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Circulating particles carotenoids are associated with rehabilitation recovery in Parkinson's disease

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ABSTRACT

Circulating nanoparticles include Extracellular Vesicles (EVs) and other circulating EV-like particles that are known mediators of the trafficking of pathologic proteins associated with Parkinson's disease (PD) progression as well as of products of the oxidative damage that characterizes the disease. In the framework of the VIRTREAD-PD trial protocol, circulating particles were isolated from the serum of 30 subjects with PD before and after an intensive rehabilitation program using a treadmill. Raman spectroscopy was used to verify modifications in the biochemistry of circulating particles and proved that an intensive motor rehabilitation program could affect the molecular composition of EV-like particles in the blood of people with PD (pwPD). Analysis performed before and after 8 weeks of intensive rehabilitation demonstrate significant changes in the biomolecules associated with EV-like particles and in carotenoid content. The presence of antioxidants linked to circulating particles was proved to be informative for the profiling of pwPD at admission and prognostic of the rehabilitation recovery.

In conclusion, our data support the hypothesis that circulating particles have a dual role in PD, both in the neurodegenerative processes and in the response to rehabilitation. Besides, the Raman fingerprint and the spectral signature of carotenoids can represent measurable biomarkers of rehabilitation.

1. Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder characterized by bradykinesia, tremor, rigidity, and postural instability [1]. It is the second most common neurodegenerative disorder and it is estimated to affect about 10 million people worldwide with a prevalence of about 2 % in the over-80 population.

From a biological point of view, the mechanisms underlying PD progression can be summarized into two main events: i) accumulation and aggregation of misfolded protein strains of α-synuclein (αsyn) within the brain tissue with neurotoxic effect; ii) disruption of the physiologic redox potential and oxidative damage to key cellular component leading to cell death [2]. These two mechanisms are closely interconnected, with the deposition of pathological protein aggregates of αsyn strictly related to brain oxidative damage and increased apoptotic events, that ultimately contribute to tissue degeneration and disease progression.

Clinically, treatments include dopamine-based pharmacologic therapies that can ameliorate patients' motor symptoms but cannot stop either neurodegeneration or disease progression that may lead to serious complications. In selected advanced cases, Deep Brain Stimulation represents a surgical treatment option for delivering an adjustable signal inside the dysfunctional nigrostriatal circuitry that characterizes the

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Abbrevaitions

AUC	Area Under the Curve
AVR	Augmented Virtual Reality
BDI	Beck's Depression Inventory
CV	Canonical Variable
EVs	Extracellular Vesicles
HY scale	Hoehn and Yahr scale
LDA	Linear Discriminant Analysis
LOOCV	Leave-One-Out Cross-Validation
mBI	Modified Barthel Index
MDS-UPDRS	Movement Disorder Society-Unified Parkinson's Disease Rating Scale
MoCA	Montreal Cognitive Assessment
NTA	Nanoparticle Tracking Analysis
PCA	Principal Component Analysis
PD	Parkinson's Disease
POMA	Performance Oriented Mobility Assessment
POMA-B	Performance Oriented Mobility Assessment – Balance
POMA-G	Performance Oriented Mobility Assessment – Gait
pwPD	People with Parkinson's Disease
ROS	Reactive Oxygen Species
RS	Raman Spectroscopy
TT	Treadmill Training

disease [3].

Rehabilitation has long been considered an adjuvant to pharmacological treatments with the aim of maximizing functional abilities, improving quality of life, and minimizing secondary complications. More recently, rehabilitation has been recognized as a pivotal intervention for people with PD (pwPD), with intensive exercise improving walking speed and balance [4]. However, PD is characterized by a relatively large phenotypic heterogeneity, and it is unclear whether specific clinical subtypes may adapt differently to the rehabilitative intervention. Clinical evidence on rehabilitation efficacy has long relied on poor quality studies, largely heterogeneous, based on empirical experience. A pressing need in rehabilitation medicine is the identification of specific biomarkers ("rehabilomics"), i.e, measurable indicators, that can resume individuals' biological characteristics to tailor a personalized approach in rehabilitation and/or verify the therapeutic effect of a particular intervention or clinical decision [5].

In this regard, Extracellular Vesicles (EVs) are membrane-bound nanoparticles released within the bloodstream by all body tissues, known to be characterized by typical protein markers (i.e. tetraspanins) that can help recapitulate their biogenetic pathway, and by common physicochemical properties [6]. Nonetheless, EV is a general term that includes nanoparticles with molecular and structural heterogeneity that make them difficult to be separated by particles (EV-like particles) with similar size and density in the bloodstream, including lipoproteins [6,7]. The role of EVs and EV-like particles in PD pathogenesis has been extensively proposed [8], envisioning circulating nanoparticles as vehicles and key players in the transfer of αsyn throughout the body, in the spreading of misfolded αsyn, tau, and other factors and in the disease propagation [9–11]. However, EV-like particles do not carry just pathological proteins, but also bioactive mediators of brain remodelling, and were suggested as valuable sources of biomolecular information about muscle-brain crosstalk in rehabilitation [12,13]. Specifically, in pwPD undergoing rehabilitation, circulating nanoparticles can have a dual role being involved in both the neurodegenerative processes and in the response to rehabilitation therapy. For example, physical exercise can dramatically change the concentration and biochemistry of blood-derived EVs [14,15], reduce chronic oxidative stress (increased mitochondria biogenesis and autophagy up-regulation), and stimulate the synthesis of neurotransmitters and trophic factors [16]. The release of multiple factors, like protein, microRNA, and DNA, can also be shuttled as cargo of circulating nanoparticles [17]. Interestingly, changes in EV cargo were specifically attributed to oxidative stress as reactive oxygen species (ROS) production, and detoxification can be mediated by EV-like particles, but ROS can play a role also in the production of EVs themselves [18].

In the effort to understand the complex interplay among biomarkers, rehabilitation, and neurodegeneration, the primary aim of this study was to identify markers of neuroplasticity and brain remodelling potentially associated with the beneficial effects of an intensive motor rehabilitation program, by detecting changes in EVs and EV-like particles isolated from the bloodstream of pwPD. The present work was conducted within the wider framework of the VIRTREAD-PD protocol (Clinicaltrials ID: NCT05902065), a randomized controlled single-blind trial that compares intensive treadmill training (TT) rehabilitative program with and without augmented virtual reality (AVR) in pwPD characterized by gait disturbances [19]. Taking into account previous considerations, one of the main questions was to assess whether and how the biochemical content of EV-like particles may change according to the rehabilitation program and its outcome, exploring also the identification of potential prognostic markers.

2. Materials and methods

2.1. Patient recruitment and sample processing

In the framework of the VIRTREAD-PD trial protocol (Clinicaltrials ID: NCT05902065), a cohort of 42 pwPD was recruited at IRCCS Don Gnocchi of Fondazione Don Gnocchi (Florence, Italy), after approval by the local Institutional Review Board (Comitato Etico Regione Toscana – Area Vasta Centro) on the July 5, 2022 (Approval Number: 20915_spe). Adult patients were enrolled after signing written informed consent, according to previously published inclusion/exclusion criteria [19]. Recruited subjects were randomized into two groups to take part in different types of rehabilitative intervention: the conventional TT (control group) and the TT integrated with AVR (the TT + AVR arm; experimental group). Blood was collected at recruitment (T0) and post-rehabilitation (T1, after 8 weeks of treatment) for all subjects. Serum was separated by centrifugation for 10 min at 2500×*g* at room temperature, then aliquoted and stored at −80 °C until use. EV-like particles were isolated from 30 pwPD with a combinatorial procedure based on size exclusion chromatography and ultracentrifugation (see Supplementary Material). The isolation could not be performed on all recruited subjects due to limited volumes of serum collected.

The characterization of EV-like particles was performed by Nanoparticle Tracking Analysis (NTA) to evaluate their size distribution and their concentration. Mean concentration and mean size were calculated based on NTA results for all samples at both time points, to determine any statistical difference between T0 and T1 samples, without clustering data according to the rehabilitation protocol. EV-associated molecules were detected by dot blot to verify the enrichment of blood-derived EVs in the nanoparticle suspension (Detailed method description in Supplementary Material).

2.2. Clinical and instrumental assessment

For a comprehensive overview of the clinical and instrumental assessments, along with their respective administration timelines, please refer to the study protocol published by Lombardi et al. [19]. The objective of this investigation was to explore the correlation between biological, clinical, and functional outcomes. To address this objective,

data from the assessments conducted at T0 and T1 using the following scales and tests was analysed.

1. The Performance Oriented Mobility Assessment (POMA) [20] in both its gait (POMA-G) and balance (POMA-B) subscales.
2. The MDS-UPDRS part III [21,22].
3. The modified Barthel Index (mBI) [23].
4. The Montreal Cognitive Assessment (MOCA) [24].
5. The Beck's Depression Inventory (BDI) [25].

Furthermore, the study utilised the OptoGait system (Optogait, Microgate S.r.l., Bolzano, Italy) in conjunction with two pairs of photocells (WITTY, Microgate Srl; Bolzano, Italy; 0.001-s accuracy) to assess the overground gait parameters. Participants were instructed to walk at both a self-selected speed and a high speed, defined as the maximal speed that can be safely maintained over a limited distance. All gate parameters were measured using the central 4 m corridor, excluding the acceleration and deceleration periods registered at the commencement and cessation of walking phases, respectively. This methodological approach has been employed to ensure the measurement of steady-state walking data. In the present study, the following gait parameters were considered: gait speed at self-selected walking speed (Gait Speed Self) and at maximal speed (Gait Speed Max), step and stride lengths at self-selected walking speed (Step Length Self and Gait Cycle Self) and at maximal speed (Step Length Max and Gait Cycle Max), and cadence, defined as the number of steps per minute at self-selected (Cadence Self) and maximal (Cadence Max) speed.

2.3. Raman analysis

Samples were prepared for Raman spectroscopy (RS) analysis by depositing 5 μl of freshly isolated EV-like particles onto a calcium fluoride slide and left at room temperature until completely dried. Drops were then analysed using a Raman spectroscope (LabRAM Aramis, Horiba Jobin Yvon S.A.S, Lille, France) equipped with a 532 nm laser source, daily calibrated with a silicon reference substrate. Particle characterization was performed following a previously optimized protocol [26]. Briefly, acquisition parameters were as follows: 30 s acquisition time, 1800 grooves/mm diffraction grating, 400 μm entrance slit and 600 μm hole, in the spectral ranges 600-1800 cm^{-1} and 2600-3200 cm^{-1}. An average of 10 spectra were acquired for each sample at the border of the dried drop, in random spots, taking advantage of the "coffee ring" effect, allowing each spectrum to describe the biochemical features of small particle clusters and possible co-isolated factors. In parallel, Raman spectra of the pure molecules of β-carotene and lutein were obtained using the same acquisition parameters here described (Supplementary Material and Supplementary Fig. 4).

2.4. Data analysis

Data were analysed using LabSpec6 (Horiba, France) and OriginLab23b software (Origin Lab, Northampton). Raman data were processed to remove the contribution of fluorescence, baseline corrected, and normalized to allow comparison between subjects.

The area under the curve (AUC) of the most relevant Raman peaks was also calculated. The angular coefficients of the line joining the corresponding AUC values at T0 and T1 (AUC slope) for each sample were also calculated. Multivariate statistical analysis was performed to reduce data dimensions and evaluate the ability of the Raman fingerprint to discriminate between the considered samples. Briefly, Principal Component Analysis (PCA) was used to reduce data dimensions. To evaluate the diversity between pre- and post-rehabilitation samples, the first 15 principal components (PCs) were extracted and used to obtain a classification model by linear discriminant analysis (LDA), which provided the Canonical Variable (CV) scores. A leave-one-out cross-validation (LOOCV) analysis was performed on the resulting CV. To further evaluate statistical differences between T0 and T1 mean spectra, a non-parametric Wilcoxon paired signed rank test was applied to the CV scores.

Then, keeping age and disease duration as co-variates, partial correlation analysis was performed between Raman data and the main clinical scales used to profile patients before (to assess their status) and after (to monitor their status and evaluate the prognostic significance) rehabilitation. Detailed description of clinical assessments and statistical methods is reported in Supplementary Material.

ROC curve analysis was used to evaluate the accuracy of our model to distinguish between responders and non-responders to rehabilitation by observing the AUC of carotenoid peaks in relation to functional improvement (MBI).

3. Result

3.1. Baseline characteristics of subjects

A cohort of 30 pwPD was successfully recruited and considered for the identification of the biochemical profile of circulating particles. For all subjects, demographic and clinical information were obtained as summarized in Table 1.

3.2. Clinical results

Table 2 presents the median values (with minimum, maximum, and interquartile range) for clinical, cognitive, and gait-related outcomes at two time points (T0 and T1) in the considered cohort of participants, along with their associated p-values. None of the variables was normally distributed thus non-parametric statistics Wilcoxon signed-rank test for paired samples was employed. The median (IQR) MDS-UPDRS part III score was found to be 33 (14/50; 15) at T0 and 35 (9/57; 13.5) at T1 ($p = 0.23$), while the median BDI score remained unchanged at 11 (T0: 3/28; 8, T1: 2/38; 10, $p = 0.44$). The median MBI score remained at 100, with minor alterations in range ($p = 0.31$), and the median MoCA score shifts from 23 (19.1/29.2; 2.56) to 23.3 (14.8/30.2; 5.46) ($p = 0.47$). Minor alterations were evident in the POMA-B and POMA-G scores, with POMA-B at 13 (5/16) at both time points ($p = 0.566$) and POMA-G showing a slight increase from 9 (5/11) to 10 (5/12) ($p = 0.211$). The total median POMA score shifts from 22 (13/26; 4) to 22.5 (11/27; 4.17) ($p = 0.18$). Several gait parameters showed significant improvement. Specifically, the median self-selected cadence escalates from 108 (89.4/128; 16.5) steps per minute at T0 to 112 (82.7/134; 8.96) at T1 ($p = 0.007$), while median maximum cadence raised from 125 (102/164; 16.9) to 128 (99.6/178; 14.1) ($p = 0.029$). Median self-selected gait speed increased from 0.99 m/s (0.53/1.42; 0.44) to 1.15 m/s (0.450/

Table 1
Subject recruitment. The main demographic parameters of recruited subjects are reported. Information about their clinical history is provided. MDS-UPDRS III: Movement Disorder Society- Unified Parkinson's Disease Rating Scale third part; HY: Hoehn and Yahr scale; TT: treadmill training; AVR: augmented virtual reality.

Subject recruitment	
Mean age ± 1SD	Years: 73.2 ± 8.9
Sex	Males: 10 Females: 20
Disease form at onset	
Bradykinesia	7
Resting tremor	15
Mixed form	8
MDS - UPDRS III	14–57
HY	HY2: 18 subjects; HY3: 12 subjects;
Mean Disease duration ± 1SD	Years: 8.55 ± 5.34
Rehabilitation treatment arm	TT (control group): 15 subjects; TT + AVR (experimental group): 15 subjects

Table 2
Clinical and instrumental assessments before and after the treatment. Data from 30 participants are reported. Median values with minimum, maximum, and interquartile range for clinical, cognitive, and gait-related outcomes at two time points (T0 and T1) are reported. * Significance is set for $p < 0.05$. MDS-UPDRS = Movement Disorder Organization - Unified Parkinson's Disease Rating Scale; BDI= Beck's Depression Inventory; MBI= Modified Barthel Index; POMA-B= Performance Oriented Mobility Assessment Balance; POMA-G = Performance Oriented Mobility Assessment Gait.

Outcomes	T0	T1	*p*
MDS-UPDRS III	33 (14/50; 15)	35 (9/57; 13.5)	*0.23*
BDI	11 (3/28; 8)	11 (2/38; 10)	*0.44*
MBI	100 (38/100; 2.75)	100 (88/100; 2)	*0.31*
MoCA	23 (19.1/29.2; 2.56)	23.3 (14.8/30.2; 5.46)	*0.47*
POMA-B	13 (5/16; 3)	13 (5/16; 2)	*0.57*
POMA-G	9 (5/11; 2)	10 (5/12; 3)	*0.21*
POMA tot	22 (13/26; 4)	22.5 (11/27; 4.17)	*0.18*
Cadence Self (steps/min)	108 (89.4/128; 16.5)	112 (82.7/134; 8.96)	**0.007*
Cadence Max (steps/min)	125 (102/164; 16.9)	128 (99.6/178; 14.1)	**0.029*
Gait Speed Self (m/s)	0.995 (0.53/1.42; 0.44)	1.15 (0.45/1.49; 0.42)	**0.03*
Gait Speed Max (m/s)	1.34 (0.61/2.70; 0.54)	1.50 (0.55/2.22; 0.58)	**0.04*
Step Length Self (cm)	56.2 (34.6/75.3; 18.7)	62.4 (31.9/76; 19.5)	*0.06*
Step Length Max (cm)	66 (33.6/101; 23)	70.6 (33.4/97.3; 25.3)	*0.13*
Gait Cycle Self (% s)	1.11 (0.93/1.35; 0.18)	1.07 (0.9/1.55; 0.08)	**0.03*
Gait Cycle Max (% s)	0.970 (0.73/1.19; 0.14)	0.93 (0.66/1.25; 0.11)	**0.05*

1.49; 0.42) ($p = 0.03$). Median maximum gait speed exhibited a marked improvement, rising from 1.34 m/s (0.61/2.7; 0.54) to 1.50 m/s (0.550/2.22; 0.58) ($p = 0.04$). According to Baudendistel et al. [27], the gain obtained in gait speed reached the minimally clinically important difference. While other gait parameters demonstrated trends toward enhancement, these did not attain statistical significance. Median step length (self-selected) increased from 56.2 cm (34.6/75.3; 18.7) to 62.4 cm (31.9/76; 19.5) ($p = 0.0$ 61), and median maximum step length increased from 66 cm (33.6/101; 23) to 70.6 cm (33.4/97.3; 25.3) ($p = 0.129$). Furthermore, the median gait cycle self-selected time decreased from 1.11 (0.930/1.35; 0.183) to 1.07 (0.9/1.55; 0.082) ($p = 0.03$). Conversely, the median maximum time of the gait cycle decreased from 0.970 (0.730/1.19; 0.135) to 0.93 (0.66/1.25; 0.108) ($p = 0.05$).

3.3. Circulating particles characterization

Blood samples were collected before and after the treadmill-based rehabilitation protocol from 30 pwPD and circulating particles were successfully isolated from all samples. Although the proposed protocol based on commercial size exclusion chromatography isolation columns is often reported as valuable method for EV isolation, the presence of co-isolates is well known and particles of similar size and density can be isolated in combination or associated to EVs [7]. For this reason, the presence of small EVs and lipoproteins was verified using specific markers, as well as broader characterization techniques like NTA analysis. The isolation was achieved with high reproducibility, as attested by the characterization results reported herein.

NTA analysis was used to verify the size distribution and the concentration of EV-like particles in people recruited before and after rehabilitation (Supplementary Fig. 2). Comparing T0 and T1 samples, a significant variation was observed in the mean concentration of EV-like particles (T0: $9.6 \times 10^9 \pm 1.1 \times 10^{10}$ particles/ml; T1: $1.3 \times 10^{10} \pm 1.4 \times 10^{10}$ particles/ml; $p < 0.05$ after Wilcoxon Signed Pair Rank non-parametric test). No difference in the mean size of circulating particles was observed (T0: 145 ± 32 nm; T1: 153 ± 23 nm) for the considered patients (Supplementary Fig. 2). The presence of small EVs (such as exosomes) in both T0 and T1 samples was also confirmed by the positivity of the dot blot for FLOT-1, TSG101, Alix and CD9, well-known EV markers (Supplementary Fig. 3). In parallel, the evaluation of ApoA1 was also performed, confirming the presence of lipoproteins in the considered nanoparticle suspension (Supplementary Fig. 3).

3.4. Changes in the Raman fingerprint of particles after rehabilitation

RS analysis was successfully performed on 30 T0-T1 pairs of samples from pwPD undergoing intensive rehabilitation.

The average Raman spectrum for each of the 30 samples from pwPD before and after intensive rehabilitation was obtained in the spectral range 600–1800 cm^{-1} and 2600-3200 cm^{-1}. All samples showed a good signal-to-noise ratio and the expected peaks and bands related to proteins (Amide I 1600–1690 cm^{-1}; Amide III 1200–1300 cm^{-1}) and nucleic acids (720–820 cm^{-1}), with the prominent spectral contribution of the lipidic component of EV-like particles in the spectral range 2600–3200 cm^{-1} (Fig. 1A). Data confirm the repeatability of the analysis compared to the data reported by our group in 2019 [26].

To verify changes in the biomolecular cargo of particles in pwPD before and after rehabilitation, differences in the molecular fingerprints were extracted by subtracting the T0 average spectrum from the T1 average spectrum (Fig. 1D). Functional chemical groups were assigned based on previously consolidated findings [28]. The data showed differences in the presence and intensity of peaks related to nucleic acids (975 cm^{-1}) which were found to be more represented in T0 average spectrum. Peaks related to lipids (2849 cm^{-1} and 2896 cm^{-1}) and carotenoids (1154 cm^{-1} and 1514 cm^{-1}; Fig. 1B and C) seemed to be predominant in the spectrum of T1 samples (Fig. 1D). Besides, the comparison of the present data to those publishes in 2019 [26], confirmed that the contribution of carotenoids was not detectable nor in pwPD before the rehabilitation intervention, nor in the control subjects of comparable age in the circulating EV-like particles. On the contrary, peaks become clearly visible after the completion of the intensive rehabilitation program (Fig. 1B and C).

Based on these observations, on the remarkable variations in the carotenoid peaks (Fig. 1B and C), and on the theoretical role of oxidative stress in PD pathogenesis, to further investigate the contribution of carotenoids to the particle spectra, the area under curve (AUC) of the peaks related to carotenoids (1154 ± 10 cm^{-1} and 1514 ± 10 cm^{-1}) were calculated for each subject (Supplementary Fig. 1). In parallel, the AUC of the peak of Amide I (1665 ± 20 cm^{-1}) was also calculated and considered as a reference band as it accounts for the secondary structure of proteins (Fig. 2A). The AUC of both carotenoid peaks showed a higher mean value at T1 when comparing T0 and T1 data, but no significant differences were observed between the calculated areas. The AUC of the Amide I peak resulted to be unchanged in all subjects, as expected (Fig. 2A). The angular coefficient of the line joining the corresponding AUC values at T0 and T1 for each sample was also calculated, showing considerable variability in the changes related to the carotenoid content (Supplementary Fig. 4).

Finally, to verify the possibility of discriminating EV-like particles isolated from serum of pwPD before and after rehabilitation by their Raman spectrum, multivariate statistical analysis was performed. The calculated PCs describe the differences between the considered Raman spectra and represent the percentage of data variance (PC1 = 76.5 %; PC2 = 9.6 %). The comparison by Wilcoxon Paired Signed Rank Test of the CV scores resulting from the LDA classification model demonstrated significant differences when comparing T0 and T1 time-points ($p < 0.001$; Fig. 2B), thus demonstrating a significant biochemical change in the EV-like particles composition after rehabilitation in the considered cohort of pwPD.

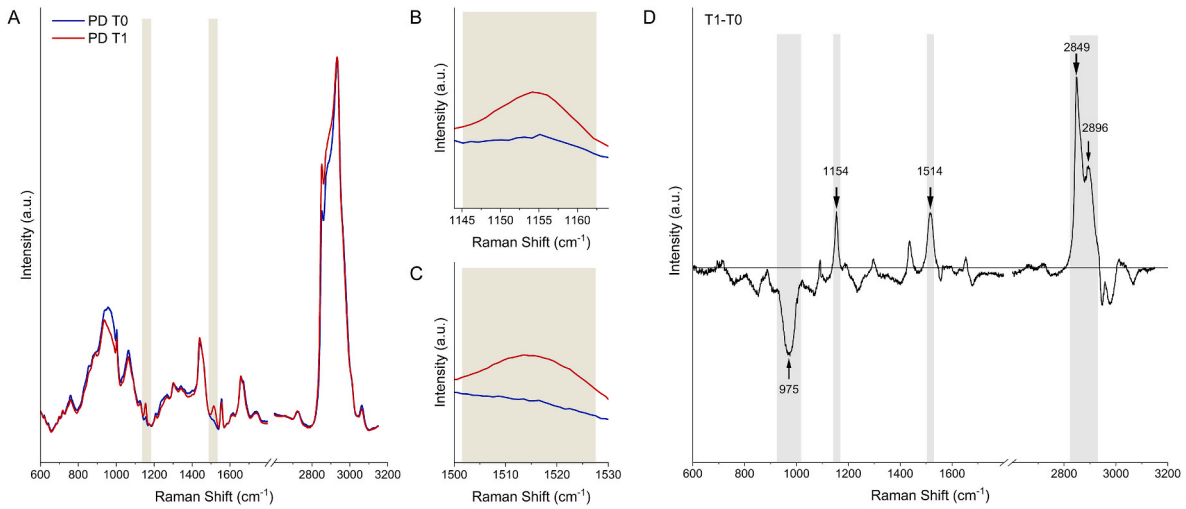

Fig. 1. Raman fingerprint of EV-like particles before and after rehabilitation. (A) Average Raman spectra of circulating EV-like particles obtained from serum of 30 pwPD before (T0; blue line) and after (T1; red line) rehabilitation. (B) Magnification of peak at 1154 cm-1. (C) Magnification of peak at 1514 cm-1. (D) Subtraction spectrum (T1-T0) of mean Raman spectra. Arrows indicate the most relevant peaks in the subtraction spectrum.

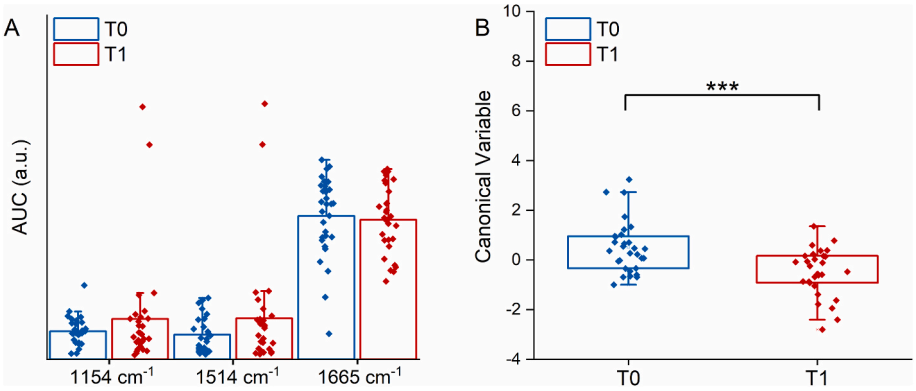

Fig. 2. Area under curve and multivariate analysis. (A) Comparison of the average area under curve (AUC) of peaks related to carotenoids (1154 cm^{-1} and 1514 cm^{-1}) and Amide I (1665 cm^{-1}) before (T0) and after (T1) the rehabilitative treatment. (B). Box plot reporting the score of the canonical variable obtained after the multivariate statistical analysis comparing Raman spectra of EV-like particles before (T0) and after (T1) rehabilitation. ***$p < 0.001$ after Wilcoxon Paired Signed Rank Test. Each dot represents one subject. T0: blue bars and symbols; T1: red bars and symbols.

3.5. Correlation between Raman data and clinical assessment

The correlation analysis was performed to better interpret the Raman results in the framework of the clinical assessment of the participants. First, the contribution of carotenoids in the spectrum of EV-like particles in the considered cohort at T0 was evaluated by correlating it with age, sex and disease stage for all subjects, showing a direct correlation between the intensity of the peaks at 1154 cm^{-1} and the individual age (Pearson correlation coefficient: 0.38; $p = 0.03$). No correlation was found with sex, subtype of PD at onset, and staging of the disease measured by HY scale.

Then, keeping age and disease duration as co-variates, partial correlation analysis was performed between the AUC of carotenoid and Amide I peaks and the main clinical scales used to assess pwPD before (profiling the patients' status) and after (monitoring of patients' status and prognostic significance) rehabilitation. As shown in Supplementary Table 1, the AUC of the carotenoid peaks (1154 cm^{-1} and 1514 cm^{-1}) both in T0 and T1 samples was significantly correlated with some of the major scales used to profile pwPD, with significant results in the correlation with the Performance-Oriented Mobility Assessment (POMA scale, measure of patient's gait/POMA-G and balance/POMA-B abilities) as well as to the modified Barthel Index (MBI scale, measure of patient's disability). Specifically, the AUC of carotenoids at T0 was found to be negatively correlated to the POMA-B scores at T0 (Partial Corr. Coeff. −0.59, $p < 0.001$ for AUC 1154 cm^{-1} and -0.48, $p = 0.01$ for AUC 1514 cm^{-1}). Both carotenoid peak intensities correlated with the total POMA score at T0 (Partial Corr. Coeff. −0.55, $p < 0.001$ for AUC 1154 cm^{-1} and -0.46, $p = 0.02$ for AUC 1514 cm^{-1}) (Fig. 3A and B), but not with the POMA-G score at T0. Considering the clinical relevance of the POMA scores, it is worth noting that the carotenoid cargo of EV-like particles measured at T0 (AUC 1154 cm^{-1} T0) correlates with the total POMA score at T1 (Partial Corr. Coeff. −0.43, $p = 0.02$), and with the POMA-G at T1 (Partial Corr. Coeff. −0.43, $p = 0.03$), suggesting a potential prognostic value of the antioxidants for pwPD (Fig. 3C).

Moreover, the antioxidant content of EV-like particles (AUC 1154 cm^{-1} and AUC 1514 cm^{-1}) at T0 correlated negatively with the MBI at T0 (Partial Corr. Coeff. −0.61, $p < 0.001$ for AUC 1154 cm^{-1} and -0.43, $p = 0.02$ for AUC 1514 cm^{-1}), suggesting that low levels of carotenoids in the bloodstream are indicative of a less severe disability of the patient, before starting the rehabilitation treatment. Of note, the amount of carotenoids measured in pwPD at T1 still negatively correlates with the disability status of the patient (Partial Corr. Coeff. −0.45, $p = 0.02$ for both AUC 1154 cm^{-1} and AUC 1514 cm^{-1}), confirming the possibility to monitor the disability profile of the patient by the antioxidant cargo of EV-like particles. Even more relevant from a clinical point of view, the measure of the spectral contribution of carotenoids associated with

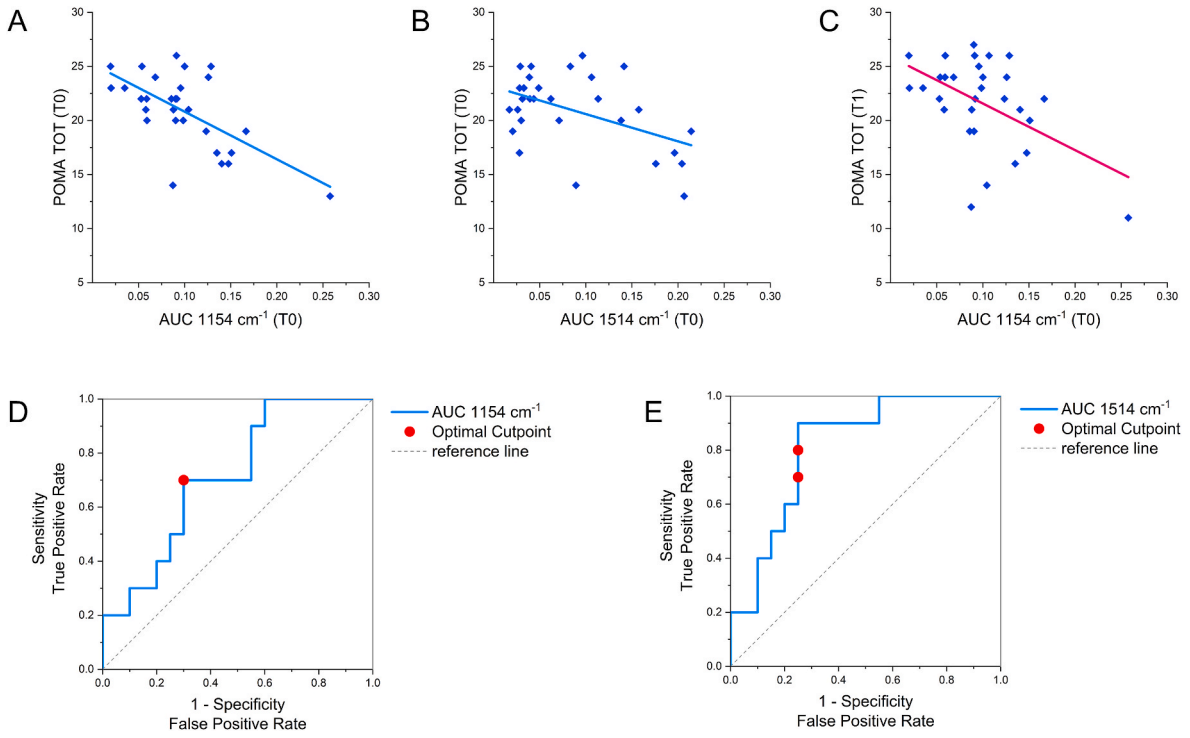

Fig. 3. Correlation analysis between Spectroscopic Raman data and clinical assessment. A–B: Scatter plots representing the negative correlation between the total POMA scale scores at T0 and the intensity of the 1154 cm^{-1} (A) and 1514 cm^{-1} (B) peaks of carotenoids related to EV-like particles. C: Scatter plots representing the negative correlation between the total POMA scale scores at T1 (after rehabilitation) and the intensity of the 1154 cm^{-1} peak of carotenoids related to EV-like particles. D–E: ROC curves calculated for the AUC of carotenoids to discriminate between responders and non-responders based on the variation in the disability scale MBI.

circulating particles at T0 negatively correlates with the MBI at discharge (Partial Corr. Coeff. −0.46, $p = 0.01$ for AUC 1154 cm^{-1} and -0.42, $p = 0.03$ for AUC 1514 cm^{-1}) meaning that the low levels of carotenoids at admission could be predictive of the less severe disability of the patient both at admission and at discharge. Moreover, the antioxidant content of EV-like particles measure with AUC 1154 cm^{-1} at T0 correlates positively (Partial Corr. Coeff. 0.58, $p < 0.0001$ for AUC 1154 cm^{-1}) with the variation in the MBI score between admission and discharge, i.e. a measure of the patient recovery after rehabilitation, but the correlation is not significant for AUC 1514 cm^{-1}. This last observation is in agreement with the correlation found between the spectral variation in the carotenoid content (angular coefficient of the line joining the integrated values – SLOPE 1154 and SLOPE 1514) and the clinically measured change in disability profile (variation in the MBI scores - ΔMBI): Partial Corr. Coeff. of 0.71 ($p < 0.0001$) and of 0.67 ($p < 0.0001$) for the angular coefficient of the AUC 1154 cm^{-1} and AUC 1514 cm^{-1}, respectively. These data make us hypothesize that an increment in the antioxidant content can be related to increased disability of pwPD.

Thus, we divided pwPD in responders and non-responders to rehabilitation treatment according to the variation in the MBI scores (responders: ΔMBI >0, 10 subjects, median 0.021; non-responders: ΔMBI ≤0, 20 subjects, median 0) and we used the spectral measure of carotenoids obtained at T0 (AUC), to calculate ROC curves and test the ability of carotenoids in discriminating between responders and non-responders, as defined. The use of carotenoid AUC values at T0 is essential to establish their predictive role. In parallel, the use of MBI scores for the ROC curve analyses allowed us to distinguish between similar but clinically different functional levels, observing variations in the global assessment that follows rehabilitation, not restricted only to the motor or functional aspect.

As shown in Fig. 3D and E, the AUC of 1154 cm^{-1} and 1514 cm^{-1} can be considered indicative of the response or non-response to rehabilitation with an accuracy of 72 % (95 % Confidence interval: 51 %–92 %) and 82 % (95 % Confidence interval: 63 %–100 %), respectively. These measures allowed us also to identify the cut-off values for AUC 1154 cm^{-1} and AUC 1514 cm^{-1} that correspond to 0.1 and 0.09, respectively. Based on the present data, these measurements indicate that pwPD who start the rehabilitation program with a Raman fingerprint of EV-like particles with calculated AUC of carotenoids lower that the identified cut-off values are expected to be better responders to the rehabilitation intervention than people with higher carotenoid content.

Finally, the AUC of both carotenoid peaks in T0 samples negatively correlates with the instrumental assessments of the performance of patients described by Cadence Max, Gait Speed Max, and Step Length Max measured at T0 and are prognostic of the bad performance for the same parameters evaluated at the end of the rehabilitation program (Supplementary Table 1).

Considering the AUC related to Amide I, that describes the secondary structure of proteins associated with the EV-like particles, it resulted to be negatively correlated to MDS-UPDRS III at T0 (Partial Corr. Coeff −0.67, $p < 0.001$) and to the BDI score at T0 (Partial Corr. Coeff −0.5, $p = 0.01$), that measure motor symptoms and mood of the patient, respectively. Although these data require further investigation to clarify the molecules involved in the reported observation, they are not surprising if we take into consideration that all proteins, including pathological and misfolded proteins (i.e. αsyn), can contribute to the spectral signal of Amide I. On the contrary, it was unexpected that the AUC 1665 cm^{-1} (Amide I) at T0 negatively correlates with the POMA-G value at T0 (Partial Corr. Coeff −0.49, $p = 0.01$), suggesting that the spectral signature of circulating particles comprises the information regarding proteins that might be determinants of the gait imbalance of pwPD. The patient's profiling based on the Amide I region seems also to be prognostic of a bad performance of the subject after the rehabilitation training evaluated by the total POMA score at T1 (Partial Corr. Coeff −0.42, $p = 0.03$), and balance worsening (Partial Corr. Coeff −0.39, $p = 0.04$), but these data need further investigation.

Finally, CV values at T0 showed relationship with the max cadence at T0 and gait cycle max at T0, suggesting again a direct connection between the cargo of EV-like particles in blood and the clinical status of pwPD.

4. Discussion

In PD, oxidative stress is one of the leading mechanisms associated with neurodegeneration, even though the complex mechanisms that contribute the loss of dopaminergic neurons remain to be fully elucidated [2].

In the present work, data demonstrate that rehabilitation can modify the oxidative state of the organism, and changes in the oxidative cargo of circulating particles are related to the individual response to intensive rehabilitation programs. Specifically, the present work highlights that intensive rehabilitation impacts the biochemical composition of circulating particles in pwPD, demonstrating their role in the complex systemic response to rehabilitation. In this regard, our data suggests that rehabilitation impacts the oxidative cargo of blood derived particles that can be related to the individual response to an intensive rehabilitation program. Summarizing our main findings and focusing on the POMA-G scale (primary clinical outcome of the present study), the AUC 1665 cm^{-1} at T0 negatively correlates with POMA-G at T0 whereas the AUC 1154 cm^{-1} at T0 negatively correlates with POMA-G at T1 (but not at T0), suggesting that the peak of Amide I (AUC 1665) might be an indicator of the disease pathology, whereas the peak of carotenoids might represent a prognostic factor for gait improvement after rehabilitation. Moreover, both carotenoid peaks (1154 cm^{-1} and 1514 cm^{-1}) at T0 negatively correlate with instrumental gait parameters (in particular step length and gait speed) and functional ability at T0 and T1, supporting the hypothesis that the carotenoid concentration can reflect the disease severity. Despite the limited cohort considered, it is worth noting that we were able to identify a cut-off value for the spectral signal of carotenoids that can discriminate pwPD in responders and non-responders to the rehabilitation treatment, with moderately good accuracy (>70 %).

In PD, EVs and EV-like particles are known to be carriers of molecules associated with neurodegeneration. The biochemical profile of circulating nanoparticles was already proven to be related to the disease stage with the Raman fingerprint of blood-derived EVs representing a biomarker for the profiling of pwPD. Previously, we demonstrated that the Raman fingerprint of circulating nanoparticles in the serum of pwPD is significantly different from that of age and sex-matched healthy controls. In that work, the presence of carotenoids associated to EV-like particles isolated using the same protocol proposed in the present study was not observed [26]. The present data confirm our previous observations and shed light on the ability of EV-like particles to mediate the response to rehabilitation by mobilizing molecules involved in oxidative homeostasis.

The cargo of EVs or EV-like particles released in oxidative conditions was proved to include both oxidized molecules and antioxidant molecules, representing a potential source of oxidative stress biomarkers [29]. Our results suggest that the antioxidant reservoir of individual subjects and their association with circulating nanoparticles can be related to their clinical status and to their response to an intensive rehabilitation treatment. Besides, a change in the antioxidant cargo of EV-like particles can be induced by intensive rehabilitation. The most interesting result is related to the role of carotenoids, known scavengers of ROS, that can circulate associated with EV-like particles and whose concentration was proved to be related to the individual responsiveness to the proposed rehabilitation program for pwPD.

Carotenoids are a huge class of molecules that can be subdivided into different groups based on their chemical structure. Many of them are included in the human diet and directly linked to fruit and vegetable consumption. Carotenoids exert fundamental antioxidant and anti-apoptotic effects in neurodegenerative disorders, resulting to be the main lipophilic antioxidants counteracting lipid-oxidating molecules in brain tissue. Carotenoids are known to be lipophilic molecules, typically circulating in blood together with lipoproteins. Recent literature has also demonstrated that carotenoids can be stabilizing agents for aggregated proteins like amyloids [30]. It was reported that the carotenoid precursor, Vitamin A, has depolymerizing effects on α-syn aggregates *in vitro* [31], whereas retinoic acid (RA; i.e. Vitamin A metabolite) seems to be directly involved in dopaminergic neurotransmission control and in regulation of the nigrostriatal pathway [31]. A study by Zhou and colleagues, demonstrated that RA administration attenuates both degeneration and loss of dopaminergic neurons in substantia nigra, alleviating motor dysfunctions in mice models of PD [31–33]. Also, the concentration of serum carotenoids was proved to decrease with PD progression [34], although a detailed nutritional assessment is often lacking in these studies.

It has to be noted that we cannot conclude whether carotenoids are transferred in the bloodstream by small EVs or by lipoproteins of comparable size and density, or even by lipoproteins associated with EVs (the so-called biomolecular corona). Association of carotenoids to EVs or lipoproteins should be further investigated and could shed light on the mechanisms underlying our observations. Nonetheless, from a clinical point of view, antioxidants associated with circulating particles were shown to be involved in the patients' response to the rehabilitation treatment and their abundance in the bloodstream seems to be related to a more severe disease status and a negative prognosis for the rehabilitation recovery. Although these observations need further validation in wider independent cohort, our data are in line with previous literature about the oxidative stress related to PD progression making us hypothesize that the mobilization of the carotenoids is indicative of a more oxidative status of the patient [35]. Further experimental studies are needed to verify this hypothesis and clarify the mechanisms underlying the present observations and the molecular basis of the reported correlations between redox status and rehabilitation outcomes. Indeed, the role of carotenoids in the considered cohort can have multiple explanations, including the role of carotenoids as biomarkers of oxidative load or their protective role in the disease progression and rehabilitation response. On the contrary, considering the connection between oxidative stress and rehabilitation recovery, a work by Cocco and colleagues recently proved changes in oxidative stress parameters between baseline and at the end of the rehabilitation program in people with a subacute stroke [36]. However, to the best of our knowledge, measurable changes in antioxidant cargo of EV-like particles were not associated with the patient status and to the rehabilitation outcome in pwPD. Further studies should be designed to better characterize carotenoids in the blood of pwPD, taking into account multiple quantification methods, such as mass spectrometry and chromatographic analysis, and a wider cohort of subjects, in order to validate the current results in an independent population.

Despite the limited cohort of considered subjects, our data suggest a correlation between the biomolecular profile of EV-like particles, their antioxidant content and the individual response to rehabilitation. We can speculate that the biomolecular profile of circulating particles can be predictive of the effectiveness of the rehabilitation treatment in terms of changes in the disability profile (variation in the MBI scores), and in an improvement in the gait pattern. Although the mechanisms underlying the present observations are difficult to be interpreted, we can speculate that circulating carotenoids are involved in the response of pwPD to intensive rehabilitation and that the amount of mobilized antioxidant reservoir can impact a patient's recovery, being a potential tool for monitoring individual clinical status and rehabilitation's effectiveness. However, future investigation about the role of carotenoids associated to circulating particles, as well as an in dept-characterization of their mechanism of action in motor and cognitive rehabilitation is needed.

Future multicentered clinical trials on the impact of multiple rehabilitation interventions on wider cohorts of pwPD are necessary to further validate our observations and verify the proposed cut-off values

for carotenoids. Besides, it is worth noting that no changes in the nutrition habits were reported in the present cohort, and pharmacological treatment was stable according to the study protocol. Future studies should consider nutrition data as part of the patients clinical profiling at enrolment, and follow up.

The present study supports the role of circulating particles in the neurodegenerative processes of PD, but also in the individual response to motor rehabilitation. Moreover, Raman spectroscopy was effective in the identification of specific molecular changes related to the rehabilitation response and allowed us to identify carotenoids as crucial mediators of the treatment response. Our data provide evidence about the possibility to use the Raman fingerprint of EV-like particles and the spectral signature of carotenoids as a measurable biomarker of rehabilitation efficacy, even though future studies should consider also disease progression as a variable by including longer follow up periods. Considering that previous studies proved variations in carotenoid concentration in serum during PD progression [34], the variability in the Raman data could be due to PD pathogenesis and further studies should be dedicated to the monitoring of neurodegeneration during disease progression.

In conclusion, the present study leads to two major conclusions: i) the effectiveness of the rehabilitation treatment is linked to the redox status of the patient and ii) changes in the redox status may be related to changes in the rehabilitation outcomes. It is also possible that modifying the cargo of circulating particles may impact the redox and clinical status of the patients, opening the way towards new pharmacological treatments, but this observation goes beyond the scope of our study and should be addressed by dedicated trials.

CRediT authorship contribution statement

Alice Gualerzi: Writing – original draft, Visualization, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Martina Gerli:** Writing – original draft, Visualization, Investigation, Formal analysis. **Aurora Mangolini:** Writing – review & editing, Investigation. **Silvia Picciolini:** Writing – review & editing, Visualization, Supervision, Formal analysis. **Francesca Rodà:** Writing – review & editing, Visualization, Supervision. **Valentina Mangolini:** Writing – review & editing, Investigation. **Stefano Doronzio:** Writing – original draft, Investigation, Data curation. **Diego Longo:** Writing – original draft, Investigation, Data curation. **Giulio Cherubini:** Writing – review & editing, Investigation. **Cristina Polito:** Writing – review & editing. **Chiara De Santis:** Writing – review & editing. **Silvia Ramat:** Writing – review & editing. **Francesca Cecchi:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Gemma Lombardi:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Marzia Bedoni:** Writing – review & editing, Funding acquisition, Supervision, Resources.

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Declaration of competing interest

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The Graphical Abstract was created with BioRender.com with permission.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2025.103841.

Data availability

Data will be made available on request.

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