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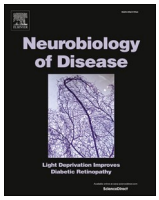

Stage-dependent phosphoproteome remodeling of Parkinson's disease blood cells

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ABSTRACT

The complexity and heterogeneity of PD necessitate advanced diagnostic and prognostic tools to elucidate its molecular mechanisms accurately. In this study, we addressed this challenge by conducting a pilot phosphoproteomic analysis of peripheral blood mononuclear cells (PBMCs) from idiopathic PD patients at varying disease stages to delineate the functional alterations occurring in these cells throughout the disease course and identify key molecules and pathways contributing to PD progression. By integrating clinical data with phosphoproteomic profiles across various PD stages, we identify potential stage-specific molecular signatures indicative of disease progression. This integrative approach allows for the discernment of distinct disease states and enhances our understanding of PD heterogeneity.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder responsible for both motor and non-motor disturbances, whose neuropathological hallmarks are the loss of dopaminergic nigral cells and the accumulation of ⟨-synuclein positive Lewy bodies. However, PD is complex and heterogeneous, arising from multiple impaired cellular pathways (e.g., mitochondrial dysfunction, impairment of protein clearance, neuroinflammation, oxidative stress), with a profound individual variability either in clinical presentation or the pathogenic background. Accordingly, there is an urgent need for a molecular and unbiased patients characterization, which may improve current limitations in diagnosing and staging of the disease, while offering novel candidate targets for therapeutic interventions, eventually within a precision medicine perspective (Marras et al., 2024; Höglinger et al., 2024). Although they primarily affect the central nervous system, molecular events underlying PD can be accurately tracked in peripheral tissues (Karayel et al., 2022; Virreira Winter et al., 2021). Accordingly, the potential utility of peripheral blood-based biomarkers as minimally invasive tools to define, stratify and monitor PD patients has been recently demonstrated (Marras et al., 2024; Tan et al., 2020). Peripheral blood mononuclear cells (PBMCs), which include several immune cell subpopulations, have emerged as a unique spatial window mirroring systemic alterations of the disease. Indeed, they can participate in inflammatory and reactive processes bearing on neurodegeneration (Lauritsen and Romero-Ramos, 2023; Schirinzi et al., 2022) or exhibit some of the pathogenic milestones of PD, such as the accumulation of pathological a-synuclein species (Avenali et al., 2021; Petrillo et al., 2020). Moreover, since phosphorylation events directly reflect cellular changes due to neurodegeneration (Tenreiro et al., 2014), PBMCs might also serve as a highly promising source of phosphoproteins useful as non-invasive biomarkers. Here, we employed high-sensitive mass spectrometry (MS)-based phosphoproteomics for a pilot study aimed to globally and unbiasedly characterize the (phospho)proteome profile of PBMCs from control subjects and PD patients at different disease stages. Despite the limited size of the patient cohort, our study demonstrates

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that global phosphoproteomic analysis of PBMCs can improve our understanding of the PD disease and identify molecules and pathways targetable for biomarker or therapy purposes. We showed that PD remodeled both the proteome and phosphoproteome of PBMCs, impacting the abundance of crucial hallmark proteins and mitochondrial metabolic enzymes. Moreover, both the proteome and the phosphoproteome profiles of PD PBMCs differed in patients grouped according to increasing disease severity. Using our recently implemented SignalingProfiler pipeline (Venafra et al., 2024), we discovered how key signaling pathways may be rewired at different disease stages and affect PD biological features. Through a machine-learning approach, we identified a few molecular targets, mainly involved in lysosome and phagosome biogenesis, that could be used for disease staging or drug development.

2. Materials and methods

2.1. Study population

The study included $n = 20$ PD patients and $n = 10$ sex/age-matched controls recruited at the Neurology Unit of Tor Vergata University Hospital (Rome, Italy) in 2023. PD was diagnosed according to MDS 2015 Postuma's criteria. We excluded patients with: main inherited forms of PD (e.g., GBA, LRRK2, PARKIN, PINK1, SNCA, etc.), dementia (according to MDS criteria) or cortical syndromes, red flags for atypical parkinsonism (e.g., supranuclear gaze palsy, prominent dysautonomia). Controls were control volunteers without history and clinical signs of neurological diseases. Subjects with main acute/chronic infectious/inflammatory/internal diseases and/or abnormal blood cell count were excluded. For each subject, demographics, anthropometrics, and medical history were collected. PD patients were assessed under the effect of habitual antiparkinsonian therapy ("on" state) through the Hoehn and Yahr scale (H&Y) and the MDS-UPDRS part III; the levodopa equivalent daily dose (LEDD) was also calculated. Contemporary to clinical assessment, all participants underwent venous blood sampling (15 ml), in the morning, after overnight fasting (morning drugs allowed). The study was approved by the local EC (protocol n° 16.21) and agreed with the principles of Helsinki declaration. All participants signed an informed consent.

2.2. PBMCs isolation

At the donation site, all human donor blood samples were fully anonymized and informed consent was obtained from all human subjects. PBMCs were isolated from fresh blood and collected by Ficoll-gradient protocol. Briefly, 5 ml of blood were stratified on 3 ml of Ficoll per tube. Samples were centrifuged at 1220 $\times g$ for 20 min without breaking. After centrifugation, the interphase was recovered, transferred into new tubes and washed with PBS1$\times$ + 2 %FBS twice. To lyse red-blood cells, 1 ml of RBC lysis buffer 1$\times$ to each tube and samples were incubated for 10 min at 4 °C. Next, samples were diluted with PBS1X + 2 % FBS and centrifuged 1220 $\times g$ for 10 min. After centrifugation, the supernatant was discarded and the cells were washed with PBS1X. Cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. Proteomic and (phospho)proteomic sample preparation

See supplementary materials for details.

2.4. Mass spectrometry analysis

See supplementary materials for details.

2.5. Proteome and phosphoproteome data processing

See supplementary materials for details.

2.6. Proteome and phosphoproteome bioinformatics data analysis

See supplementary materials for details.

2.7. PD-groups specific networks' generation with SignalingProfiler

See supplementary materials for details.

3. Results

3.1. MS-based (phospho)proteomics characterization of PBMCs from PD patients

First, we aimed to investigate the protein changes in peripheral blood mononuclear cells (PBMCs) of Parkinson's disease (PD) patients. Thus, we performed state-of-the-art mass spectrometry (MS)-based (phospho) proteomics of PBMCs collected from 20 PD patients and 10 age-matched control voluntary blood donor subjects (Fig. 1A). Patients were staged according to the Hoehn and Yhar (H&Y) scale and assessed for motor severity through the MDS-UPDRS (Movement Disorders Society – Unified PD Rating Scale) part III score; the individual levodopa equivalent daily dose (LEDD) was calculated (clinical data summarized in Table 1).

Patients-derived PBMCs were isolated and their proteome and phosphoproteome profiles analyzed by a label-free quantification approach coupled with a data-independent acquisition method. Our strategy enabled us to identify about 8000 proteins (Table S1) and 13,000 class I (localization probability score > 0.75) phosphopeptides (Table S2) mapping to 2754 protein groups (**Fig.S1A-B**). Next, we investigated whether the (phospho)proteome remodeling of PBMCs could reflect PD pathophysiology. Thus, we performed a principal component analysis (PCA) to investigate whether unbiased proteome and phosphoproteome profile enables segregation of patient-derived PMBCs according to their disease status. As shown in Fig. 1B-C**,** with the exception of some patients (PD5 in the proteome analysis; PD3/4/18 in the phosphoproteome analysis), component 1 clearly discriminates case from control PBMCs at both the proteome and phosphoproteome levels, revealing a disease-specific molecular remodeling of PBMCs. To quantitatively evaluate the impact of PD on the PBMCs (phospho)proteome layer, we performed a Student's *t*-test analysis of the proteome and phosphoproteome data between control and patient PBMCs. This approach resulted in the identification of 1755 proteins (Table S1) and 983 phosphosites mapping to 181 protein groups (Table S2) that were differently modulated between control and patients. Overall, we found that about 20 % of the measured proteome and 8 % of the phosphoproteome were significantly changed in PD PBMCs. Combined analysis of the phosphoproteome and proteome data showed that phosphorylation sites were evenly detected, irrespective of protein abundance, and that for 87 % of the quantified phosphorylation sites, the corresponding protein was also quantified (**Fig. S1C**). When normalizing for the protein level, more than 50 % of the phosphosites still remained significantly modulated. Only 2 % of the proteins have a joint modulation of phosphorylation and total protein levels, while the regulation of the remaining proteins was mainly due to the modulation of the total level (**Fig.S1D**). Interestingly, the abundance of PD-related proteins, as annotated in the KEGG database, was significantly changed in PD PBMCs as compared to the control ones. Indeed, critical proteins for PD pathogenesis, including SNCA, LRRK2, PARK7 and SEPTIN5, were globally upregulated in PD PBMCs, whereas proteins associated with mitochondrial metabolism displayed a downregulation pattern, with few exceptions (COX6B1, COX4A11, NDUFA4) (Fig. 1D).

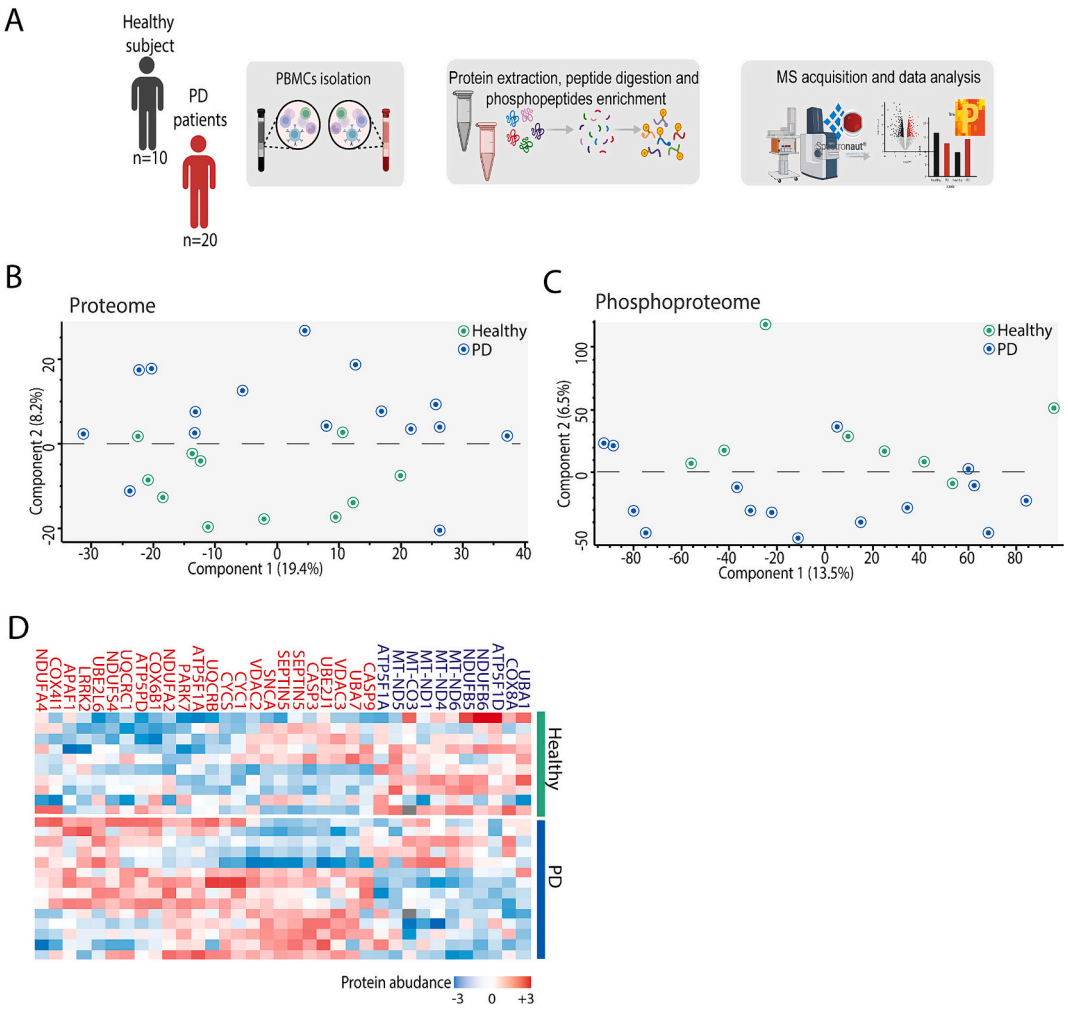

Fig. 1. MS-based (phospho)proteomics characterization of PBMCs of Parkison's disease patients. **(A)** Schematic representation of the experimental workflow. PBMCs from healthy and PD patients were isolated from peripheral blood by using Ficoll gradient. Cells were lysed and protein extracts were digested and processed. Phosphopeptides and peptides were loaded onto StageTips (Evotips) and acquired by high sensitivity LC-MS/MS (Evosep/Bruker timsTOF Ultra). Data acquisition and downstream bioinformatic analysis were performed in Spectronaut and Perseus environments. (**B**—**C**) Principal Component Analysis (PCA) of the proteins **(B)** and phosphosites **(C)** quantified across the replicates. **(D)** Heatmap displaying the intensity of Parkinson's disease related proteins in control and PD patients.

3.2. Global pathway modulation in PBMCs from PD patients

Next, we used gene ontology term enrichment analysis to characterize the PD global effects on protein and phosphoprotein levels. We took advantage of the 2D annotation enrichment analysis algorithm, based on nonparametric Mann-Whitney test (FDR < 0.05) (Cox and Mann, 2012). We found that leukocyte migration, cell motility, and immune responses were among the top positively enriched processes both at the proteome and phosphoproteome levels in PD PBMCs as compared to the control ones (Fig. 2A). Additionally, proteins involved in vesicle-related processes were upregulated at both the proteome and phosphoproteome layers in PD PBMCs. Indeed, peripheral blood contains extracellular vesicles (e.g. exosomes and microvesicles) which are released by various cell types, including neurons, and carry proteins, such as ⟨-synuclein, nucleic acids, and other molecules (Stuendl et al., 2021). Moreover, we observed that PD has a specific and significant impact on lysosomal proteins (Fig. 2B), suggesting an impairment of protein clearance also in peripheral tissues aside from the CNS. Interestingly, metabolic enzymes involved in glucose metabolism, such as LDHA, ENO1, and GAPDH, were found to be upregulated in both omic layers in PD PBMCs as compared to the controls (Fig. 2C). Importantly, glucose metabolism deregulation has been recently described as a peculiar feature of PD, accounting for the higher rate of glucose intolerance and type 2 diabetes mellitus in patients (Sánchez-Gómez et al., 2021). Therefore, our observations suggest that a strong remodeling of the glycolytic rate occurs in peripheral tissues of PD patients. In contrast, biological processes related to RNA processing, splicing, and chromatin modifications are down-regulated in PD patients both at the proteome and phosphoproteome level, confirming previous reports (Lu et al., 2014).

3.3. Staging PD patients according to the PBMC (phospho)proteome profile

Prompted by these results, we next asked whether our unbiased (phospho)proteomic analysis could enable the stratification of PD patients according to the disease severity. Interestingly, principal component analysis (**Fig. S2A**—**B**) and unsupervised hierarchical clustering (Fig. 3A-B) separated PD patients into three subgroups, at both the proteome and phosphoproteome levels. By analyzing the MDS-UPDRS part III and H&Y scores of patients belonging to these three subgroups, we noticed that the (phospho)proteome-based classification reflected the fold change differences of the mean values of these clinical parameters and, thus, the disease severity (Fig. 3C-D). Accordingly, we labeled the group 1 as the "mild group", the group 2 as the "intermediate group", and the group 3 as the "severe group". Statistical analysis

Table 1
Clinical features of PD patients and healthy samples.

SUBJECT	SEX	AGE	DURATION	LEDD	MDS-UPDRS-III	H&Y
	M	76	6	525	20	3
PD1						
PD2	F	74	1	0	9	2
PD3	M	76	1	0	30	2
PD4	M	71	21	1275	N.A.	4
PD5	F	77	6	300	39	4
PD6	F	63	3	751	32	3
PD7	F	65	5	326	23	2
PD8	M	78	10	920	28	3
PD9	M	72	14	2020	44	3
PD10	M	68	1	1891	44	3
PD11	M	71	2	425	27	2
PD12	M	77	6	525	30	2
PD13	M	50	5	720	33	3
PD14	M	69	7	600	33	3
PD15	M	69	2	320	28	2
PD16	F	74	8	1577	37	3
PD17	M	83	16	1331	49	3
PD18	M	72	23	790	62	3
PD19	M	67	14	1130	45	2
PD20	M	68	5	618	36	2.5
CTRL1	F	65	–	–	–	–
CTRL2	F	67	–	–	–	–
CTRL3	M	78	–	–	–	–
CTRL4	F	64	–	–	–	–
CTRL5	M	54	–	–	–	–
CTRL6	F	77	–	–	–	–
CTRL7	M	66	–	–	–	–
CTRL8	F	68	–	–	–	–
CTRL9	M	78	–	–	–	–
CTRL10	M	77	–	–	–	–

revealed that 3508 proteins and 455 phosphosites were significantly modulated across patients at different disease stages (Table S3–4). Despite the heterogeneity among the patients, this modulation of the (phospho)-proteome did not correlate with the mean of the individual LEDD of the PD groups (**Fig. S2C**).

The highest number of significant proteins and phosphosites were observed between the mild and the severe groups (**Fig. S2D-E**). When comparing the proteome of mild patients with those of the severe ones, we identified proteins significantly involved in biological processes associated with PD pathophysiology, such as depression, locomotion and inflammation (**Fig. S2F**). Unsupervised hierarchical clustering of statistically modulated proteins and phosphosites enabled to cluster molecules according to their abundance in the three PD groups of patients. Specifically, proteins upregulated in the severe group were enriched for vesicle-related processes, actin cytoskeleton dynamics and cell motility (Fig. 3E). In contrast, proteins involved in oxidative phosphorylation, DNA repair pathways, and splicing were upregulated in mild and intermediate patients. At the phosphoproteome level, proteins involved in the cAMP, G-protein coupled receptor, focal adhesion, ERK1/2 signaling pathways as well as immune response process were hyperphosphorylated in the severe group, while proteins belonging to histone modification, DNA repair, and RNA splicing processes appear to be hypophosphorylated (Fig. 3F). Combined analysis of the phosphoproteome and proteome data showed that among the 532 significantly modulated phosphosites between mild and severe groups, 355 had a joint increase or decrease of the total protein level, while 177 were modulated only at the phosphorylation level (**Fig. S2G)**. In this protein pool, we found that some have great consistency with PD pathogenesis. Specifically, the phosphorylation of the residue S258 of the MFF protein, a crucial mitochondrial fission factor, was significantly increased in the severe group (Fig. 3G). whereas the phosphorylation level of S32 of SCAMP3, a NEDD4 substrate involved in the vesicle trafficking, was lower in the severe group than in mild. Moreover, several members of the Rab protein family were differently phosphorylated between the two groups. For instance, we found that the S72 of Rab7A, a substrate of the LRRK1 kinase (Malik et al., 2021), was highly phosphorylated in the severe group as compared to the mild counterpart. Accordingly, these findings collectively suggest an abnormal key kinase activity in PD shaping the disease course.

3.4. *Signaling pathways modulation in PBMCs of PD patients*

Our results revealed that the PD severity was correlated with the dysregulation of crucial signaling pathways. Prompted by these results, we took advantage of our recently developed *SignalingProfiler* pipeline (Venafra et al., 2024) to systematically generate stage-specific signaling networks of PD PMBCs. Briefly, *SignalingProfiler* is a highly flexible computational approach that combines omics data with prior evidence reported in the literature to build context-specific networks. We generated three PD-group-specific signaling networks, by employing a three-steps strategy. First, we inferred the activity of modulated signaling proteins from phosphoproteomic data by looking at the changes in phosphorylation of their target phosphosites (for kinases) and of their regulatory phosphosites (for kinases, transcription factors, or other signaling proteins) (Fig. 4**A, S3A**—**B,** Table S5**)**. Interestingly, while CSNK2A1, CSNK1E, TFGBR1, BMPR1A, PAK1, and PLK2–3 kinases were upregulated in PBMCs of patients with mild phenotypes, WEE1, PRKD2, and SLK resulted in being more active in the groups with higher severity (Fig. 4A). Second, for each group we connected in a directed and signed network the kinases to transcription factors and other signaling proteins modulated in their phosphorylation levels. Finally, these PD group-specific networks were connected to 29 PD-relevant phenotypes chosen from the SIGNOR database (Lo Surdo et al., 2023) (e.g., apoptosis, myelination, etc.). The choice underlying the PD-relevant phenotypes

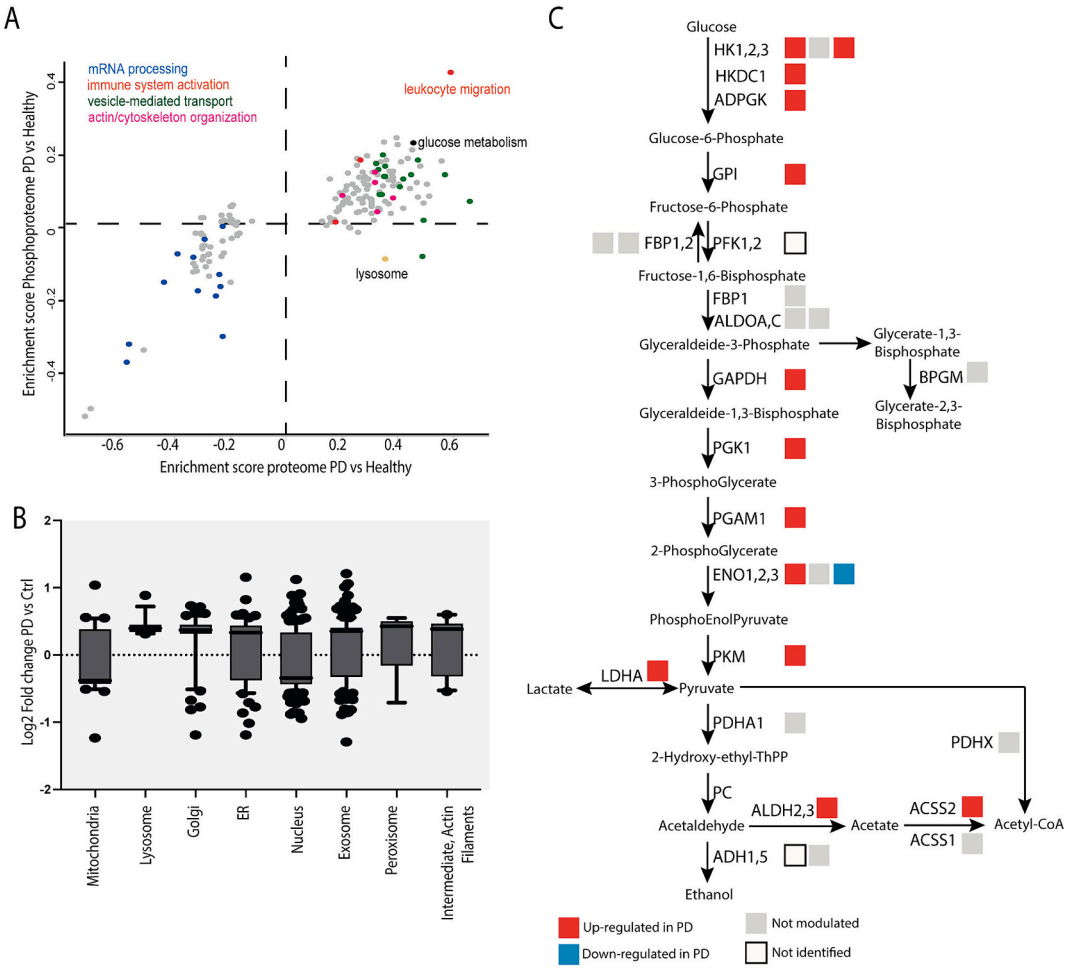

Fig. 2. Global pathway modulation in PBMCs from PD patients. **(A)** Scatterplot relative to the two-dimensional annotation enrichment analysis. Pathways modulated in PBMCs of PD patients at the proteome level in comparison with the phosphoproteome are plotted (Benjamin Hochberg FDR < 0.05). Each dot represents a KEGG pathway or GO-Biological Process (GO-BP) term. Related pathways or GO-BP are labeled with the same color, as reported in the legend. Position scores of the pathways reflects the fold change (Log2) between PD and control at the proteome (x-axis) and phosphoproteome (y-axis). **(B)** Box-plot reporting the fold change (Log2) between PD and control PBMCs of the proteins grouped according to their cell compartment annotation. **(C)** Schematic representation of the glycolytic process. For each enzyme the color of the relative square indicates the up- (red), down- regulation (blue) in PBMCs of PD patients compared to control ones. Grey and white squares indicate not significant modulated or not identified enzymes respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

relied on growing evidence showing that PBMCs can reflect some of the PD pathogenic milestones (Avenali et al., 2021; Petrillo et al., 2020). Our approach resulted in three signaling networks reflecting the pathways modulation in the mild, intermediate, and severe groups of patients at mechanistic levels. We merged the networks (see Methods) to identify remodeled signaling axes as the severity of the disease increases (Fig. 4B). As displayed in Fig. 4B, we were able to generate a PD graph, where every protein node is divided into three portions representing the protein activity state in each group, and edges represent molecular interactions occurring in at least one severity group. Interestingly, actin cytoskeleton reorganization, a process impaired with the progression of the disease due to the accumulation of protein aggregates, is regulated by the CSNK2A1 – PAK1 axis in the three groups (Orange edges in Fig. 4B), however, the proteins in the axis appear inhibited as the PD severity increases (Zhang et al., 2022). Similarly, signaling axes regulating the protein metabolism (e.g., protein degradation or synthesis, and autophagy) were not present in the severe group (Maiese, 2020). On the other hand, among the severe-specific axes, there is the CSK – SRC axis responsible for the activation of synaptic vesicle recycling observed in the severe group in both *SignalingProfiler* analysis and pathway enrichment analysis (Fig. 3E), while CDKs, a protein family already associated with PD development (Nguyen et al., 2002), activate neurodegeneration in intermediate and severe stages through uncharacterized players, such WEE1-CDK2 and CDK4-CDK7 axes. Overall, this systemic approach provided some insights into key potential regulators of known dysregulated pathways and phenotypes with the increase of PD severity.

3.5. *Identification of novel potential molecular markers for PD*

Next, we leveraged machine learning techniques to further analyze our proteomic data with the aim of identifying novel proteins serving as disease status markers for PD. Thus, the proteins significantly modulated between PD and controls were used to train a support vector machine algorithm in the Perseus software performing a leave-one-out cross-validation. Using this strategy, we obtained a list of proteins categorized as potential PD biomarkers (Table S6). Among the promising candidates, we identified already known PD circulating biomarkers, such as Gelsolin and Clusterin (Anastasi et al., 2021). The top candidate proteins enriched biological processes related to metabolic pathways, lysosome, phagosome, and synaptic vesicle cycle (Fig. 4C). Interestingly, most of the predicted candidate biomarkers enriching the metabolic pathways related term were represented by glycolitic enzymes, such as ENO1,

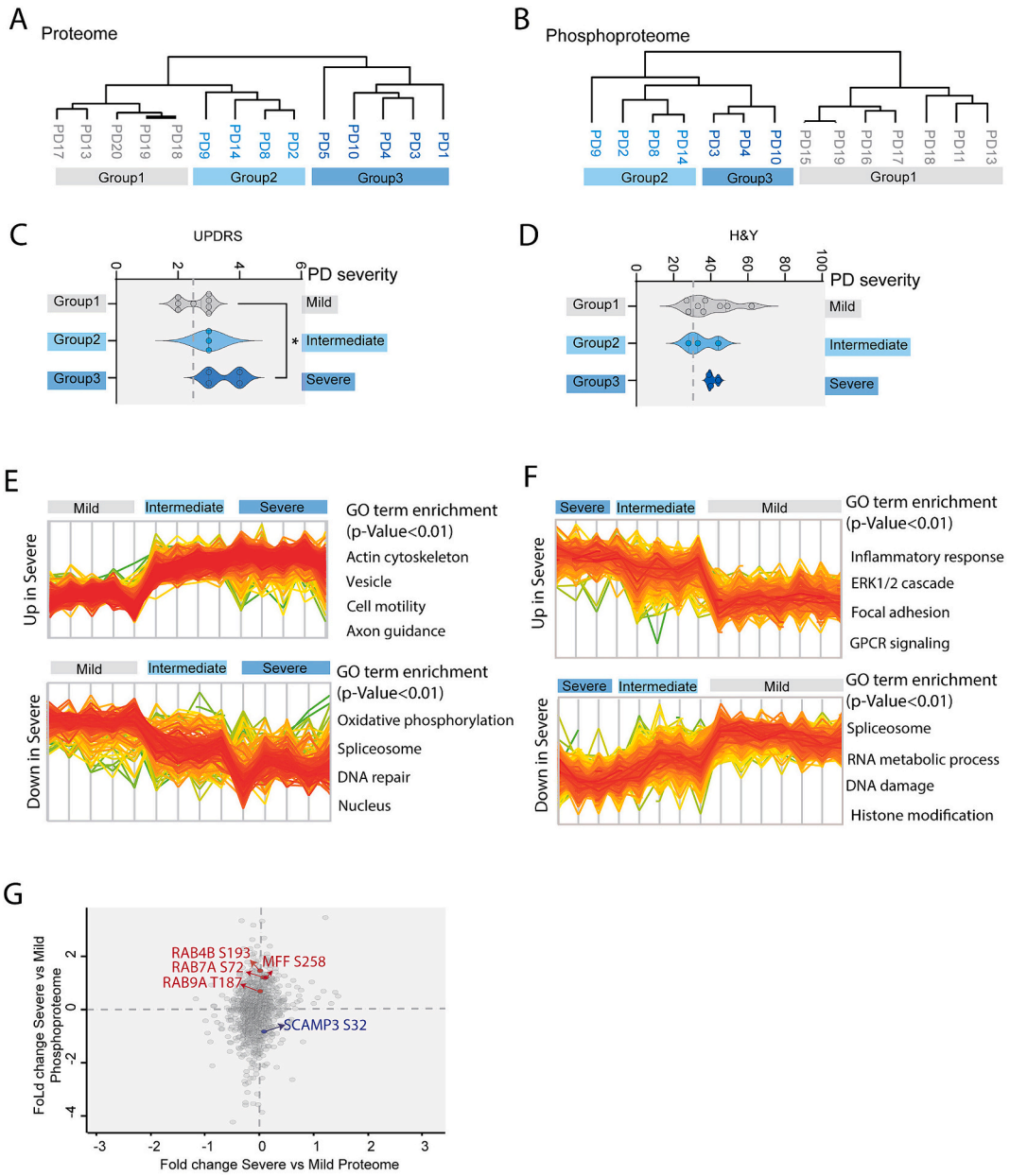

Fig. 3. Stratification of severity of Parkinson's disease patients according to their (phospho)proteome profiles. **(A-B)** Hierarchical clustering of PD patients according to the proteome (A) and phosphoproteome (B) data. **(C—D)** Violin plots reporting the score of H&Y and UPDRS scores of PD patients. **(*E*-F)** Profiling of intensity of the two main clusters of the proteins (E) and phosphosites (F) modulated at different stages of PD and their relative GO-term enrichment analysis. **(G)** Scatterplot reporting the proteins that are modulated at the phosphoproteome level only between the severe and mild groups. Crucial phosphorylation events already linked to Parkinson's disease have been highlighted.

GAPDH, HK3, and PKM2, confirming the relevance of glycolysis deregulation to PD, even as a biomarkers source. Next, we investigated the correlations between the protein intensity of the top 100 candidate PD biomarkers and the clinical parameters (MDS-UPDRS part III and H&Y scores). By performing a R^2 correlation analysis, we found that the expression level of several candidate biomarkers is correlated ($R2 > 0.5$; p-value<0.001) with the motor state (Fig. 4 **D**). We found that PGRMC1, SKAP2, TST and GPD2 were the highest correlated proteins. Taken together, our data thus revealed that machine learning-based biomarker prediction could be a promising strategy to identify novel molecular targets for PD.

4. Discussion

The relevance of blood mononucleate cells in PD has recently emerged, revealing that different immune activation patterns accompany the clinical-pathological progression of the disease (Harms et al., 2021; Magistrelli et al., 2020). To the best of our knowledge, this is the first pilot study employing MS-based phosphoproteomics of PBMCs of PD patients to define different disease stages and identify potential functional changes occurring in the peripheral immune cells.

Despite the PD cohort has a limited size, our unbiased analysis revealed that the (phospho)proteome profile could efficiently discriminate between control and patient PBMCs, suggesting that both the proteome and phosphoproteome are drastically affected in PD, consistently with a global remodeling of crucial cellular pathways. Namely, we

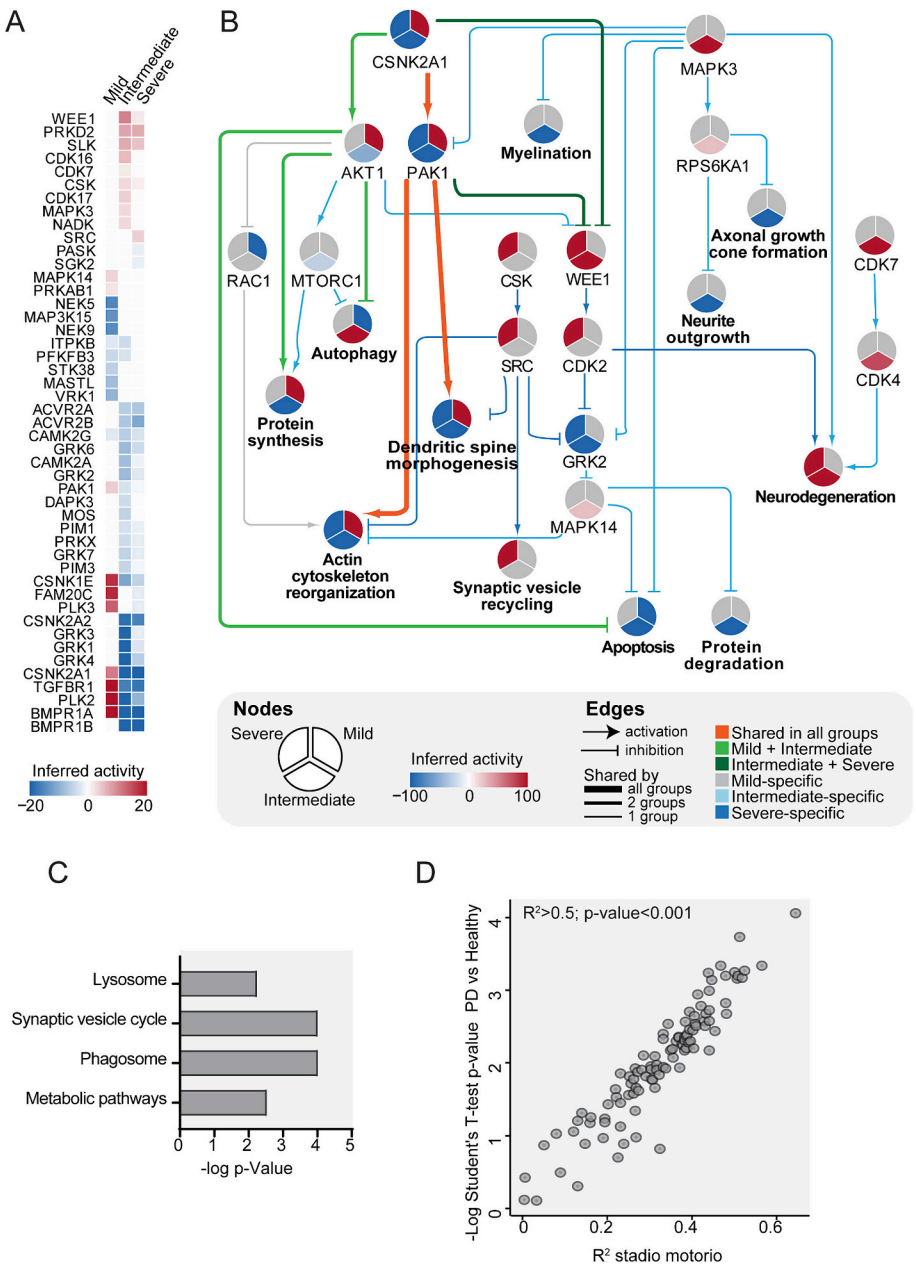

Fig. 4. Diagnostic signaling axes and biomarkers in PBMCs of PD patients. **(A)** Heatmap reporting the inferred activity of modulated kinases. Blue and red represent inhibited and activated proteins in mild, intermediate, and severe PD groups with respect to control, respectively. **(B)** SignalingProfiler-generated network connecting signaling proteins modulated in their activity in at least one group with PD-relevant phenotypes. Each pie chart sector represents the protein activity modulation in mild, intermediate, or severe groups (red activated, blue inhibited, grey unmodulated). Edges' width and color report the number and type of groups sharing the interaction. **(C)** Bar plot reporting the GO-biological processes/cell compartment enriched by the predicted candidate PD biomarkers. **(D)** R^2 correlation analysis between the top 100 candidate PD biomarkers and the H&Y scores. Each dot represents a candidate biomarker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

found that the lysosomal proteins and the glycolytic metabolic enzymes were upregulated, as previously reported (Avenali et al., 2021; Mächtel et al., 2023; Naeem et al., 2022; Smith et al., 2018); the proteins involved in mitochondrial metabolism were instead reduced.

Some of the proteins and phosphosites upregulated in PD PBMCs belong to classical pathogenic driving forces of PD, including SNCA (⟨-synuclein), LRRK2, PARK7 (DJ-1). As well we also observed a global increase of proteins involved in the vesicle-related processes, which may be linked with the blood presence of extracellular vesicles (e.g., exosomes and microvesicles) coming from the nervous tissue (Ebanks et al., 2020). Pathways related to nucleic acid metabolism (e.g., RNA processing, splicing, and chromatin modifications) were instead downregulated, mirroring the events occurring in the Substantia Nigra of patients (Garcia-Esparcia et al., 2015) or at a systemic level (Lu et al., 2014; Whittle et al., 2024).

Collectively, these observations suggest that PBMCs can recapitulate the biological milestones of PD and serve as a model for the central PD pathology (Schirinzi et al., 2022; Avenali et al., 2021; Petrillo et al., 2020; Jankovic and Tan, 2020).

Our quantitative shotgun (phospho)proteomic workflow enabled us to stratify PD patients according to their disease stage, indicating that (phospho)proteome profile of circulating immune cells is correlated with the disease severity. Interestingly, PBMCs from more advanced patients exhibited an upregulation and the hyperphosphorylation of

proteins associated with actin cytoskeleton dynamics, cell motility, and vesicle-related processes (eg. S72 of RAB7), suggesting an increase of immune-inflammatory activity at later disease stages (Tansey et al., 2022; Oliveira Da Silva and Liz, 2020). Crucial mitochondrial proteins (MFF) were also found hyperphosphorylated in more severe patients, implicating the leukocytic mitochondrial metabolism in the disease progression. In early-stage disease patients, we observed an upregulation and the hyperphosphorylation of oxidative phosphorylation and DNA repair pathway proteins, which reveal the establishment of cytoprotective and antioxidant responses, typically characterizing the first phase of the disease (Shadfar et al., 2023). However, when interpreting these data, we cannot exclude a direct effect of dopaminergic therapies per se on the (phospho)proteomic profile, although no significant correlations resulted with LEDD.

Our newly implemented SignalingProfiler workflow allowed for assessing the activity pattern of crucial kinases differentially operating among the disease stages. Specifically, CK1, CK2 kinases as well as TFGBR1, BMPR1A and PLK2 were upregulated in early-stage patients, whereas PAK1 kinase was downregulated in those more advanced. This approach outlined disease stage-specific signaling networks, revealing the potential critical role of the CSNK2A1-GYS1 axis in leading PBMCs remodeling along the PD course.

Finally, integrating our MS-based approach with machine learning techniques enabled us to pinpoint potential disease biomarkers, even reflecting motor severity. Gelsolin and the clusterin resulted as the most promising. Gelsolin is an actin regulatory protein, highly expressed in human leukocytes participating in cytoskeletal functions, which also interacts with α-synuclein within the pathogenic dynamics leading to Lewy bodies accumulation (Welander et al., 2011). Clusterin, instead, is an extracellular chaperone selectively expressed by the monocytes recognizing misfolded proteins, including pathological α-synuclein, and facilitating their bulk clearance and subsequent degradation (Filippini et al., 2021). Other metabolic enzymes were also candidate biomarkers, with particular relevance for those related to glycolysis. However, dedicated studies, testing diagnostic accuracy and clinical-prognostic value, are definitely due. As a pilot experience, this study is limited by the small sample size, the cross-sectional design, and the exiguity of clinical parameters; accordingly, findings must be interpreted cautiously and need replication on larger, independent cohorts before translating on a clinical ground. As strengths, instead, we recruited a strictly-selected population to avoid potential confounders and involved patients with progressive motor severity to open a larger window on the disease. However, our unbiased analysis identified stage-specific (phospho)proteomic signatures in PBMCs of PD patients, which support the high value of such an easily accessible tissue in the biological characterization of the disease and the subsequent discovery of biomarkers and targets to improve diagnosis, staging and therapeutic interventions.

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CRediT authorship contribution statement

Giorgia Massacci: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Veronica Venafra:** Methodology, Formal analysis, Data curation. **Maximilian Zwiebel:** Formal analysis, Data curation. **Maria Wahle:** Formal analysis, Data curation. **Rocco Cerroni:** Formal analysis, Data curation. **Jacopo Bissacco:** Formal analysis, Data curation. **Livia Perfetto:** Formal analysis, Data curation. **Vito Michienzi:** Formal analysis, Data curation. **Alessandro Stefani:** Formal analysis, Data curation. **Nicola Biagio Mercuri:** Formal analysis, Data curation. **Tommaso Schirinzi:** Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francesca Sacco:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Nothing to report.

Data availability

Data will be made available on request.

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References

Anastasi, F., et al., 2021. Proteomics profiling of neuron-derived small extracellular vesicles from human plasma: enabling single-subject analysis. Int. J. Mol. Sci. 22, 2951.
Avenali, M., et al., 2021. Profiling the biochemical signature of GBA-related Parkinson's disease in peripheral blood mononuclear cells. Mov. Disord. 36, 1267–1272.
Cox, J., Mann, M., 2012. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. BMC Bioinformatics 13 (Suppl. 1), 1–11.
Ebanks, K., Lewis, P.A., Bandopadhyay, R., 2020. Vesicular dysfunction and the pathogenesis of Parkinson's disease: clues from genetic studies. Front. Neurosci. 13, 1381.
Filippini, A., et al., 2021. Extracellular clusterin limits the uptake of α-synuclein fibrils by murine and human astrocytes. Glia 69, 681–696.
Garcia-Esparcia, P., Hernández-Ortega, K., Ansoleaga, B., Carmona, M., Ferrer, I., 2015. Purine metabolism gene deregulation in P arkinson's disease. Neuropathol. Appl. Neurobiol. 41, 926–940.
Harms, A.S., Ferreira, S.A., Romero-Ramos, M., 2021. Periphery and brain, innate and adaptive immunity in Parkinson's disease. Acta Neuropathol. (Berl.) 141, 527–545.
Höglinger, G.U., et al., 2024. A biological classification of Parkinson's disease: the SynNeurGe research diagnostic criteria. Lancet Neurol. 23, 191–204.
Jankovic, J., Tan, E.K., 2020. Parkinson's disease: etiopathogenesis and treatment. J. Neurol. Neurosurg. Psychiatry 91, 795–808.
Karayel, O., et al., 2022. Proteome profiling of cerebrospinal fluid reveals biomarker candidates for Parkinson's disease. Cell Rep. Med. 3, 100661.
Lauritsen, J., Romero-Ramos, M., 2023. The systemic immune response in Parkinson's disease: focus on the peripheral immune component. Trends Neurosci. 46, 863–878.
Lo Surdo, P., et al., 2023. SIGNOR 3.0, the SIGnaling network open resource 3.0: 2022 update. Nucleic Acids Res. 51, D631–D637.
Lu, B., Gehrke, S., Wu, Z., 2014. RNA metabolism in the pathogenesis of Parkinson's disease. Brain Res. 1584, 105–115.
Mächtel, R., Boros, F.A., Dobert, J.P., Arnold, P., Zunke, F., 2023. From lysosomal storage disorders to Parkinson's disease – challenges and opportunities. J. Mol. Biol. 435, 167932.
Magistrelli, L., et al., 2020. Relationship between circulating CD4+ T lymphocytes and cognitive impairment in patients with Parkinson's disease. Brain Behav. Immun. 89, 668–674.
Maiese, K., 2020. Dysregulation of metabolic flexibility: The impact of mTOR on autophagy in neurodegenerative disease. Int. Rev. Neurobiol. 155, 1–35.

Malik, A.U., et al., 2021. Deciphering the LRRK code: LRRK1 and LRRK2 phosphorylate distinct Rab proteins and are regulated by diverse mechanisms. Biochem. J. 478, 553–578.
Marras, C., et al., 2024. Transitioning from subtyping to precision medicine in Parkinson's disease: a purpose-driven approach. Mov. Disord. Mds, 29708. https://doi.org/10.1002/mds.29708.
Naeem, U., et al., 2022. Glycolysis: the next big breakthrough in Parkinson's disease. Neurotox. Res. 40, 1707–1717.
Nguyen, M.D., Mushynski, W.E., Julien, J.-P., 2002. Cycling at the interface between neurodevelopment and neurodegeneration. Cell Death Differ. 9, 1294–1306.
Oliveira Da Silva, M.I., Liz, M.A., 2020. Linking alpha-synuclein to the actin cytoskeleton: consequences to neuronal function. Front. Cell Dev. Biol. 8, 787.
Petrillo, S., et al., 2020. Systemic activation of Nrf2 pathway in Parkinson's disease. Mov. Disord. 35, 180–184.
Sánchez-Gómez, A., Díaz, Y., Duarte-Salles, T., Compta, Y., Martí, M.J., 2021. Prediabetes, type 2 diabetes mellitus and risk of Parkinson's disease: a population-based cohort study. Parkinsonism Relat. Disord. 89, 22–27.
Schirinzi, T., et al., 2022. Pattern of mitochondrial respiration in peripheral blood cells of patients with Parkinson's disease. Int. J. Mol. Sci. 23, 10863.
Shadfar, S., Parakh, S., Jamali, M.S., Atkin, J.D., 2023. Redox dysregulation as a driver for DNA damage and its relationship to neurodegenerative diseases. Transl. Neurodegener. 12, 18.
Smith, A.M., et al., 2018. Mitochondrial dysfunction and increased glycolysis in prodromal and early Parkinson's blood cells. Mov. Disord. 33, 1580–1590.
Stuendl, A., et al., 2021. α-Synuclein in plasma-derived extracellular vesicles is a potential biomarker of Parkinson's disease. Mov. Disord. 36, 2508–2518.
Tan, E.-K., et al., 2020. Parkinson disease and the immune system — associations, mechanisms and therapeutics. Nat. Rev. Neurol. 16, 303–318.
Tansey, M.G., et al., 2022. Inflammation and immune dysfunction in Parkinson disease. Nat. Rev. Immunol. 22, 657–673.
Tenreiro, S., Eckermann, K., Outeiro, T.F., 2014. Protein phosphorylation in neurodegeneration: friend or foe? Front. Mol. Neurosci. 7.
Venafra, V., Sacco, F., Perfetto, L., . SignalingProfiler 2.0: A Network-Based Approach to Bridge Multi-Omics Data to Phenotypic Hallmarks. http://biorxiv.org/lookup/doi/10.1101/2024.01.25.577229. https://doi.org/10.1101/2024.01.25.577229.
Virreira Winter, S., et al., 2021. Urinary proteome profiling for stratifying patients with familial Parkinson's disease. EMBO Mol. Med. 13, e13257.
Welander, H., et al., 2011. Gelsolin co-occurs with Lewy bodies in vivo and accelerates α-synuclein aggregation in vitro. Biochem. Biophys. Res. Commun. 412, 32–38.
Whittle, B.J., et al., 2024. Early-stage idiopathic Parkinson's disease is associated with reduced circular RNA expression. Npj Park. Dis. 10, 25.
Zhang, K., Wang, Y., Fan, T., Zeng, C., Sun, Z.S., 2022. The p21-activated kinases in neural cytoskeletal remodeling and related neurological disorders. Protein Cell 13, 6–25.