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PROJECT COMPLETION REPORT

1. **Project Number** : 5423
2. **Title of the Project** : Identification and characterization of Ubiquitin and SUMO modified exosomal proteins from Parkinson's Disease patient's blood
3. **Funding Agency Name** : ICMR
4. **Project Reference Number provided by the Funding Agency**: No.5/4-5 Ad-hoc/Neuro/217/2020-NCD1
5. **Principal Investigator (Name & Address):** Dr. Madhusoodanan UK, Additional Professor, Biochemistry**,** SCTIMST, Thiruvananthapuram –
6. **Co-Investigators (Name & Address):** Dr. Syam K, Professor, Neurology, SCTIMST, Thiruvananthapuram – 695011
7. **Collaborating Institutions** : NIL
8. **Date of Commencement** : 22-03-2021
9. **Duration** : 3 years
10. **Date of Completion** : 20-03-2024
11. **Objectives as approved** :

- Isolation and characterization of brain derived exosomes from plasma of PD patients
- Identification of exosomal proteins, Ubiquitinated and SUMOylated proteins by mass spectrometry
- Evaluate weather identified panel of proteins could be used as biomarker in PD

12. **a. Deviation made from original objectives if any, while implementing the project and reasons thereof:**

b. Modification in brain derived exosomal isolation strategy: As proposed in the methodology, brain-derived exosomes were isolated using Protein A magnetic beads coupled with LiCAM antibody, or Streptavidin agarose beads coupled with Biotinylated-LICAM antibody. However, significant concentration of Immunoglobulins from plasma was found to be copurifying with exosomes in this method. Moreover, the yield of exosome was very low. Since this low yield and contamination was affecting the downstream proteomic analysis, differential centrifugation (Ultracentrifugation) was used to isolate total exosomes from plasma.

This resulted in improved total exosome concentration and quality required for proteomic analysis.

c. Modifications in sample preparation for proteomic analysis:

To immunoprecipitate Ubiquitin and SUMO conjugated proteins, we initially conducted a western blot to confirm their presence in exosomes. Although the presence of these modified proteins was apparent from the gel **(Figure 1)**, the immunoprecipitation resulted in very low quantities, insufficient for mass spectrometry analysis. The mass spectrometric analysis resulted in detection of mostly immunoglobulins alone than specific modified proteins (**Figure 1**). Due to limitations in obtaining more than 10 ml of blood (5 ml plasma) from PD patients, scaling up the initial plasma was not feasible to obtain the necessary quantity of modified proteins for proteomics analysis. Therefore, we proceeded with proteomics analysis using total exosomal proteins instead of focusing exclusively on the modified proteins.

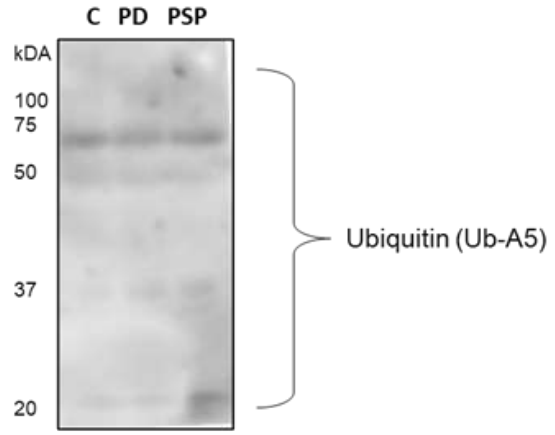
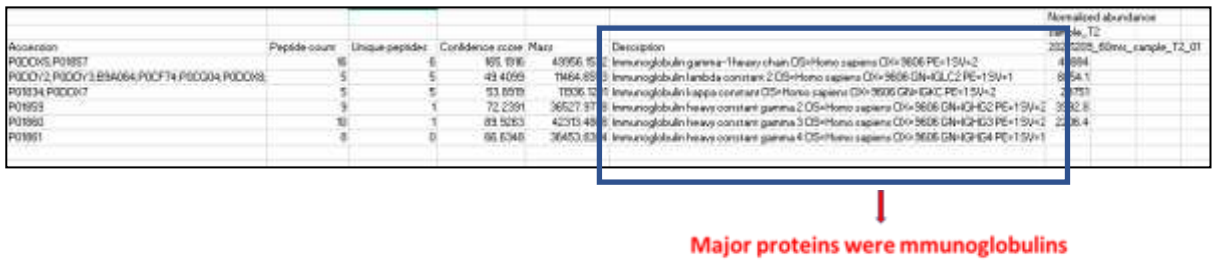


Figure 1: *Western blot analysis of plasma exosomal Ubiquitin (Ubiquitin A-5 antibody, Cat no: SC166553, 1:1000 dilution) isolated from* ***C:*** *control,* ***PD:*** *Parkinson's Disease and* ***PSP:*** *progressive supranuclear palsy.*



13. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs:

Objective 1

Isolation and characterization of brain derived exosomes from plasma of PD patients

Subject recruitment

All experiments were conducted after obtaining the approval of the Institutional Ethics Committee of SCTIMST (IEC/1383) and written consent of the subjects. Patients with Parkinson's disease who satisfy the standard diagnostic criteria (United Kingdom Parkinson's Disease Society Brain Bank [UKPDSBB] Clinical diagnostic criteria) was selected in the age group 18- 80 years irrespective of gender. The healthy volunteers who are not suffering from any neurodegenerative diseases were recruited for the study.

(1) Clinical diagnosis of PD as per UKPDSBB diagnostic criteria.

(2) Age more than 18 years

Exclusion criteria:

(1) Cognitive or psychiatric dysfunction sufficiently severe enough to impair the patient's ability to provide informed written consent.

(2) Any red flags or additional neurological signs raising suspicion of atypical Parkinsonism.

Inclusion criteria for healthy volunteers:

(1) Gender matched to patients

(2) Normal neurological examination

Exclusion criteria for healthy volunteers:

Positive family history of PD or other neurodegenerative disease, smokers.

Proforma: Patient details is recorded in the following proforma

PROFORMA

Title of the study: "Identification and characterization of Ubiquitin and SUMO modified exosomal proteins from Parkinson's Disease patient's blood"

Principal Investigator: ***Dr. Madhusoodanan U.K.***
Co-PI: ***Dr. Syam K***
Demographic Details:
Subject ID: ***Subject type: Patient / Control***
Age: ***Gender:***
Geographical Locality:
Rural / Urban:
Level of Education:
Weight
Height: ***BMI:***
Co-morbid conditions / medical history (other than PD, T2DM, Hypertension etc)

For Patients with PD:

Age of Onset: ***Side of Onset:***

First symptom: ***Duration of disease:***

Current Symptoms: ***Current disease Phenotype:***

<u>Past Treatment</u>

SI No	*Medication*	*Maximum dose tried*	*Duration of exposure*

<u>Ongoing treatment:</u>

SI No	*Medication*	*Dosage schedule*	*Total daily dosage*

Presence of motor fluctuations

Dyskinesias

Details of motor complications

Non-motor Symptoms: ***Non-motor Symptoms Scale Score:***

Domains affected:

UPDRS 1: ***UPDRS II:***

UPDRS III: ***UPDRS IV:***

Sample Collections Details

SI No	*Blood sample*	*Date and time of Collection*	*Date and time of Transportation*	*Analysis and remarks*

A total of 213 plasma samples were collected from patients at diagnosis of Parkinson's disease (PD; n =145), Progressive supra nuclear palsy (PSP; n = 59), Multiple system atrophy (MSA; n=2), amyotrophic lateral sclerosis (ALS; n = 7) and healthy controls (n = 27). The clinical and demographic details of sample collected is shown in **Table 1**

Table 1. Demography and clinical data of control and Parkinson's disease (PD) patients

	Controls	PD patients
n (gender)	N=27 (15F, 12M)	N=145 (46F, 99M)
Age (range)	38.2±9.3	56.8±10.4
Age at onset (years)	-	50.3±11.5
Disease duration (years)	-	7.3±5.5
MoCA	-	25.2±4.3
H&Y scale	-	2.04 ± 0.78
UPDRS scale III	-	20.7 ± 11.8

MoCA - Montreal Cognitive Assessment; H&Y- Hoehn and Yahr; UPDRS - Unified Parkinson's Disease Rating Scale; N - total number of samples, F - Female, M - Male

Sample collection: 10 ml blood sample was collected in EDTA tubes. The whole blood sample was centrifuged at 3500 rpm for 10 min and the supernatant (plasma) was stored as aliquots labelled with the unique study code at -80^{o}C, immediately.

Exosome Isolation and characterization: Exosome was isolated from plasma using ultracentrifugation (Hitachi Micro-ultracentrifuge). The exosome pellet was resuspended in 50 ul PBS and stored at -80°C. The presence of exosome was confirmed by western blot analysis with primary antibodies: HSP 90α, TSG 101, Albumin, and negative marker Calnexin **(Figure 2A)**. The isolated exosomes were homogeneous as evident from the Transmission electron microscopic image **(Figure 2B)** and Dynamic Light Scattering (DLS) (**Figure 2C**).

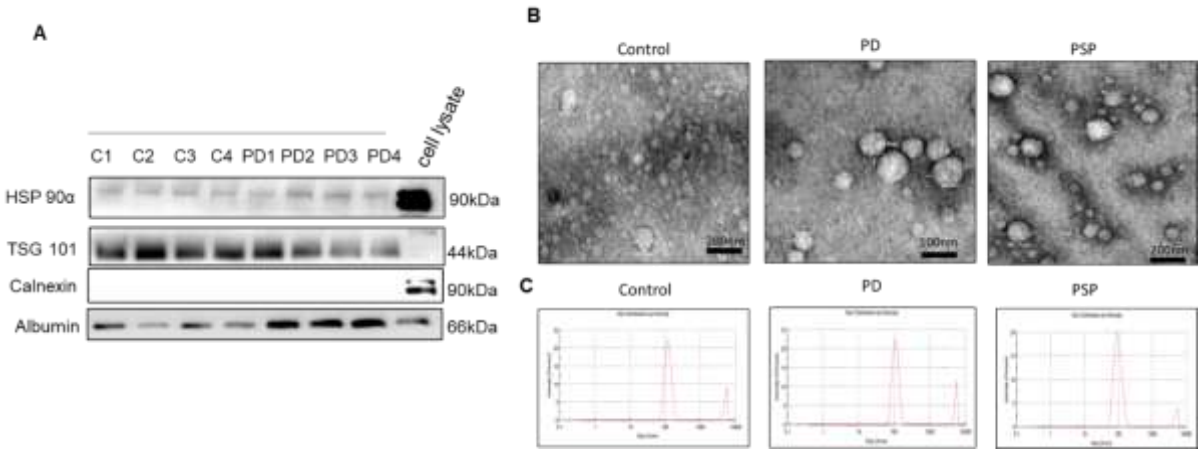


Figure 2: *Isolation and characterisation of exosomes.* ***A.*** *Western blot analysis of exosomes isolated from healthy control and PD patients using* HSP 90α*, TGS101, Albumin and Calnexin.* ***B.*** *TEM image of isolated exosomes from healthy control, PD and PSP.* ***C.*** *DLS analysis of isolated exosomes from healthy control, PD and PSP.*

Objective 2

Identification of exosomal proteins, Ubiquitinated and SUMOylated proteins by mass spectrometry

Exosomes were isolated from plasma of control (n=15; 6F and 9M) and PD (n=15; 6F and 9M) patient samples using ultracentrifugation as described above. The quality of the protein was checked by Coomassie Blue Staining after SDS PAGE (**Figure 3A**). For western blotting, equal amounts of exosome (12µg) were separated by 10% gel and transferred to PVDF membrane and western blot (**Figure 3B-3C**).

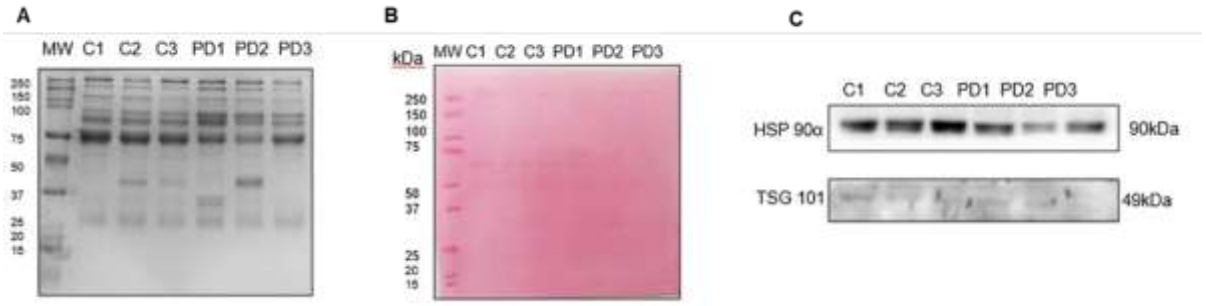


Figure 3: *Characterization of exosomes by western blot analysis* ***A.*** *Coomassie staining of exosomal proteins.* ***B.*** *Ponceau S-stained image of membrane after transfer of proteins for western blot.* ***C.*** *Western blot analysis showing the presence of exosomal proteins (HSP90α and TSG101) in control and PD samples. MW: molecular weight marker, C1-C3 are healthy controls; PD1-3 are PD patient's samples.*

Proteomics analysis

Total 30 samples [control (n=15; 6F and 9M) and PD (n=15; 6F and 9M)] were subjected to mass spectrometry. Approximately 80 µg (80 µL, concentration - 1 µg/µL) of plasma exosome from each sample (control and PD), was subjected to in-solution tryptic digestion. Capillary LC of tryptic peptides was performed with Waters NanoAcquity UPLC chromatographic system (Waters, Manchester, UK) coupled to Synapt G2 High-Definition MS™ System (HDMSE System, Waters), controlled and processed by Mass Lynx 4.1 SCN781 software. Mass spectrometry was performed at the proteomics facility, The Rajiv Gandhi Centre for Biotechnology (paid service: 7000 Rs/Sample).

Approximately 65 proteins and 1978 peptides were identified among the control and patient group. Among the proteins identified, about 14 proteins were shortlisted after manually deducting the albumin, immunoglobulin, plasma proteins and other compliment proteins identified in the proteomic data **(Table 2).** Gene ontology (GO) analysis was performed with the shortlisted proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software. With the *Homo sapiens* genome as statistical background, the proteins were classified based on the molecular function, biological processes and cellular

component (**Figure 4**). The list of KEGG pathway associated with the identified proteins is shown in **Table 3.**

Table 2: *Proteins identified in the plasma exosomes of control and PD patients.*

Sl. No.	**Accession**	**Max fold change**	**Highest mean condition**	**Lowest mean condition**	**Description**
1	Q14790	1.711674	PD	CTL	Caspase-8
2	Q86VF7	4.508243	CTL	PD	Nebulin-related-anchoring protein
3	P41227	1.595743	CTL	PD	N-alpha-acetyltransferase 10
4	Q9UHR6	1.327459	CTL	PD	Zinc finger HIT domain-containing protein 2
5	P0CG39	1.365636	CTL	PD	POTE ankyrin domain family member J
6	P10909	1.237184	CTL	PD	Clusterin
7	Q6S8J3; A5A3E0; P0CG38; Q9BYX7	1.23919	CTL	PD	POTE ankyrin domain family member E
8	Q96C28	1.307176	PD	CTL	Zinc finger protein 707
9	Q9UPN9	1.017738	PD	CTL	E3 ubiquitin-protein ligase TRIM33
10	P02787	1.439272	PD	CTL	Serotransferrin OS=Homo sapiens
11	P01023	1.26934	CTL	PD	Alpha-2-macroglobulin
12	O75636	1.093599	PD	CTL	Ficolin-3
13	Q9UKV3	1.665433	PD	CTL	Apoptotic chromatin condensation inducer in the nucleus
14	**P23528**	**1.163006**	**PD**	**CTL**	**Cofilin-1**

Table 3: *KEGG pathway analysis (using DAVID) of proteins identified in the plasma exosomes of control and PD patients.*

ID	**Gene Name**	**Pathway**
Q9UKV3	Apoptotic Chromatin Condensation Inducer 1(ACIN1)	Nucleoplasmic transport and mRNA surveillance pathway and
Q14790	Caspase 8 (CASP8)	p53 signalling pathway, apoptosis, necroptosis
P10909	Clusterin (CLU)	Complement and coagulation cascade
P23528	Cofilin 1(CFL1)	Axon guidance, Fc gamma mediated phagocytosis, regulation of actin cytoskeleton

P02787	Transferrin (TF)	HIF1 signalling pathway, Ferroptosis and mineral absorption
Q96C28	Zinc finger protein 707 (ZNF707)	HIV1 infection

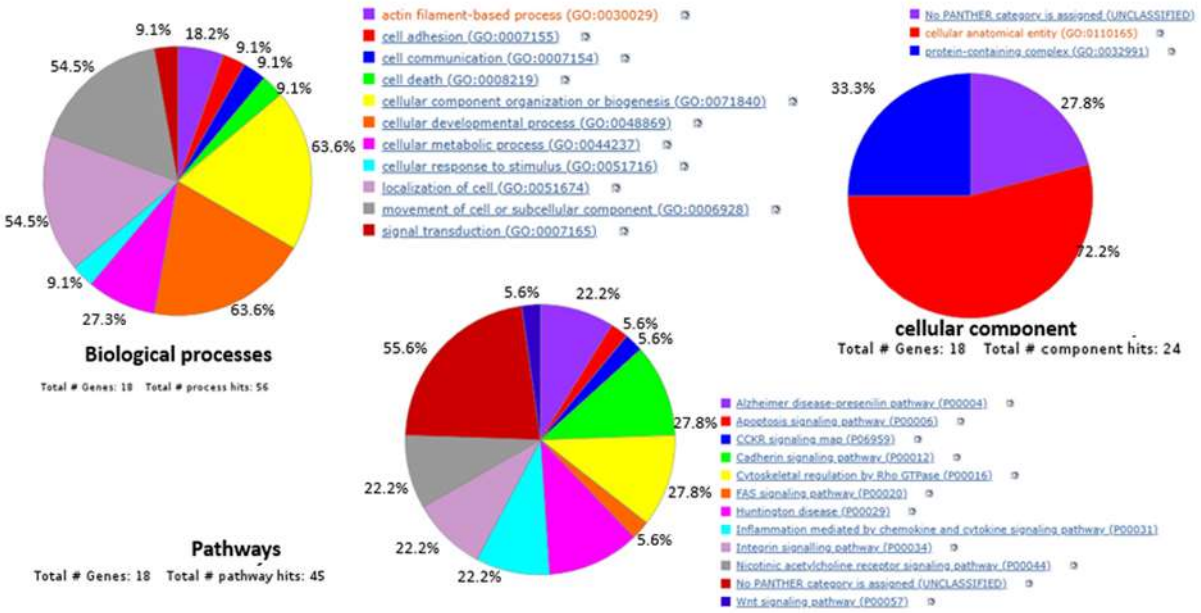


Figure 4: *PANTHER class analysis of protein based on biological process, cellular components and pathway.*

Objective 3

Evaluate weather identified panel of proteins could be used as biomarker in PD

Sample collection, exosome isolation:

Exosomes were isolated from plasma of control (n=114; 63F and 51M) and PD (n=279; 85F and 194M) patient samples using ultracentrifugation as described above. The demographic and clinical details of PD and controls are shown (**Table 4**).

Table 4: *Demography and clinical data of PD and controls*

	Control	PD
N (Gender)	114 (63F, 51M)	279 (85F,194M)
Age	47.1±11.6	57.8±9.9
UPDRS Scale III	-	21.5±12.9

ELISA: For differentially expressed Clusterin (CLU) and Cofilin 1(CFL1) is being evaluated.

14. Detailed analysis of results:

The proteins identified by mass spectrometry were subjected to Gene Ontology (GO) analysis by Annotation, Visualization and Integrated Discovery (DAVID) functional Annotation Bioinformatic analyzer v 6.7. Statistical insights into biological processes, cellular components, and pathways were derived from the PANTHER database. A total of 65 proteins and 1978 peptides were identified across both control and patient groups. After manually excluding albumin, immunoglobulin, plasma proteins, and other complement proteins found in the proteomic data (**Table 2**), 14 proteins were shortlisted. **(Table 2).** Clusterin (CLU) and Cofilin 1(CFL1) was selected from the above list for further investigation due to previous studies suggesting its direct involvement in PD (Yan et al., 2020; Yan et al., 2022). Given its direct association with α-Syn aggregation and PD pathogenesis, we speculated that this could be the ideal candidate to be found in exosomes and detectable in circulating plasma exosomes in PD.

Reference:

Yan M, Meng L, Dai L. Zhang X, Chen G, Zheng Y et al., (2020). Cofilin 1 Promotes the Aggregation and Cell-to-Cell Transmission of α-Synuclein in Parkinson's Disease. *Biochem. Biophys. Res. Commun. 529*, 1053–1060.

Yan M, Xiong M, Dai L, Zhang X, Zha, Y, Deng X, Yu Z, Zhang Z (2022). Cofilin 1 Promotes the Pathogenicity and Transmission of Pathological α-Synuclein in Mouse Models of Parkinson's Disease. *NPJ Park. Dis. 8*, 1.

15. Summary sheet of not more than 2 pages under following heads:
(Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

Title: Identification and characterization of Ubiquitin and SUMO modified exosomal proteins from Parkinson's Disease patient's blood.

Introduction: The membrane-encapsulated extracellular vesicles or exosomes released from normal and diseased cells carry functional proteins, RNA, DNA, and metabolites. Central nervous system derived exosomes in blood are attractive target for clinical diagnosis, therapeutic response and biomarker discovery for neurodegenerative diseases since exosomes cross the blood brain barrier. Consistently, detectable amount of disease associated proteins have been found in exosomes, in the body fluids of patients. The aim is to derive a set of differentially expressed exosomal protein panels among PD patients using biochemical and proteomics approach.

Rationale: Although the potential to use exosomes and their contents as biomarkers have been studied in various diseases, its use in CNS disorders like Parkinson's Disease (PD) is still in

early stage. Owing to the ubiquitous presence and stability in various body fluids, exosomal proteins could serve as a source of potential biomarkers as their contents reflect the characteristic disease status of the tissue of their origin.

Objectives:

- Isolation and characterization of brain derived exosomes from plasma of PD patients.
- Identification of exosomal proteins, Ubiquitinated and SUMOylated proteins by mass spectrometry.
- Evaluate whether identified panel of proteins could be used as biomarker in PD.

Methodology:

Subject recruitment: Patients with Parkinson's disease who satisfied the standard diagnostic criteria (UKPDSBB) was selected for the study. Patients suffering from atypical parkinsonism (multiple system atrophy-MSA, progressive supranuclear palsy-PSP) and non-motor disease (Amyotrophic lateral sclerosis-ALS) was selected based on the standard diagnostic criteria. People who are not suffering from any neurodegenerative disease was included as healthy controls.

Sample collection: 10 ml blood sample was collected in EDTA tubes and the plasma separated was stored immediately in aliquots in -80°C.

Exosome isolation: Total plasma exosomes were isolated by differential centrifugation using ultracentrifuge.

Characterization of exosomes: SDS-PAGE followed by western blot was performed using following exosomal specific antibodies TSG101, Hsp90α, nonspecific marker Albumin, and a negative marker Calnexin. The homogeneity and size distribution were assessed by Dynamic Light Scattering. Transmission electron microscopy was performed to confirm the morphological features of the isolated exosomes.

Exosomal protein identification: Mass spectrometry, **ESI-LC (MS/MS),** analysis was used to identify the exosomal proteins.

Results:

Isolation of exosomes: Exosomes were isolated from plasma of patients (Parkinson's disease, MSA, PSP and ALS) and healthy controls by differential centrifugation (ultracentrifugation) method. SDS-PAGE coupled with Coomassie staining showed the presence of number of proteins in the exosome. The presence of exosomes in the eluted fraction was confirmed by western blot using exosome specific markers (TSG101 and Hsp90α). The morphological characteristics of exosomes was analyzed using transmission electron microscopy (TEM) which showed cup shaped morphological structures in the size range of 50-150 nm. The homogeneity and particle distribution of exosomes were analyzed by dynamic light scattering (DLS) which showed a homogenous peak with average particle size of 109.2nm.

Exosomal protein identification by Mass Spectrometry: ESI-LC (MS/MS) was performed with isolated exosomes from Parkinson's disease and control samples. Approximately 14 samples were identified that are specifically altered in PD when compared to control samples.

Translational Potential:

The unique exosomal protein "cargo" proteins, may be used as a blood-based screening method for suspected PD cases, progression or response to therapeutic intervention. The incidence of

noncommunicable disease including neurodegenerative diseases is a major health burden in India. Increased levels of unique proteins found in PD patients exosomes compared to controls could suggest that they play important role in PD pathogenies and progression. Further validation is required through ELISA assays with a larger sample size, along with comparison to related disorders such as MSA/PSP and non-motor disorders like ALS, to confirm the specificity of these proteins as a marker for PD.

16. Contributions made towards increasing the state of knowledge in the subject:

- Validation of dysregulated Clusterin (CLU) and Cofilin 1(CFL1) through ELISA assays along with comparison to related disorders such as MSA/PSP and non-motor disorders like ALS, has to be performed to confirm the specificity of CLU and CFL1 as a marker for PD. Additionally, conducting ELISA assays with samples from individuals with young onset PD (YPOD), varying disease durations, and severity, to determine the significance of increased Cofilin 1 levels in these conditions.

17. Conclusions summarising the achievements and indication of scope for future work:

- Conducting longitudinal follow-up cohorts to confirm the biomarker potential.
- Western blot analysis for exosomal Cofilin 1 to directly visualize the increased levels in a set of few samples.
- Additionally, other proteins identified through mass spectrometry, including Caspase 8, Clusterin, Transferrin, ZNF707, and ACIN1 (**Table 3**), could be potential biomarkers in the future, require further investigation of their involvement in PD pathogenesis.
- Mechanistic studies to understand the specific roles played by Cofilin 1 in PD pathogenesis using in vitro PD cellular model, animal model or iPSC cells derived from patients.

18. Science and Technology benefits accrued:

a. List of research publications with complete details:

NIL

b. Manpower trained on the project:

i.	**Research Scientists or Research Fellows**	**:**	**One Junior Research Fellow**
ii.	**No. of PhD's produced**	**:**	**One PhD (ongoing)**
iii.	**Other Technical Personnel trained**	**:**	**NIL**
c.	**Patents taken, if any**	**:**	**NIL**
d.	**Products developed, if any**	**:**	**NIL**

20. Abstract: (In 300 words for possible publication in ..... Bulletin)

Parkinson's disease (PD) diagnosis remains challenging, particularly in early stages, prompting a search for specific, minimally invasive biomarkers. Circulating exosomes, containing various biomolecules, have emerged as promising candidates for neurodegenerative disease including PD due to their ability to cross the blood-brain barrier. This study aimed to identify potential PD specific biomarkers and possibly to elucidate underlying disease mechanisms. Mass spectrometry analysis of exosomal proteins revealed elevated levels of Cofilin 1 and Clusterin in PD patients compared to controls. Future research directions include further validation with larger sample sizes, longitudinal studies, and comparison with related disorders like MSA/PSP to establish the specificity of Cofilin 1 and Clusterin. Additionally, investigation of other identified proteins like Caspase 8, Clusterin, Transferrin, ZNF707, and ACIN1 may uncover additional biomarkers and expand our understanding of PD mechanisms. Overall, this study contributes to the identification of one reliable blood-based biomarker, Cofilin 1, for PD diagnosis. This research also opens up new directions for understanding PD mechanisms, sheds light on the molecular pathways underlying the disease, and developing diagnostic tools and potentially leading to novel therapeutic strategies.

21. Procurement/Usage of Equipment: NONE

a. Details of Equipment:

Sl. No.	Name of Equipment	Make/ Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown

b. Suggestions for disposal of equipment(s):

NONE

Madhusoodanan UK

03-07-2024

(Name and Signature of PIs with date)

Routing: *Signed copy of "Project completion Report" by PI →* root@sctimst.ac.in, rpc@sctimst.ac.in