using the D'Agostino-Pearson omnibus normality test. The statistical analysis was conducted using parametric statistical analysis. We looked at variances using the F-test before making pairwise comparisons. One-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's post hoc tests were used for the statistical studies. The Grubbs and ROUT tests were used to find the significant outlier values. For the clinical correlation analysis, we conducted a Pearson correlation coefficient between the UPDRS scores of patients with PD and healthy controls. A Pearson's correlation coefficient of $p > |0,2|$ and an FDR multiple-testing adjusted $P < 0,05$ were considered statistically significant.

Protein expression data from MS were utilized as predictor outcomes for the condition in order to generate ROC curves. The predictors' performance was assessed using the R package pROC v.1.18.5, and the curves were graphically shown using ggplot2 v.3.4.4.

Data were collected at random, prepared, inspected, and then blindly assessed. At $p \leq 0,05$, the statistics were deemed significant. Data was expressed as mean $\pm$ standard error of the mean (SEM).

Role of Funders

Funders did not have any role in study design, data collection, data analyses, interpretation, or writing of report.

RESULTS

Overview of the differentially expressed proteins and phosphorylated proteins between controls and PD groups.

To investigate the differential LRRK2 phospho-signature, we have assessed, for the first time, the proteome and phosphoproteome of PBMCs derived from patients with PD, including idiopathic PD patients (n = 37) and patients with PD carrying the G2019S mutation of the LRRK2 gene (n = 29). The study also includes the data of non-manifesting carriers of the G2019S LRRK2 mutation (n = 18) and controls (n = 36). Importantly, samples per group were collected from three independent hospitals' cohorts in Spain (Hospital Clínic de Barcelona, Hospital Marqués de Valdecilla in Santander and Hospital de Donostia in San Sebastian), providing an inherent solid and clinically well-characterized dataset (Fig. 1a and see Table 1). We performed DIA-MS and successfully identified 4615 peptides and 13000 phospho-peptides. Analysis of the proteomic data showed that the patients with PD carrying the LRRK2 mutation were the most distinct group, having the highest number of significantly upregulated (frequency = 110) and downregulated proteins (frequency = 162) respect to the controls (CTs). Interestingly, the iPD group resembles the CTs, since the number of differentially expressed proteins is very low. In this line, the disparity in proteomics between the L2NMC G2019S and L2PD G2019S patients was also slight (Fig. 1b).

On the other hand, the phosphoproteomic analysis indicated that the groups that showed more elevated differences were the iPD versus the L2PD G2019S patients, indicating that these two patient types exhibit differences in the biochemical signaling involving phosphorylation. Notably, compared to CTs, differentially phosphorylated proteins, in the L2NMC G2019S group were more abundant than in the L2PD G2019S group, suggesting that the degree of phosphorylation's could influence the 'phenoconversion' leading to the appearance of the motor symptoms (Fig. 1c).

Similarly, we generated UpSet plots to observe the overlap between differentially expressed proteins (DEPs) and phospho-proteins (DPPs) across the different groups' comparisons. Unlike traditional Venn diagrams, UpSet plots use a matrix-based layout to effectively visualize the number of shared proteins or phospho-proteins (Y axis, # of DEPs or DPPs in intersections), between comparatives specified below the X axis, by connecting orange square dots. If no DEPs or DPPs were shared between a given comparison and any of the others, that comparison was excluded from the plot, and the square dots remain in grey. Analyzing the proteomic data, we observed that there are common DEPs in the intersection of Controls (CT) vs L2NMC G2019S and iPD vs L2NMC G2019S, as well as CT vs L2PD G2019S and iPD vs L2PD G2019S, this overlapping suggest that certain DEPs are due to the mutation, rather than the disease itself. Contrariwise, fewer shared DEPs were observed between the iPD vs L2PD and iPD vs L2NMC, indicating potential distinct molecular pathways involved in these conditions (Fig. 2a).

Regarding the phosphoproteomic data, we observed a substantial amount of differentially phosphorylated proteins in common between the CTs vs iPD and iPD vs L2PD G2019S comparison, implying that the L2PD G2019S phosphoproteome is more similar to the CT than to the iPD conditions (Fig. 2b). In the comparison L2NMC vs iPD we observed that there are 65 DPPs and in the L2NMC vs L2PD comparison there are only 10 DPPs. Interestingly, in the intersection of these two comparisons, we found 3 phosphosites, the Ser235 and Ser236 residues at ribosomal protein S6 (RPS6), and the Ser1233 residue at TCOF1 (Fig. 2c). RPS6 is a component of the 40S small ribosomal subunit[25] and controls cell growth and proliferation through the selective translation of specific mRNAs [26]. RPS6 is also involved in the ribosome biogenesis [27]. Interestingly, TCOF 1 is a nucleolar protein that regulates RNA polymerase I action over enzymes responsible for ribosomal processing and modification[28]. Therefore, these three phosphorylations are hallmarks of PD pathology, suggesting a shared ribosomal-related mechanism in both L2PD G2019S and iPD patients.

Proteome analysis reveals an enrichment in differential expression of ribosomal proteins.

We next conducted a pathway enrichment analysis using KEGG annotations based only on the proteomic data, which revealed distinct signaling pathways altered across the different groups.

In the CT vs L2NMC G2019S comparison, we observed significant enrichment in inflammatory related pathways, the cytoskeleton organization and the ribosome function (Fig. 3a). In the CT vs L2PD G2019S comparison, enriched pathways again included inflammatory pathways, protein processing in endoplasmic reticulum and ribosomal pathways (Fig. 3b). When comparing CT vs iPD, we observed changes in inflammatory pathways, Falconi anaemia and ribosome pathways (Fig. 3c). Furthermore, comparing L2NMC G2019S vs L2PD G2019S, enriched pathways included were the Neutrophil trap formation, RNA polymerase and ribosomal pathways (Fig. 3d). Finally, in the iPD vs L2PD comparison, we also observed significant enrichment in ribosome related pathways along with focal adhesion or MAPK signaling (Fig. 3e).

Interestingly, the ribosome related pathways were enriched across all the above-mentioned comparisons, suggesting that the ribosomal and ribosomal-related proteins play a key role in the pathogenesis of PD and more specifically in the “phenoconversion” of non-manifesting carriers of the LRRK2 mutation. These indicates that ribosome pathways are progressively altered in PBMCs from non-manifesting state of the disease and show a higher impact in the PBMCs from iPD patients. Hereon, we defined them as our candidate proteins to validate and explore their implications.

Phosphoproteomic data suggest pSer235-RPS6 as a potential candidate for disease manifestation.

Applying the same cut-off strategy to analyze the phosphoproteome and given that ribosomal pathways were enriched in all comparisons, we examined whether there were also phosphorylation changes in the ribosomal proteins. The volcano plots revealed that pSer235-RPS6 was downregulated in the L2NMC G2019S versus CTs ($\log_2FC = -1,30$; adjusted $P = 9,01E-04$) (Fig. 4a). No significant changes were observed in L2PD G2019S (Fig. 4b) or putting together all the LRRK2 mutants (Fig. 4c). However, when comparing the L2NMC G2019S vs L2PD G2019S phosphoproteome, we observed again pSer235-RPS6 as a differential phosphosite upregulated in the L2PD G2019S condition (Fig. 4d) ($\log_2FC = 1,17$; adjusted $P = 3,44E-04$), with no changes in the iPD vs CT condition (Fig. 4e) and downregulated in the iPD vs L2NMC G2019S comparison (Fig. 4f) ($\log_2FC = -4,57$; adjusted $P = 3,44E-04$).

When we expressed the levels of pSer235-RPS6 versus the total levels of RPS6, we confirmed a downregulation in L2NMC in comparison to CTs, and the pSer235-RPS6 upregulation in L2PD and iPD (Fig. 4g). Interestingly, the next juxtaposed Ser236 residue of the same protein was not differentially phosphorylated among any comparisons (Fig. 4h). Surprisingly, the proteome data reveal that total RPS6 protein levels are downregulated in L2PD G2019S patients compared to controls (Fig. 4i).

To evaluate the diagnostic potential of pSer235-RPS6, we performed ROC curve analyses for the pairwise comparison L2NMC vs L2PD and L2NMC vs iPD. Specifically, the area under the curve (AUC) is 0,828 for the L2NMC G2019S vs L2PD and AUC 0,811 for the L2NMC vs iPD (Fig. 4j-k). These AUC values indicate a high discrimination power postulating pSer235-RPS6 as a potential clinical group classifier.

Our findings collectively demonstrate that RPS6 is differentially phosphorylated at the Ser235 residue in the L2NMC G2019S versus controls and iPDs, and importantly between in the L2NMC G2019S vs L2PD G2019S comparison, suggesting that pSer235-RPS6 is a good candidate to monitor disease phenoconversion, and even for an eventual disease progression evaluation.

Increased pS235-RPS6 normalized by total RPS6 levels correlate with UPDRS Scores across patients.

To evaluate the potential relationship between RPS6 phosphorylation and clinical manifestations of PD, several correlations were performed. First, we assessed the correlation between the pSer235-RPS6 / total RPS6 ratio and the clinical scores derived from the Unified Parkinson's disease Rating Scale (UPDRS).

Here we observed that the pSer235-RPS6/total RPS6 ratio correlated positively with UPDRS scores reflecting cognitive state (UPDRS I; $\rho = 0.26$, $P = 0.0053$), activities of daily living (UPDRS II; $\rho = 0.25$, $P = 0.0067$), motor examination (UPDRS III; $\rho = 0.25$, $P = 0.0069$), and therapy complications (UPDRS IV; $\rho = 0.31$, $P = 0.0008$) across the full population (Fig. 5a–d; **Supplementary Fig. 2**). Importantly, pSer235-RPS6/total RPS6 does not correlate with the age of the patients (**Supplementary Fig. 3**), indicating that the observed differences are unlikely to be driven by aging.

Stratified analysis in the G2019S-LRRK2 mutation carriers revealed a positive moderate significant correlation between pSer235-RPS6/total RPS6 ratio and UPDRS-I scores in L2PD patients ($\rho = +0.45$, $P = 0.018$), and a negative moderate correlation in L2NMC individuals ($\rho = -0.43$, $P = 0.07$). This indicates that higher levels of pS235-RPS6 are correlated with increased burden or severity of non-motor symptoms in L2PD patients, already manifesting motor symptoms. The correlation is not significant when considering only the iPD group ($\rho = +0.2$, $P = 0.25$) (Fig. 5e).

Together, these findings suggest that elevated pSer235-RPS6/total RPS6 ratio, is possibly linked to motor and non-motor clinical severity, particularly in symptomatic LRRK2 PD patients.

LRRK2 kinase activity does not seem to mediate the phosphorylation at Serine 235/236 of RPS6.

To elucidate whether LRRK2 is a direct kinase for Ser235/236 RPS6, we cultured PBMCs and treated them with 100 nM of the LRRK2 inhibitor MLI2 [29]. We used DMSO as vehicle. Cell lysates were resolved by western blot (WB) and the levels of p-Ser235/236 RPS6 were assessed. Note that this commercial antibody detects both phosphorylated residues, instead of only p-Ser235 RPS6. We also assessed the total levels of the RPS6 among the groups, factor that could influence the levels of its phosphorylated form (Fig. 6a). Interestingly, we confirmed higher levels of pSer235/236-RPS6 in the iPD cells compared with the other groups (CTs, L2NMC G2019S, L2PD G2019S), but this increase was independent of the kinase activity of LRRK2, since MLI2 did not have any effect. In the same experiment, we observed a tendency of elevated p-Ser235/236 RPS6 in L2PD G2019S cells in comparison to L2NMC G2019S cells, also insensitive to MLI2, but it was not statistically significant. (Fig. 6b-c). This may be due to the masking effect of the stable signal from the adjacent Ser236 residue, detected also with this dual epitope phospho-antibody, since DIA-MS analysis identified Ser235 as the selectively regulated residue, as is depicted in Fig. 4g-h.

LRRK2 inhibition was confirmed by assessing the levels of pThr73-Rab10, a direct LRRK2 substrate [30], in the PBMCs treated with MLI2 in all 4 groups (Fig. 6d-e).

These findings indicate that enhancement of pSer235-RPS6 in PBMCs is most prominent in iPD patients, discriminating them clearly from the LRRK2 G2019S mutation carriers.

PBMCs from patients with iPD release more IL-6 into the media.

The plasma levels of the cytokine IL-6 have been correlated with the severity of the motor symptoms in PD patients [31–33]. Moreover, RPS6 is involved in IL-6 cytokine secretion and IL-6 uptake after activating its own membrane receptor, which promote the indirect phosphorylation of RPS6 through the PI3K/Akt/mTOR pathway, overall leading to a positive feedback loop[34] (Fig. 6f). For these reasons we next assessed the levels of IL-6 released by the four PBMCs groups cultured for two hours, by ELISA. In the media coming from CTs' PBMCs the mean concentration of IL-6 was 0,84 pg/mL, in the G2019S L2NMC derived the IL-6 concentration was of 8,82 pg/mL, in the G2019S L2PD

derived was of 15,57 pg/mL and in the iPD derived the IL-6 mean concentration was 122 pg/mL (Fig. 6g). Overall, these results show a marked increase of IL-6 release by the iPD-derived PBMCs, confirming their more marked proinflammatory phenotype versus the L2PD groups.

Treatment with iPD PBMC media resulted in increased pS6 levels in primary astrocytes.

To get further insight into the putative effects of the proinflammatory PBMCs derived from iPD patients and explore their contribution to brain inflammation [35], we investigated the effect of their secretome on to mouse cultured primary astrocytes, which are important drivers of neuroinflammation.

Taking advantage that both cell types grow in RPMI medium, we incubated primary mouse astrocytic cultures (DIV10) with the PBMCs-derived medium from the 4 groups, for 24 hours (Fig. 7a). Strikingly, iPD derived-PBMCs medium induced an upregulation of pSer235/236 RPS6 in astrocytes (GFAP+ cells) (Fig. 7b, c), without affecting GFAP intensity or numbers. (Fig. 7d). However, when we correlated the intensities of pSer235/236 RPS6 and GFAP among the groups, we observed a strong positive correlation in the iPD group (P-value of < 0,0001 and $r = 1$). We also observed a positive correlation, but not significant, in both L2NMC (P-value = 0,67 and $r = 0,45$) and the L2PD patients (P-value = 0,45 and $r = 0,75$) (Fig. 7e).

Altogether, these results suggest that the secretome of iPD-derived PBMCs can upregulate pSer235/236-RPS6 in receiving astrocytes, possibly due to the elevated levels of IL-6, and correlating with astrogliosis.

DISCUSSION

Here, we comprehensively analysed the proteome and phosphoproteome of PBMCs from healthy controls and patients with L2NMC, L2PD, and iPD, identifying phosphorylation of ribosomal protein S6 at Ser235 (pSer235-RPS6) as a prominent feature associated with PD manifestation. pSer235-RPS6 levels were increased in iPD and L2PD compared with non-manifesting mutation carriers and were accompanied by elevated IL-6 release in iPD-derived PBMCs. Moreover, conditioned media from iPD PBMCs induced RPS6 phosphorylation in primary astrocytes, supporting a functional link between peripheral immune alterations and central nervous system–relevant signaling.

An important observation from our proteomic analyses was that PBMCs from L2PD G2019S patients displayed more pronounced changes in the proteomic content compared with controls than those from iPD patients, suggesting molecular distinctions between LRRK2-associated and idiopathic PD. This aligns with previous reports describing heterogeneous neuropathology among LRRK2 mutation carriers, despite similar clinical presentations and variable Lewy pathology [36]. Notably, when comparing L2NMC and L2PD groups, differences were more evident at the phosphoproteomic level than at the level of protein expression, indicating that phosphorylation-dependent signaling changes may play a key role in phenoconversion. While overlapping differentially expressed proteins between LRRK2 carriers and controls likely reflect mutation-driven effects, the identification of shared phosphosites in both L2NMC vs iPD and L2NMC vs L2PD comparisons suggests common disease-associated signaling alterations. Among these, phosphorylation of RPS6 at Ser235 and of TCOF1 at Ser1233, both involved in ribosomal and nucleolar function [37, 38], point to dysregulation of ribosome-related processes in PD PBMCs.

Consistently, ribosomal and ribosome-related pathways were enriched across all pathological comparisons, supporting the hypothesis that altered translational control may represent a convergent mechanism in PD. This is in agreement with previous studies showing disruption of protein synthesis machinery in vulnerable brain regions in PD [39]. Ribosomal proteins are central regulators of cellular homeostasis and selective translation [40], and our

findings demonstrate for the first time that such alterations are also evident in peripheral immune cells, providing an accessible system to study disease-associated translational dysregulation.

Beyond molecular profiling, we explored the clinical relevance of altered RPS6 phosphorylation. Increased pSer235-RPS6 correlated with UPDRS scores when considering the full population. Moreover, pSer235-RPS6 did not correlate with age. UPDRS I, which accounts for non-motor symptoms, positively correlated with pSer235-RPS6 in manifesting patients but not in non-manifesting carriers supporting its association with disease state rather than genetic background. Together, these findings suggest that activation of translational signaling in PBMCs reflects disease manifestation rather than LRRK2 mutation status per se.

Importantly, pharmacological inhibition of LRRK2 kinase activity with MLi-2 effectively reduced phosphorylation of the canonical substrate Rab10[18, 41] but did not affect RPS6 phosphorylation in any group. This indicates that pSer235-RPS6 in PBMCs is regulated independently of direct LRRK2 kinase activity, pointing instead to alternative upstream regulators.

Given the immune nature of PBMCs and the reported role of RPS6 in cytokine production [42], we investigated whether altered phosphorylation was associated with an inflammatory phenotype. IL-6 levels were markedly elevated in iPD-derived PBMC cultures compared with controls and LRRK2 mutation carriers, consistent with previous studies highlighting systemic inflammation as a hallmark of PD [31–33]. Although increased circulating IL-6 has been repeatedly reported in PD [33, 43], the cellular sources underlying this elevation remain poorly defined; our data support PBMCs as a potential contributor to the elevated serum IL-6 observed in patients. These data suggest that translational and inflammatory signaling pathways are concurrently engaged in peripheral immune cells during disease manifestation. Together with the lack of effect of direct LRRK2 inhibition on pSer235-RPS6, this points to alternative upstream regulators, such as IL-6–driven PI3K/Akt/mTOR signaling [44].

IL-6 levels increased progressively across groups from controls to iPD, raising the possibility of a feed-forward loop in which inflammatory signaling enhances translational activity in PBMCs, thereby sustaining immune activation. While speculative, such a mechanism could contribute to the chronic low-grade inflammation observed in PD and potentially influence disease progression[45–47]

To assess whether peripheral immune alterations could influence central nervous system signaling, we exposed primary murine astrocytes to PBMC-conditioned media. Astrocytes play a central role in neuroimmune communication and blood–brain barrier regulation, making them a relevant target for systemic inflammatory signals. Media from iPD PBMCs increased RPS6 phosphorylation in GFAP-positive astrocytes without inducing overt astrogliosis, suggesting activation of intracellular signaling rather than reactive gliosis. This is consistent with findings from human iPSC-derived PD astrocytes, which exhibit a proinflammatory state with elevated IL-6 production capable of inducing neuronal toxicity in midbrain dopaminergic neurons via IL-6 receptor–dependent STAT3 signaling [35]. These findings support a functional link between peripheral immune dysregulation—mainly IL-6 upregulation—and CNS-relevant signaling pathways.

Several limitations of this study should be acknowledged. The phospho-specific antibody used for validation detects both Ser235 and Ser236, precluding discrimination between these sites at the protein level, although DIA-MS data identified Ser235 as the selectively regulated residue. Additionally, while we demonstrate associations among RPS6 phosphorylation, IL-6 release, and astrocyte signaling, causal relationships remain to be fully established. Finally, neonatal hippocampal astrocyte cultures may not fully recapitulate responses of mature or region-specific astrocytes relevant to PD pathology [48]. Nevertheless, this model provides an initial functional validation, and future

studies using region-specific or human iPSC-derived astrocytes will be important to further define translational relevance.

CONCLUSIONS

Together, our findings support a model in which PD manifestation is associated with a distinct peripheral immune signaling state characterized by coordinated activation of translational and inflammatory pathways. By integrating unbiased phosphoproteomics with functional and clinical validation, we identify phosphorylation of ribosomal protein S6 at Ser235 and elevated IL-6 release as convergent features in PBMCs of the disease state across idiopathic and LRRK2-associated PD. Importantly, these alterations track with clinical severity, discriminate manifest from non-manifest disease, and occur independently of direct LRRK2 kinase activity, indicating that they reflect broader disease-associated signaling programs rather than mutation-specific effects. The ability of iPD-derived PBMC secretomes to induce RPS6 phosphorylation in astrocytes further suggests a mechanism by which peripheral immune dysregulation may engage central nervous system–relevant pathways, providing a functional link between systemic inflammation and neuroinflammatory signaling. Taken together, these data position peripheral translational control as a biologically meaningful and accessible readout of disease manifestation in PD, with potential implications for patient stratification, monitoring of phenoconversion, and the development of therapies targeting immune–translational crosstalk in neurodegeneration.

Abbreviations

AAO

Age at onset

Akt

Protein kinase B

ANOVA

Analysis of variance

AUC

Area under the curve

BBB

Blood–brain barrier

BH

Benjamini–Hochberg

CNS

Central nervous system

CT

Control

DIA

MS–Data–independent acquisition mass spectrometry

DMSO

Dimethyl sulfoxide

DPP

Differentially phosphorylated protein

DEP

Differentially expressed protein

ELISA
Enzyme-linked immunosorbent assay
ENT
Effective number of tests
FDR
False discovery rate
GFAP
Glial fibrillary acidic protein
GSEA
Gene set enrichment analysis
GWAS
Genome-wide association study
HRP
Horseradish peroxidase
iPD
Idiopathic Parkinson's disease
IL
6-Interleukin-6
iPSC
Induced pluripotent stem cell
KEGG
Kyoto Encyclopedia of Genes and Genomes
LRRK2
Leucine-rich repeat kinase 2
L2NMC
LRRK2 G2019S non-manifesting carriers
L2PD
LRRK2 G2019S-associated Parkinson's disease
MAPK
Mitogen-activated protein kinase
MoCA
Montreal Cognitive Assessment
MS
Mass spectrometry
mTOR
Mechanistic target of rapamycin
PBMC
Peripheral blood mononuclear cell
PD
Parkinson's disease
PI3K
Phosphoinositide 3-kinase
PRIDE
Proteomics Identifications Database
RAB10

Ras-related protein Rab-10
ROC
Receiver operating characteristic
RPS6
Ribosomal protein S6
pSer235
RPS6–Ribosomal protein S6 phosphorylated at serine 235
SEM
Standard error of the mean
SNc
Substantia nigra pars compacta
TNF
Tumor necrosis factor
UPDRS
Unified Parkinson's Disease Rating Scale
WB
Western blot

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All participants provided written informed consent, and the study was approved by the Hospital Clínic de Barcelona local ethics committee.

AVAILABILITY OF DATA AND MATERIALS

The datasets supporting the conclusions of this article are available in the Proteome Xchange Consortium via the PRIDE partner repository [19] (<https://www.ebi.ac.uk/pride/>) with the dataset identifiers PXD050865 for the proteome and PXD050944 phospho-proteome analyses. DOI: 10.1093/nar/gkab1038

COMPETING INTERESTS

The authors report no competing interests.

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AUTHORS' CONTRIBUTION

AC-G, PG-S, and AC contributed equally to this work, designed and performed experiments, analysed data, and co-wrote the manuscript.

JS-B, GC-C, L.M. assisted with experimental procedures and data acquisition.

AG, JI, JR-M, MF, RD, HH-E, AV-A, EM, IC, MR-S, AC-P, MJM, ET and MS contributed to patient recruitment, clinical assessment, and sample collection.

RF-S coordinated the sample collection and reviewed the manuscript.

JA and MJR funding and review the manuscript.

JF-I, ES, and CM jointly supervised the project, design experiments and contributed equally to its design and interpretation.

CM is the corresponding author and oversaw the manuscript writing and final approval.

All authors reviewed and approved the final version of the manuscript.

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