Medicine
Neuroscience

Plasma extracellular vesicle synaptic proteins as biomarkers of clinical progression in patients with Parkinson's disease: A follow-up study

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Abstract

Synaptic dysfunction plays a key role in Parkinson's disease (PD), and plasma extracellular vesicle (EV) synaptic proteins are emerging as biomarkers for neurodegenerative diseases. This study assessed the efficacy of plasma EV synaptic proteins as biomarkers in PD and their association with disease progression. In total, 144 participants were enrolled, including 101 people with PD (PwP) and 43 healthy controls (HCs). The changes in plasma EV synaptic protein levels between baseline and 1-year follow-up did not differ significantly in both PwP and HCs. In PwP, the changes in plasma EV synaptic protein levels were significantly associated with the changes in unified PD rating scale (UPDRS) part II and III scores. Moreover, PwP with elevated levels (first quartile) of any one plasma EV synaptic proteins (synaptosome-associated protein 25, growth-associated protein 43 or synaptotagmin-1) had significantly greater disease progression in UPDRS part II score and the postural instability and gait disturbance subscore in UPDRS part III than did the other PwP after adjustment for age, sex, and disease duration. These results indicate the promising potential of plasma EV synaptic proteins as clinical biomarkers of disease progression in PD. However, a longer follow-up period is warranted to confirm their role as prognostic biomarkers.

eLife assessment

This **useful** study presents data regarding the presence of synaptic proteins in the extracellular vesicle pool present in the blood of Parkinson's patients and non-parkinson neurological outpatients, trying to correlate changes in such levels with the progression of Parkinsonś symptoms. The results are semi-quantitative and preliminary, suggesting that these biomarkers might be **useful** in the follow up of a specific group of Parkinson patients. The evidence is **incomplete** at this point, and more quantitative approaches are required to propose this correlation. The isolation of extracellular vesicles was appropriate as revealed by their sizes, but they are not exclusively from neuronal origin. The presented approach is not ready to be used in the clinical setting.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease [1] that is well known for its progression, which involves increased disability and burden [2]. Worsening is noted not only in motor symptoms but also in nonmotor ones, particularly cognition. The rate of disease progression varies among people with PD (PwP). In the ongoing Parkinson's Progression Markers Initiative cohort study, approximately one-third of untreated PwP exhibited rapid progression during the first 2 years of follow-up. By contrast, the remaining PwP had a slow progressive course [3]. Unfortunately, no disease modifying therapy for halting disease progression and no predictor for assessing disease progression are available.

Synapses are sites of neuronal communication, and synaptic degeneration is an early functional pathogenic event in neurodegenerative diseases such as Alzheimer's disease (AD) and PD [4, 5]. Postmortem studies have revealed a substantial loss of dopamine terminals in the putamen and amygdala [6, 7]. In addition, the loss of glutamatergic corticostriatal synapses has been reported [8]. Mitochondrial dysfunction–related metabolic burden accounts for part of the synaptic loss in PD, and the aggregation of α-synuclein, a synaptic protein that is the major pathognomonic protein in PD, contributes to the pathogenesis of PD [9]. Assessment of synaptic proteins in the cerebrospinal fluid (CSF) can reflect synaptic loss in patients with neurological diseases and is a key area of research interest. Substantial research efforts have been focused on assessing the CSF synaptic pathology to improve the diagnosis of neurodegenerative diseases at an early stage, before neuronal loss, and to monitor clinical progression [10-12]. Several synaptic proteins are promising biomarkers of synaptic function. Synaptosome-associated protein 25 (SNAP-25) is a presynaptic protein that plays a crucial role in neuronal survival, vesicular exocytosis, and neurite outgrowth [13]. Increased CSF levels of SNAP-25 have been reported in PwP [12]. In addition, growth-associated protein 43 (GAP-43) is a presynaptic protein anchored to the cytoplasmic side of the presynaptic plasma membrane [14]. CSF levels of GAP-43 have been reported to be significantly higher in patients with AD than in HCs [15]. Synaptotagmin-1 is a calcium sensor vesicle protein that is vital for rapid synchronous neurotransmitter release in hippocampal neurons [16].

Significantly increased CSF levels of synaptotagmin-1 have been reported in patients with AD and mild cognitive impairment [17]. Regarding PD, one study reported an increase in the level of CSF SNAP-25 but not Ras-related protein 3A or neurogranin. Moreover, treated PwP exhibited higher CSF levels of SNAP-25 than did their drug-naïve counterparts [18].

However, the CSF collection process is moderately invasive, is inevitable during clinical assessment, and can result in some side effects, such as postpuncture headaches. Although studies have assessed blood biomarkers for PD diagnosis and progression, the results are conflicting. A lack of correlation between the peripheral blood content and the brain because of the blood–brain barrier (BBB) is a major obstacle to the identification of blood biomarkers for neurodegenerative diseases [19]. Assessment of peripheral blood extracellular vesicle (EV) proteins can be an alternative approach. EVs are tiny vesicles covered with a lipid membrane. They contain proteins, lipids, and nucleic acid responsible for cell-to-cell signal transmission. The integrity of EVs can be maintained when crossing the BBB [20]. Plasma EV biomarkers being rapidly developed for PD [21]. EV-cargo α-synuclein has been the most studied target that has exhibited strong potential for distinguishing PwP from HCs and other patients with atypical parkinsonism [22-26]. EV-cargo tau, β-amyloid, neurofilament light chain, brain-derived neurotrophic factor, and insulin receptor substrate have also been assessed in PwP [27-31]. These results indicate the potential role of EV content as biomarkers in PD.

The levels of synaptic proteins in blood exosomes, a specific type of EV, decrease in patients with AD and frontotemporal dementia [32]. Moreover, blood exosomal SNAP-25, GAP-43, neurogranin, and synaptotagmin-1 levels are lower in patients with AD. A combination of exosomal synaptic protein biomarkers could predict cognitive impairment [33]. Regarding PD, a cross-sectional study also had demonstrated that the synaptic proteins inside the blood neuron-derived exosomes are reduced in PwP compared with healthy controls (HCs), which can distinguish PwP with HCs with around 80% accuracy[34]. Considering the plasma EVs remain stable up to 90 days [35]; this prevents the fluctuation of free-form synaptic proteins because of transient surge or degradation. Moreover, SNAP-25 is transported in the blood by EVs [36]. However, there is no information from the cohort study of PD. Therefore, this study assessed the association between plasma EV synaptic proteins and PD progression and determined whether plasma EV synaptic proteins could be used as clinical biomarkers to predict the progression of PD.

Results

The participants' demographic data at baseline and 1-year follow-up are presented in **Table 1**. In total, 144 participants (101 PwP and 43 HCs) were followed up. No significant difference was noted in plasma EV SNAP-25, GAP-43, and synaptotagmin-1 levels at baseline and follow-up between PwP and HCs after adjustment for age and sex (**Figures 1A** [representative image] and B–D [dot plot]) (Supplementary Figure 1 for the original blot of the representative image).

We assessed the association between the changes in plasma EV synaptic proteins levels and the changes in clinical parameters in PwP through the generalized linear model (**Table 2**). The changes in the total score of unified Parkinson disease rating scale (UPDRS)-II was positively associated with the change of plasma EV synaptic proteins (SNAP-25, GAP-43, and synaptotagmin-1); the change of total score of UPDRS-III and akinetic rigidity (AR) subscore were significantly associated with the change of plasma EV GAP-43 and synaptotagmin-1. The changes in mini-mental status examination (MMSE) and Montral cognitive assessment (MoCA) scores were non-significantly associated with the changes in plasma EV synaptic protein levels.

We further assessed the association between the severity of the clinical parameters of PD at follow-up and the baseline plasma EV SNAP-25, GAP-43, and synaptotagmin-1 levels. After adjustment for age, sex, and disease duration, the plasma EV SNAP-25, GAP-43, and synaptotagmin-1 levels were nonsignificantly associated with the UPDRS-II, UPDRS-III, MMSE, and MoCA scores at follow-up (**Figure 2**; for details, refer to Supplementary Table 1). However, after we categorized the UPDRS-III scores into tremor, AR, and postural instability and gait disturbance

	HCs, n = 43	PwP, n = 101
Age (years)	65 (10.24)	69 (7.76)
Women	15	48
Baseline		
MMSE	27 (3.92)	26 (4.15)
MoCA	23 (4.63)	21 (5.70)
Disease duration (years)	-	2 (2.24)
UPDRS-II	-	8 (5.58)
UPDRS-III	-	22 (9.30)
1-year follow-up		
MMSE	28 (4.12)	27 (5.61)
MoCA	24 (5.82)	23 (6.45)
UPDRS-II	-	11 (6.41)
UPDRS-III	-	19 (9.39)

Data was presented as median (standard deviation). HC, healthy control; PwP, people with Parkinson's disease; MMSE, Mini-Mental State Examination; MoCA, Montreal cognitive assessment; UPDRS, unified Parkinson's disease rating scale.

Table 1.

Demographic data of study participants

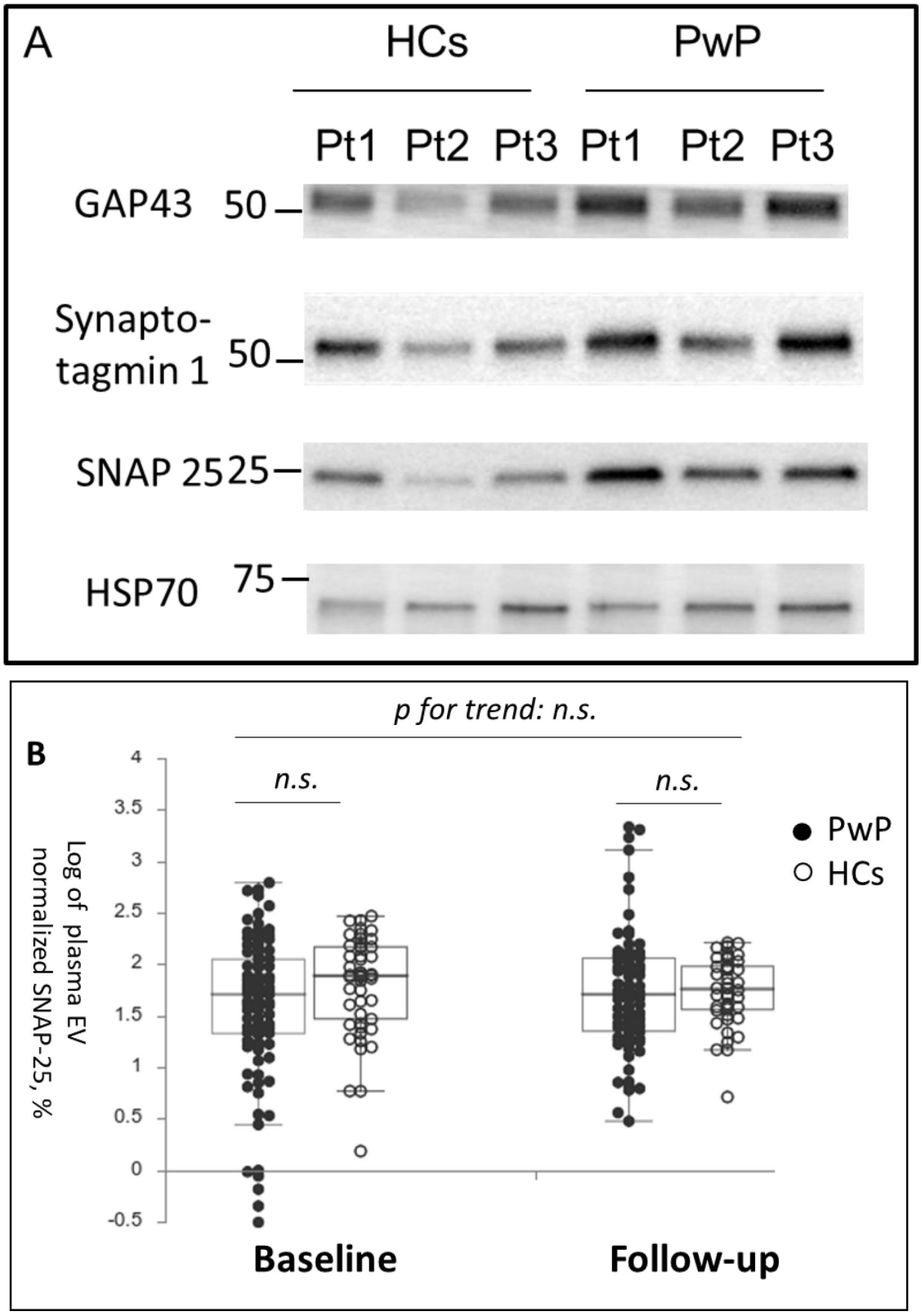

Figure 1.

Baseline and follow-up synaptic protein levels in plasma extracellular vesicles (EVs) between patients with Parkinson's disease (PwP) and healthy controls (HCs). (A) Representative protein blot images of different synaptic proteins, including SNAP-25, GAP-43, and synaptotagmin-1. Heat shock protein 70 (HSP-70) was the protein loading control. (B–D) Comparison of plasma SNAP-25, GAP-43, and synaptotagmin-1 levels between PwP and HCs at baseline and follow-up. Data are presented using a dot plot displaying the median and first and third quartile values. n.s., non-significant.

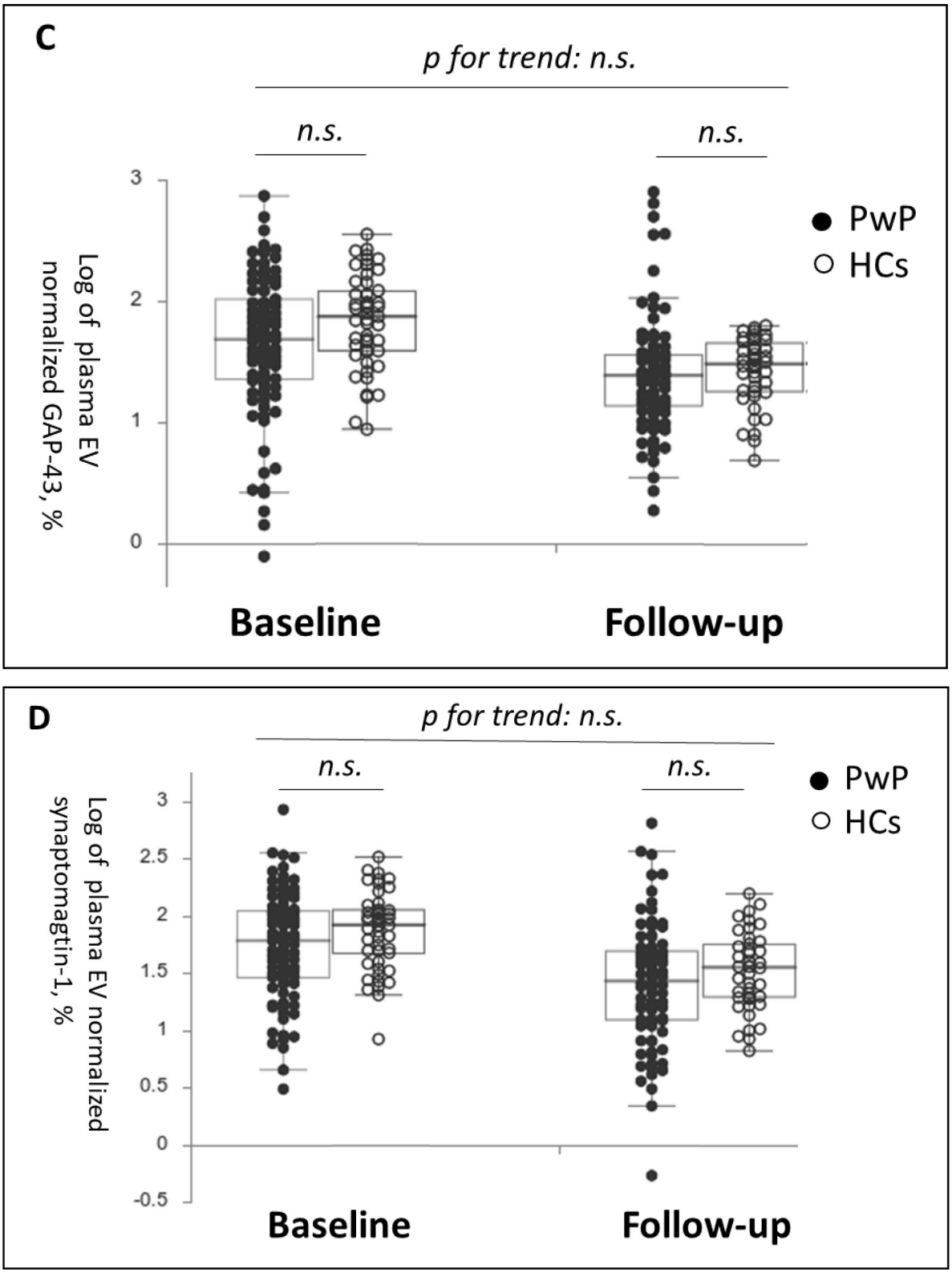

Figure 1. (continued)

	UPDRSII	UPDRSIII				MMSE	MoCA
			Tremor	AR	PIGD		
SNAP-25*Follow	0.218 **(0.049)**	0.312 (0.076)	0.004 (0.432)	0.016 (0.066)	0.009 (0.440)	-0.007 (0.932)	0.048 (0.647)
GAP-43*Follow	0.984 **(0.031)**	1.711 **(0.018)**	0.001 (0.972)	0.089 **(0.011)**	0.073 (0.115)	-0.099 (0.767)	-0.054 (0.901)
Synaptomagtin-1 *Follow	1.543 **(0.012)**	2.205 **(0.024)**	0.007 (0.815)	0.107 **(0.023)**	0.109 (0.080)	-0.361 (0.421)	-0.260 (0.661)

UPDRS, unified Parkinson Disease rating scale; AR, akinetic rigidity; PIGD, postural instability and gait disturbance; MMSE, mini-mental status examination; MoCA, Montreal cognitive assessment.

Table 2.

The association between the change of plasma EV synaptic proteins abundance (between baseline and follow-up) with the change of clinical severity in motor and cognitive domains (between baseline and follow-up) in people with Parkinson's disease. A generalized linear model was employed and the data was presented as coefficient (p value).

(PIGD) subscores, the baseline plasma EV SNAP-25 and GAP-43 levels exhibited a significant positive correlation with the PIGD subscores at follow-up; a similar trend was noted in the plasma EV synaptotagmin-1 level.

Finally, we grouped the PwP on the basis of their baseline plasma EV synaptic protein level, with a cutoff at the first quartile. Overall, PwP with elevated baseline levels of plasma EV synaptic proteins had poor total scores of UPDRS-II and UPDRS-III and poor PIGD subscores of UPDRS-III (**Table 3** and detail in supplementary Table 2). In contrast, a significant greater improvement in the tremor score was noted in the PwP with elevated baseline levels of plasma EV synaptic proteins. Moreover, PwP with elevated baseline levels of plasma EV synaptic proteins exhibited significantly greater deterioration, as assessed using the UPDRS-II scores and PIGD subscores in UPDRS-III. After adjustment for age, sex, and disease duration, repeated-measures analysis of covariance revealed that the estimated marginal means of UPDRS-II scores and PIGD subscores of PwP with elevated levels of any one plasma EV synaptic protein were significantly worse at follow-up (but not at baseline) than those of PwP without elevated plasma EV synaptic protein levels, indicating significantly faster deterioration in the former patient group (**Figure 3**).

Discussion

In this study, although no significant difference in plasma EV synaptic protein levels was noted between PwP and HCs, changes in plasma EV synaptic protein levels in PwP were associated with motor decline. In addition, baseline plasma EV synaptic protein levels were associated with clinical outcomes, as assessed using PIGD subscores at follow-up. PwP with elevated baseline levels of any one plasma EV synaptic protein (SNAP-25, GAP-43, or synaptotagmin-1) exhibited significant deterioration in activities of daily living (as assessed by UPDRS-II scores and PIGD subscores in UPDRS-III) between baseline and follow-up. These results indicate the promising potential of plasma EV synaptic proteins as biomarkers for PD, particularly its progression.

Significant worsening of UPDRS-II scores and PIGD subscores in UPDRS-III was noted in PwP with higher baseline levels of plasma EV synaptic proteins (first quartile). UPDRS-II is a crucial but underestimated parameter for assessing PwP; it can be used to assess the daily functional capability related to motor symptoms in PwP without temporary drug interruptions, as in the case of UPDRS-III [37, 38]. Assessment of the motor subtypes of PD revealed that the PIGD subtype is associated with an increased Lewy body and α-synuclein burden; rapid decline; and increased risks of cognitive impairment, falls, and mortality in comparison with the tremor-dominant (TD) subtype, which has a relatively benign course [39-42]. PwP may convert from the TD subtype to the PIGD subtype during disease progression [43]. Moreover, PIGD-related motor symptoms respond to dopaminergic medications to a lesser extent than do tremor-, akinesia-, and rigidity-related symptoms [44], which may more directly reflect the progression of the disease. We observed that PwP with elevated levels of any one plasma EV synaptic protein exhibited greater deterioration, as assessed using UPDRS-II scores and PIGD subscores. High EV synaptic protein levels conventionally indicate increased synaptogenesis; this contrasts the synaptic loss pattern noted in PD. Decreased blood exosomal synaptic protein levels have been reported in patients with AD and frontotemporal dementia [32]. However, in PwP who are at the early disease stage, compensatory synaptic sprouting and increased synaptic plasticity are noted in the striatum. This compensation, also known as motor reserve, may temporarily decrease the clinical disease burden. However, the patient would be more vulnerable to deterioration if the compensation is overshadowed by degeneration [45]. For instance, in one study, PwP who engaged in higher premorbid exercises exhibited milder motor symptoms despite having a similar gradient of striatal dopaminergic reduction at baseline; however, they exhibited more rapid deterioration [46]. Because our study mainly included PwP who had the early stage of disease (mean disease duration of less than 3 years) and a mild disease burden (baseline UPDRS-II score = 8.49), the elevated plasma EV synaptic protein levels may indicate the activation of the compensatory

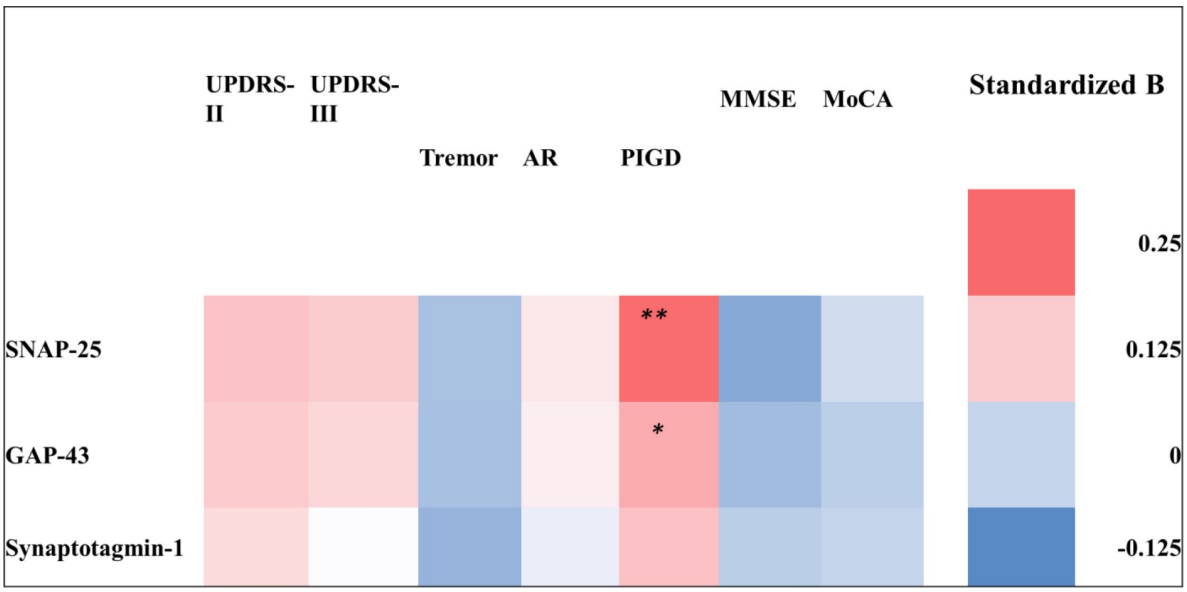

Figure 2.

Heatmap of the association between baseline plasma extracellular vesicle (EV) synaptic protein levels and clinical assessment parameters at follow-up in patients with Parkinson's disease (PwP). The logistic regression model was used to assess the baseline plasma EV SNAP-25, GAP-43, and synaptotagmin-1 levels. The motor symptoms were assessed based on the Unified Parkinson Disease Rating Scale (UPDRS)-II and UPDRS-III scores and tremor, akinetic rigidity (AR), and postural instability and gait disturbance (PIGD) subscores, and cognitive function was assessed using the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) scores. The association is presented using standardized β values. Detailed results of the regression model are provided in Supplementary Table 1. *, $p < 0.05$; **, $p < 0.01$.

Plasma EV		SNAP-25			GAP-43			Synaptotagmin-1		
		L, n=74	H, n=28	p	L, n=74	H, n=28	p	L, n=74	H, n=28	p
UPDRS-II	Baseline	8.26±5.80	9.04±4.90	**<0.001**	8.53±5.67	8.32±5.33	**<0.001**	8.46±5.70	8.50±5.24	**<0.001**
	Follow-up	10.38±6.11	13.36±6.72		10.65±6.30	12.64±6.53		10.57±6.25	12.86±6.58	
UPDRS-III	Baseline	22.31±9.54	23.89±8.65	0.259	22.22±9.66	24.14±8.25	0.244	21.97±9.37	24.79±8.93	0.145
	Follow-up	20.01±9.33	24.21±8.87		19.96±9.25	24.36±8.99		20.04±9.39	24.14±8.70	
Tremor	Baseline	0.34±0.46	0.46±0.38	**0.037**	0.34±0.34	0.46±0.37	**0.034**	0.33±0.34	0.47±0.38	**0.014**
	Follow-up	0.27±0.26	0.35±0.32		0.27±0.26	0.35±0.32		0.28±0.29	0.32±0.22	
AR	Baseline	1.04±0.46	1.09±0.44	0.300	1.03±0.47	1.11±0.42	0.325	1.03±0.46	1.13±0.45	0.195
	Follow-up	0.96±0.46	1.10±0.42		0.95±0.45	1.12±0.43		0.96±0.46	1.10±0.42	
PIGD	Baseline	0.76±0.61	0.81±0.44	**0.023**	0.77±0.60	0.77±0.45	**0.046**	0.74±0.56	0.86±0.58	**0.027**
	Follow-up	0.74±0.59	1.07±0.75		0.78±0.61	0.98±0.74		0.73±0.56	1.11±0.80	
MMSE	Baseline	25.32±4.45	25.29±3.29	0.483	25.58±4.16	24.61±4.11	0.470	25.62±3.91	24.50±4.69	0.342
	Follow-up	24.78±5.93	25.14±4.64		25.05±5.79	24.43±5.10		25.23±5.61	23.96±5.50	
MoCA	Baseline	20.68±6.00	21.21±5.00	0.834	21.14±5.77	20.04±5.62	0.899	21.12±5.33	20.07±6.69	0.926
	Follow-up	20.60±6.85	21.46±5.37		21.10±6.55	20.18±6.30		21.19±6.33	19.93±6.80	

L: 2nd to 4th quartile at baseline; H: 1st quartile at baseline; UPDRS, unified Parkinson Disease rating scale; AR, akinetic rigidity; PIGD, postural instability and gait disturbance; MMSE, mini-mental status examination; MoCA, Montreal cognitive assessment.

Table 3.

The clinical severity in people with Parkinson's disease with and without elevated (1st quartile) baseline plasma extracellular vesicle (EV) synaptosome-associated protein 25 (SNAP-25), growth-associated protein 43 (GAP-43) and synaptotagmin-1. P value indicated the inter-group comparisons for the changes.

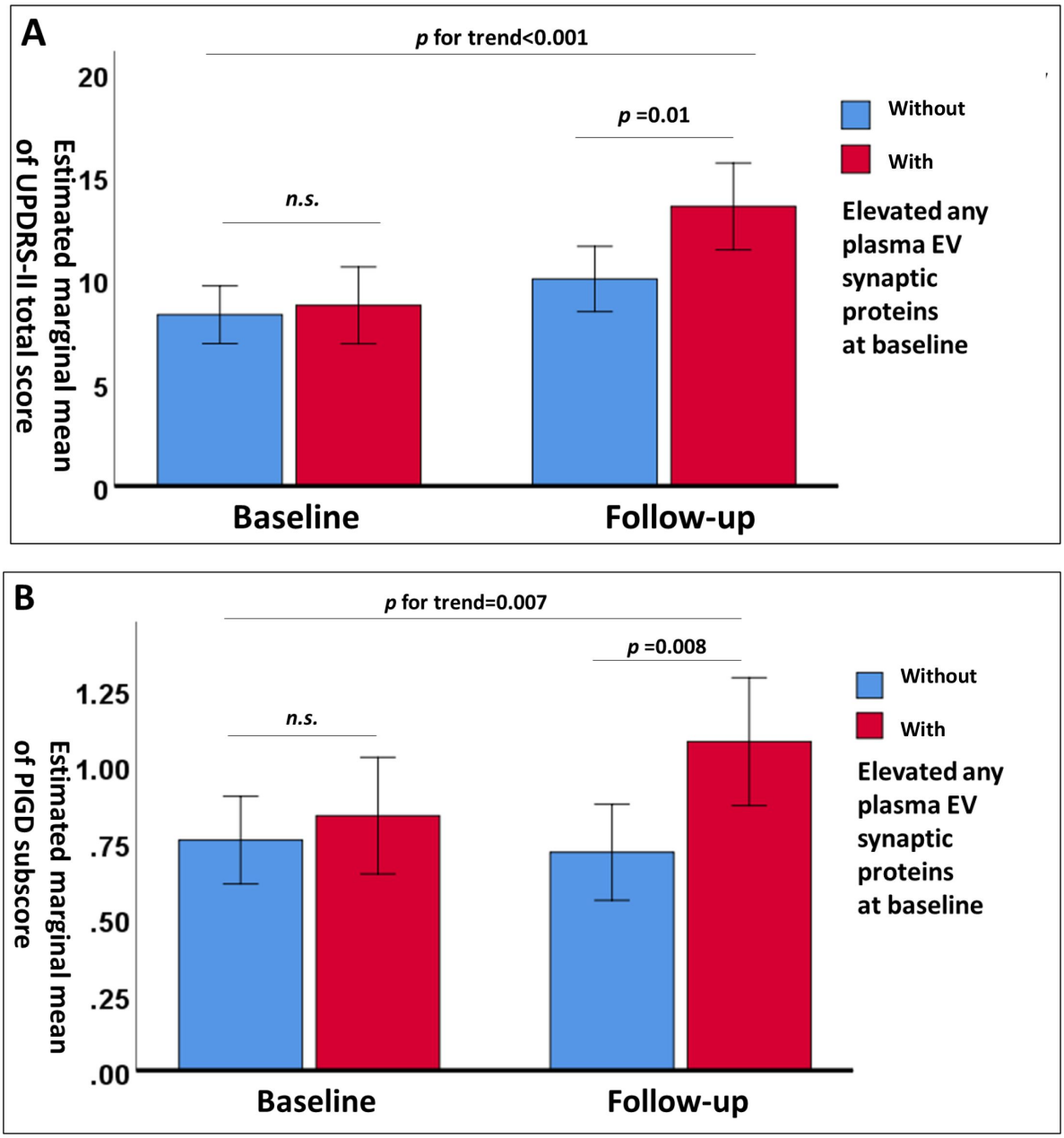

Figure 3.

Changes in estimated marginal means of Unified Parkinson Disease Rating Scale (UPDRS)-II total scores (A) and postural instability and gait disturbance (PIGD) subscores (B) after adjustment for age, sex, and disease duration in patients with Parkinson's disease (PwP) with and without elevated levels of any one plasma extracellular vesicle synaptic protein (first quartile) at baseline and follow-up. Data are presented as means with 95% confidence intervals. n.s., nonsignificant.

process. Such PwP are at an increased risk of rapid deterioration and disease progression and should be candidates for disease-modifying interventions, including pharmacological and nonpharmacological treatments.

The strength of this study is that it is the first study to assess the changes in plasma EV synaptic protein levels and determine the association between the changes in plasma EV synaptic protein levels and cognitive decline in PwP. Considering the well-established role of synaptic degeneration and plasticity in PD, the theoretical background of the use of these synaptic proteins as plasma EV biomarkers for PD is confirmed. Moreover, most synaptic proteins are neuron derived, thereby preventing contamination from nonneuronal tissues. The significant association between the changes in plasma EV synaptic proteins levels and the changes in UPDRS-II, UPDRS-III and AR subscore suggests the efficacy of these proteins in detecting motor decline in PwP, which can serve as a objective parameter for further disease-modification clinical trial. Elevated baseline plasma EV synaptic protein levels can also predict rapid deterioration in PwP, highlighting the importance of motor reserve and synaptic plasticity in the progression of PD for future research. Although any single synaptic protein cannot precisely predict the progression of PD, these protein targets may be considered for use in the biomarker panel and analyzed using an artificial intelligence–assisted artificial neural network, which is widely used to predict the outcomes of several neurological diseases [47-50].

This study has some limitations. Technically, semiquantitative assessment of plasma EV synaptic protein (SNAP-25, GAP-43, and synaptotagmin-1) levels was performed using western blot analysis. The lack of absolute values, i.e. from the results of enzyme-linked immunosorbent assay, limits further clinical application. In addition, because numerous synaptic proteins are involved in the pathogenesis of PD, the three selected proteins may not reflect all features of the synaptic condition. The selection of HSP-70 as the control for the synaptic proteins quantification of plasma EV is not undisputable. Two types of EV proteins are used for this role: membrane proteins and intravesicular proteins. Membrane proteins include CD9, CD63, and CD81; intravesicular proteins include TSG101, annexins, and chaperone proteins, such as HSP-70. Since the expression of HSP-70 is usually steady, we used the HSP-70 as the internal control, but it is a limitation of the present study. Furthermore, this study evaluated the overall plasma EVs rather than specifically focusing on neuron-derived exosomes, potentially introducing a bias towards somatic-origin EVs. Nonetheless, it is worth noting that synaptic proteins primarily originate from neurons. Even when considering neuron-derived exosomes, it's important to recognize that they are not exclusively derived from the brain, which can lead to contamination from the peripheral nervous system. The final technical issue in the present study was the relatively small size of the isolated EVs. Despite the primary focus on isolating exosomes, which are the smallest type of EVs, it's important to consider that the presence of small-sized EVs could potentially be attributed to EV fragmentation that occurs during the freezing and thawing processes. Regarding the limitation from the cohort, it is also worth mentioning that the 1-year follow-up period to assess the progression of PD was relatively short and may have been insufficient to detect significant disease progression. On the other hand, the evaluation of motor symptoms occurred in a hospital setting where we did not ask patients to stop taking their anti-PD medications due to safety concerns like the risk of falls. As a result, specific motor symptoms, particularly tremor and AR, which are more sensitive to medication compared to PIGD, may have been effectively managed by the anti-PD medications. This could potentially explain the improvement in tremor observed between the baseline and one-year follow-up, especially among PwP with elevated baseline plasma EV synaptic proteins. Additionally, synaptic dysfunction is a frequently observed phenomenon in several neurological diseases, and it is not exclusive to PD. Consequently, the HC group in our current study may have included individuals with coexisting neurological conditions, potentially explaining the lack of a significant difference between the PD group and the HCs. However, this approach also illuminates the significance of synaptic dysfunction in the advancement of PD. This insight can be invaluable for monitoring disease progression, particularly in the context of clinical trials focused on disease modification. Choosing the first quartile as a cut-off value of plasma EV

synaptic proteins is also one of the limitations of the study. While developing new biomarkers, there was no clear cut-off value as reference for the continuous variable, and percentile is considered for predicting the prognosis[51]. Further studies are required to validate this application. Lastly, the results form a mono-centric, small-scale and short-period PD cohort required further validation

In conclusion, this study revealed that changes in the levels of plasma EV synaptic proteins, namely SNAP-25, GAP-43, and synaptotagmin-1, are associated with motor decline in PwP. Elevated baseline plasma EV synaptic protein levels can predict increased deterioration of motor function, particularly PIGD symptoms, in PwP. Our results indicate that plasma EV synaptic proteins have the potential to be used as biomarkers of PD progression and detection. A longer longitudinal follow-up is warranted to clearly assess the prognostic efficacy of plasma EV synaptic proteins in PwP.

Methods

Study participants

This study included 101 PwP and 43 HCs. PD was diagnosed in accordance with the criteria used in another study [52]. Patients diagnosed as having early-to-mid-stage PD (Hoehn and Yahr stage I–III) were invited to participate in this study. HCs were excluded if they had comorbidities, such as neurodegenerative, psychiatric, or major systemic diseases (malignant neoplasm or chronic kidney disease). HCs were mainly recruited from neurological outpatient clinics; they had minor chronic health conditions, such as hypertension, diabetes, or hyperlipidemia. This study was approved by the Joint Institutional Review Board of Taipei Medical University (approval no. N201609017 and N201801043).

Clinical assessments

The participants' background data were obtained through a personal interview. Their cognitive function was assessed by trained nurses using the Taiwanese versions of the MMSE and MoCA. The severity of PD was assessed using parts I, II, and III of the UPDRS during clinic visits. PwP were assumed to be in their "on" time. Tremor, AR, and PIGD subscores were calculated from the subitems in UPDRS-III as described previously [53], with some modifications.

Plasma EV isolation and characterization

Venous blood was collected by from PwP and HCs after their outpatients clinic (non-fasting) by 21 gauge needle, and the plasma was isolated through centrifugation at 13,000 × *g* for 20 minutes immediately. Plasma was storage in the −80°C freezer before EV isolation. Plasma EVs were isolated from 1 mL of plasma by exoEasy Maxi Kit (Qiagen, Valencia, CA, USA), a membrane-based affinity binding step to isolate exosomes and other EVs without relying on a particular epitope, in accordance with the manufacturer's instructions and storaged in the −80°C freezer. The isolated plasma EVs were then eluted and stored. Usually, 400 µL of eluate is obtained per mL of plasma. The isolated plasma EVs were validated according to the International Society of Extracellular Vesicles guidelines, which include1.markers, including the presence of CD63 (ab59479, Abcam, Cambridge, UK), CD9(ab92726, Abcam, Cambridge, UK), tumor susceptibility gene 101 protein (GTX118736, GeneTex, CA, USA) and negative of cytochrome c (ab110325; Abcam, Cambridge, UK) 2. Physical characterization through the nanoparticle tracking analysis, which demonstrated the majority of the size of EV are mainly within 50-100nm 3. The morphology from the electron microscopy analysis. The validation had been described previously [29-31].

Quantification of plasma EV synaptic proteins

The isolated plasma EVs were directly lysed using protein sample buffer (RIPA Lysis Buffer, Millipore) and analyzed using protein sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibodies against SNAP-25 (GeneTex, GTX113839, 1:1000), GAP-43 (GeneTex, GTX114124, 1:5000), and synaptotagmin-1 (GeneTex, GTX127934, 1:1000) were used for the analysis. The antibodies were prepared in Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin. Secondary antibodies, including antimouse immunoglobulin G (IgG)-conjugated horseradish peroxidase (HRP; 115-035-003) and antirabbit IgG-conjugated HRP (111-035-003), were purchased from Jackson ImmunoResearch. Protein blot intensities were quantified using ImageJ software. The expression levels of plasma EV synaptic proteins were normalized to that of heat shock protein 70 (Proteintech, Cat.10995-1-AP, 1:2000). For each participant, equal volume of EV suspension (5μl) was applied to the protein quantification. To ensure that the data could be compared between different gels, all the data were normalized to the average of the control group in the same gel.

Statistical analyses

All statistical analyses were performed using SPSS for Windows 10 (version 26; SPSS Inc., Chicago, IL, USA). A linear mixed model was used to assess whether the changes in plasma EV synaptic protein levels differed between PwP and HCs after adjustment for age and sex. A generalized linear model was used to determine the association between the changes in plasma EV synaptic protein levels and the changes in clinical parameters in PwP after adjustment for age, sex, and disease duration. Multivariate logistic regression was performed to assess the association between plasma EV synaptic proteins and clinical parameters at follow-up in PwP after adjustment for age, sex, and disease duration. Repeated-measures analysis of covariance with estimated marginal means was employed to compare the changes in clinical parameters between baseline and follow-up in PwP with elevated baseline levels (first quartile) of any one plasma EV synaptic protein. Finally, p values < 0.05 were considered statistically significant.

Data Availability

Please contact the corresponding author (CT Hong). The availability of data and materials requires permission from the TMU-JIRB.

Declarations

Ethics approval and consent to participate

This study was approved by the Joint Institutional Review Board of Taipei Medical University (TMU-JIRB approval no. N201609017 and N201801043). Written informed consent was obtained from all participants for participation in the study.

Consent for publication

All authors have read and approved the final version of the manuscript. All authors agree to the present state of authorship and have signed a statement attesting to the authorship.

Availability of data and materials

Please contact the corresponding author (CT Hong). The availability of data and materials requires permission from the TMU-JIRB.

Competing interests

The authors declare that there are no competing interests.

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Authors' contributions

Study conception and design: CT Hong, L Chan, and CC Chung. Data acquisition and analysis: CT Hong, L Chan, and CC Chung. Data interpretation: CT Hong and RC Yu. Manuscript writing and revision: CT Hong, CC Chung, L Chan, and RC Yu. Provision of resources and administrative oversight: CT Hong. All authors have read and approved the final manuscript.

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Reviewer #1 (Public Review):

The study isolated extracellular vesicles (EV) from healthy controls (HCs) and Parkinson patients (PwP), using plasma from the venous blood of non-fasting people. Such EVs were characterized and validated by the presence of markers, their size, and their morphology. The main aim of the manuscript is to correlate the presence of synaptic proteins, namely SNAP-25, GAP-43, and SYNAPTOTAGMIN-1, normalized with HSP70, with the clinical progression of PwP. Changes in synaptic proteins have been documented in the CSF of Alzheimer's and Parkinson's patients. The demographics of participants are adequately presented. One important limiting, as well as puzzling aspect, is the fact that authors did not find differences between groups at the beginning of the study nor after one year, after age and sex adjustment.

- https://doi.org/10.7554/eLife.87501.2.sa1

Reviewer #2 (Public Review):

Hong and collaborators investigated variations in the amount of synaptic proteins in plasma extracellular vesicles (EV) in Parkinson's Disease (PD) patients on one-year follow-up. Their findings suggest that plasma EV synaptic proteins may be used as clinical biomarkers of PD progression.

It is a preliminary study using semi-quantitative analysis of synaptic proteins.

The authors have a cohort of PD patients with clinical examination and a know-how on EV purification. Regarding this latter part, they may improve their description of EV purification.

EV may be broken into smaller size EV after freezing. Does it explain the relatively small size in their EV preparation? Do the authors refer to the MISEV guidelines for EV purity? Regarding synaptic protein quantification, the choice of western blotting may not be the best one. ELISA and other multiplex arrays are available. How the authors do justify their choice? Do the authors try to sort plasma EV by membrane-associated neuronal EV markers using either vesicle sorting or immunoprecipitation?

Many technical aspects may be improved. Such technical questions weakened the authors' conclusions.

- https://doi.org/10.7554/eLife.87501.2.sa0

Author Response

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *The study isolated extracellular vesicles (EV) from healthy controls (HCs) and Parkinson patients (PwP), using plasma from the venous blood of non-fasting people. Such EVs were characterized and validated by the presence of markers, their size, and their morphology. The main aim of the manuscript is to correlate the presence of synaptic proteins, namely SNAP-25, GAP-43, and SYNAPTOTAGMIN-1, normalized with HSP70, with the clinical progression of PwP. Changes in synaptic proteins have been documented in the CSF of Alzheimer's and Parkinson's patients. The demographics of participants are adequately presented.*
>
> - *One important limiting, as well as puzzling aspect, is the fact that authors did not find differences between groups at the beginning of the study nor after one year, after age and sex adjustment.*

Response: Thanks for your comments. We acknowledge your observation that the absence of a discernible difference in plasma EV synaptic protein levels between the PD and control subjects constitutes a significant limitation of our study. This outcome could be attributed to the fact that the controls were recruited from the neurology outpatient clinic, representing a group that could be considered "sub-healthy." Moreover, these individuals are not exempt from aging-related neurodegenerative processes. Considering that our PD subjects are in the early stages of the disease (with a mean disease duration of less than 3 years) and that synaptic dysfunction is a broader indicator rather than specific to PD, these factors could collectively contribute to the lack of distinction between the PD and control groups.

However, our primary intention was also to explore the potential of plasma EV synaptic proteins as predictive markers for disease progression in PD. In this regard, we have identified their applicability within the current PD cohort. We are committed to conducting further follow-up with these study subjects over an extended duration to delve deeper into these findings.

We revised the following statement in the discussion part to address this issue as following "Additionally, synaptic dysfunction is a frequently observed phenomenon in several neurological diseases, and it is not exclusive to PD. Consequently, the HC group in our current study may have included individuals with coexisting neurological conditions, potentially explaining the lack of a significant difference between the PD group and the HCs. However, this approach also illuminates the significance of synaptic dysfunction in the advancement of PD. This insight can be invaluable for monitoring disease progression, particularly in the context of clinical trials focused on disease modification."

- *Tables in general are hard to follow. Specifically, Table 2 does not convey a clear message nor in the text of the Table itself, and the per 100% of change needs to be explained in the corresponding legend.*

Response: Thanks for your comment. In Table 2, our aim was to demonstrate the association between the change of plasma EV synaptic proteins with the change of clinical severity, and presented as coefficient (p value). We apologize for any prior ambiguity in the main text's description of these results and have since made revisions to enhance clarity.

Regarding the "per 100% change," this is due to the quantification of plasma EV synaptic proteins being based on a semi-quantitative Western blot method. Each measurement was normalized by the average baseline plasma synaptic protein levels of healthy controls (HCs). The term "per 100% change" denotes the increase or decrease in plasma EV synaptic protein abundance relative to the average baseline levels observed in healthy controls. We apologize for any confusion caused and removed this term. In addition, we rephrased the statement to ensure better understanding and readability in the Table legend of revised manuscript as following “The association between the change of plasma EV synaptic proteins abundance (between baseline and follow-up) with the change of clinical severity in motor and cognitive domains (between baseline and follow-up) in people with Parkinson’s disease. A generalized linear model was employed and the data was presented as coefficient (p value).”

- *It is only when PwP were classified as a first quartile that a significantly greater deterioration was found. However, in the case of tremor, the top 25% had values going from 0.46-0.47 to 0.32-0.35, whereas the lower three quarters went from 0.33-0.34 to 0.27-0.28 depending on the protein analyzed. This needs to be clarified in the text.*

Response: Thanks for your comments. As per the unified Parkinson's disease rating score (UPDRS), a higher score indicates greater severity of symptoms. Regarding tremor, we observed a general trend of improvement in both groups. PwP with elevated baseline plasma EV proteins had a trendy of worse tremor score at baseline, and the improvement was significantly better than the rest of PwP. This improvement seems to contradict the progressive nature of PD, and one possible explanation could be the alleviation of symptoms due to medication usage. The assessment of motor symptoms took place within the hospital setting, where we refrained from requesting patients to withhold their anti-PD medications due to concerns about safety issues such as falls. Consequently, certain motor symptoms might have been effectively controlled by the anti-PD medication. Traditionally, symptoms like tremor and rigidity (as reflected by the akinetic rigidity score) respond well to medications, while postural instability and gait disturbance (PIGD) are less responsive. In our cohort, we noted an improvement in tremor scores and stability in akinetic rigidity (AR) scores. Conversely, PD patients with higher baseline plasma EV synaptic protein levels exhibited notable progression in PIGD scores. These findings have been documented in the results section and discussed comprehensively within the revised manuscript as following “On the other hand, the evaluation of motor symptoms occurred in a hospital setting where we did not ask patients to stop taking their anti- PD medications due to safety concerns like the risk of falls. As a result, specific motor symptoms, particularly tremor and AR, which are more sensitive to medication compared to PIGD, may have been effectively managed by the anti-PD medications. This could potentially explain the improvement in tremor observed between the baseline and one-year follow-up, especially among PwP with elevated baseline plasma EV synaptic proteins.”

- *Table 3 is hard to read and some of the values seem repetitive, especially for tremor, AR, and PIGD. It looks as if Figure 2 represents the same information as*

> *Table 3.*

Response: Thanks for your information. We have ensured the accuracy of the results presented in Table 2. While some of the entries may appear similar, they do indeed possess distinct differences.

To enhance readability, we streamlined the information in Table 3 by removing the p-values from the intra-group comparisons between baseline and the 1-year follow-up within each domain. We retained the original p-values for trend related to the inter-group comparisons for changes. Detailed information has been relocated to the supplementary section of the revised manuscript. In Figure 2, we illustrated the relationship between baseline plasma extracellular vesicle (EV) synaptic protein levels and the clinical assessment parameters during follow-up in patients with Parkinson's disease (PwP). This portrayal is distinct from the information depicted in Table 3.

If you had concerns about the resemblance between Table 3 and Figure 3, please note that the values in Table 3 represent raw scores, while the values in Figure 3, namely the estimated marginal means, are the "adjusted" scores for UPDRS-II and PIGD at baseline and follow-up. These adjustments encompass age, sex, and disease duration. We sincerely apologize for any lack of clarity in our previous description and have since revised it accordingly.

> - *The text and figure legends are not helpful in guiding the reader to understand the presented information.*

Response: Thanks for your comments and we apologized for the unclear statement. We revised the figure legend and the main text for better understanding of the readers.

> ***Reviewer #2 (Public Review):***
>
> *Hong and collaborators investigated variations in the amount of synaptic proteins in plasma extracellular vesicles (EV) in Parkinson's Disease (PD) patients on one-year follow-up. Their findings suggest that plasma EV synaptic proteins may be used as clinical biomarkers of PD progression.*
>
> - *It is a preliminary study using semi-quantitative analysis of synaptic proteins.*

Response: Thanks for your comments. The present study represents the initial phase of our investigation into the role of plasma EV synaptic proteins within our PD cohort. Our findings have revealed the potential predictive significance of these synaptic proteins in relation to PD progression. We are committed to conducting further follow-up with these study subjects over an extended period.

Furthermore, it's important to acknowledge that the semi-quantitative approach employed to assess protein abundance was a limitation of this study. This limitation stems from the low concentration of plasma EV synaptic proteins, which restricts the feasibility of utilizing techniques such as ELISA or other quantitative methods for protein assessment. We have duly acknowledged this limitation within the scope of the present study as following “Semiquantitative assessment of plasma EV synaptic protein (SNAP-25, GAP-43, and synaptotagmin-1) levels was performed using western blot analysis. The lack of absolute values limits further clinical application.”

Moving forward, we intend to adopt alternative EV isolation methods that enable the extraction of a larger abundance of plasma EV proteins, facilitating more accurate quantitative assessments. In addition, a longer longitudinal follow-up is warranted to clearly

assess the prognostic efficacy of plasma EV synaptic proteins in PwP, which we had mentioned in the manuscript.

> - *The authors have a cohort of PD patients with clinical examination and a know-how on EV purification. Regarding this latter part, they may improve their description of EV purification. EV may be broken into smaller size EV after freezing. Does it explain the relatively small size in their EV preparation? Do the authors refer to the MISEV guidelines for EV purity?*

Response: Thanks for your comments. In the previous manuscript, we provided a relatively detailed account of the procedures related to EV isolation and validation (https://doi.org/10.1096/fj.202100787R). In the revised manuscript, we added some information about the principle of the EV isolation kit, and the validation antibody as following “Plasma EVs were isolated from 1 mL of plasma by exoEasy Maxi Kit (Qiagen, Valencia, CA, USA), a membrane-based affinity binding step to isolate exosomes and other EVs without relying on a particular epitope, in accordance with the manufacturer’s instructions and storaged in the −80。C freezer. The isolated plasma EVs were then eluted and stored. Usually, 400 μL of eluate is obtained per mL of plasma. The isolated plasma EVs were validated according to the International Society of Extracellular Vesicles guidelines, which include1.markers, including the presence of CD63 (ab59479, Abcam, Cambridge, UK), CD9(ab92726, Abcam, Cambridge, UK), tumor susceptibility gene 101 protein (GTX118736, GeneTex, CA, USA) and negative of cytochrome c (ab110325; Abcam, Cambridge, UK) 2. Physical characterization through the nanoparticle tracking analysis, which demonstrated the majority of the size of EV are mainly within 50-100nm 3. The morphology from the electron microscopy analysis. The validation had been described previously [29-31]. “

It's important to note that our primary focus was on exosomes, the smallest subtype of EVs. Through nanoparticle tracking analysis, we observed that the majority of isolated EVs fell within the diameter range of 50-150nm, exhibiting significant surface marker (i.e. CD63 and CD9) expression. Moreover, electron microscopy confirmed their vesicular morphology. These meticulously validated EVs were promptly analysed post-isolation.

However, we acknowledge that the plasma obtained from study participants might have undergone freezing prior to EV isolation. This freezing process has the potential to diminish the yield rate of EVs and result in some degree of fragmentation. We have duly included this issue as a limitation in our revised manuscript as following “The final technical issue in the present study was the relatively small size of the isolated EVs. Despite the primary focus on isolating exosomes, which are the smallest type of EVs, it's important to consider that the presence of small-sized EVs could potentially be attributed to EV fragmentation that occurs during the freezing and thawing processes.”

> - *Regarding synaptic protein quantification, the choice of western blotting may not be the best one. ELISA and other multiplex arrays are available. How the authors do justify their choice?*

Response: Thanks for your comments. We appreciate your input regarding the semi-quantitative western blot analysis not being the most optimal approach. Owing to the limited quantity of isolated plasma EVs and the significant protein abundance of synaptic proteins within these EVs, we did explore the use of an ELISA assay. However, it's worth noting that for a specific subset of the samples, the readout obtained was lower than the lower limit of detection of the ELISA kit. In response, we have incorporated this point as limitation within the discussion section of the revised manuscript as following “Semiquantitative assessment of plasma EV synaptic protein (SNAP-25, GAP-43, and synaptotagmin-1) levels was performed

using western blot analysis. The lack of absolute values, i.e. from the results of enzyme-linked immunosorbent assay, limits further clinical application."

> - *Do the authors try to sort plasma EV by membrane-associated neuronal EV markers using either vesicle sorting or immunoprecipitation?*

Response: Thanks for your comments. The current study did not specifically isolate neuron-derived extracellular vesicles (EVs), potentially introducing some bias to the results. However, it's important to note that synaptic proteins, such as SNAP-25, exhibit a high degree of neuron-specific expression, with a predominant presence in the brain (as indicated by https://www.proteinatlas.org/ENSG00000132639-SNAP25/tissue). Given this context, the limitation of not analyzing neuron-derived EVs could be mitigated to some extent. In response, we have incorporated this point as limitation within the discussion section of the revised manuscript as following "Furthermore, this study evaluated the overall plasma EVs rather than specifically focusing on neuron-derived exosomes, potentially introducing a bias towards somatic-origin EVs. Nonetheless, it is worth noting that synaptic proteins primarily originate from neurons. Even when considering neuron-derived exosomes, it's important to recognize that they are not exclusively derived from the brain, which can lead to contamination from the peripheral nervous system."

> - *Many technical aspects may be improved. Such technical questions weakened the authors' conclusions.*

Response: Thanks for your comments. We recognize that the aforementioned issues represent limitations of our current study. In response, we have incorporated these points as limitations, including the semi-quantitative assessments, the isolation of total but not neuron-derived exosomes in the plasma, and the short follow-up time within the discussion section of the revised manuscript.

> - *The discussion is pretty long to justify the data. It may be shortened by adding some information in the introduction.*

Response: Thanks for your comments. We have repositioned a statement from the second paragraph of the discussion to the introduction. This adjustment serves to enrich the background understanding of the link between synaptic dysfunction and neurodegenerative diseases.