Oligomeric α-Syn and SNARE complex proteins in peripheral extracellular vesicles of neural origin are biomarkers for Parkinson’s disease

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A B S T R A C T

Blood-based biomarkers are needed to be used as easy, reproducible, and non-invasive tools for the diagnosis and prognosis of chronic neurodegenerative disorders including Parkinson’s Disease (PD). In PD, aggregated toxic forms of α-Synuclein (α-Syn) accumulate within neurons in the brain and cause neurodegeneration; α-Syn interaction with SNARE proteins also results in synaptic dysfunction. We isolated neural derived extravesicles (NDEs) from peripheral blood of 32 PD patients and 40 healthy controls (HC) and measured the concentrations of oligomeric α-Syn and of the presinaptic SNARE complex proteins: STX-1A, VAMP-2 and SNAP-25. Oligomeric α-Syn was significantly augmented whereas STX-1A and VAMP-2 were significantly reduced in NDEs of PD patients compared to HC (p < 0.001 in all cases). ROC curve analyses confirmed the discriminatory ability of NDEs oligomeric α-Syn, STX-1A and VAMP-2 levels to distinguish between PD patients and HC. Oligomeric α-Syn NDEs concentration also positively correlated with disease duration and severity of PD. These results are promising and confirm that NDEs cargoes likely reflect core pathogenic intracellular processes in their originating brain cells and could serve as novel easily accessible bio-markers. Further studies are needed to confirm results and eventually for testing rehabilitation programs and drug treatments effects.

1. Introduction

Parkinson’s disease (PD), the most common movement disorder and the second most frequent neurodegenerative condition after Alzheimer’s disease (AD) (Kalia and Lang, 2015), currently affects approximately 2% of the older population. The disease is characterized by the accumulation of proteinaceous inclusions formed of misfolded and aggregated forms of α-Synuclein protein (α-Syn), within Lewy bodies (LB), and by the neurodegeneration of dopaminergic (DA) neurons in the substantia nigra (SN) (Spillantini et al., 1997; Lang and Lonzano, 1998a,b). According to the dying-back hypothesis, PD-associated neurodegeneration starts at synaptic terminals in the striatum and progresses along the nigrostriatal pathway, resulting in the degeneration of DA cells in the SN (Caminiti et al., 2017). Notably, synaptic alterations usually can be observed before the DA neuron loss, leading to the classification of PD as a “synapthopathy” (Bridi and Hirth, 2018).

α-Syn is a neuronal presynaptic protein of 140 amino acids, which is encoded by SNCA gene, and is localized at the presynaptic terminals. In healthy conditions, wild type α-Syn is present in its native conformation as a soluble monomer and regulates neurotransmitters release, vesicle exocytosis and homeostasis, as well as synaptic functions and plasticity (Murphy et al., 2000; Burré, 2015). Several dominant single point mutations in SNCA gene have been identified in early onset familial PD cases (Polymeropoulos et al., 1997; Appel-Cresswell et al., 2013; Pasanen et al., 2014); these mutations lead to the formation of oligomers of α-Syn, ultimately resulting in the generation of amyloid-β sheet fibrils that aggregate into LB. A number of data indicate that α-Syn oligomers, rather than α-Syn fibrils, are neurotoxic (Winner et al., 2011; Forloni et al., 2016) because they negatively regulate neuronal excitability (Kaufmann et al., 2016). Importantly, both multiplications (Farrer et al., 2004) of the SNCA locus and SNCA polymorphisms have been associated with higher risk of developing PD (Hou et al., 2019; Bi et al., 2020; Du et al., 2019) and the higher doses of α-Syn observed in patients with duplication and triplication of SNCA locus correlate with severity of
symptoms (Book et al., 2018).

Presynaptic terminals release neurotransmitters through the exocytosis of vesicles in response to an action potential and Ca$^{2+}$ influx (Sudhof, 2004); this promotes the assembly of a molecular machinery that includes the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, which is formed by VAMP-2, SNAP-25, and Syntaxyn-1. SNARE complexes assemble and disassemble thousands of times per minute to allow exocytosis of synaptic vesicles, and α-Syn is believed to act as a chaperone to promote SNARE complex formation (Burré et al., 2010). The synaptic accumulation of oligomers and other aggregated forms of α-Syn and its interaction with the components of the synaptic machinery, including the SNARE proteins, is likely at the basis of the synaptic dysfunction of PD.

Recently, attention has been focused on the possibility of using extracellular vesicles (EVs) as biomarkers to facilitate the diagnosis and the prognosis of chronic neurodegenerative diseases. EVs are cell-derived particles that are grouped into three types based on their sizes and biogenesis: exosomes (50–150 nm), microvesicles (100–1000 nm) and apoptotic bodies (up to 5 μm) (Latilhar et al., 2019). They are released from almost all cell type, carry selectively incorporated bioactive molecules; and can transmit biological information to recipient cells. For these reasons EVs are considered vehicles of intercellular communication in both physiological and pathological conditions (Simons and Raposo, 2009). Notably, central nervous system (CNS) cells EVs can cross the blood-brain barrier (BBB) and move from the brain to systemic circulation and vice-versa (Goetzl et al., 2016; Mustapic et al., 2017; Shi et al., 2014; Shi et al., 2016). Neural-derived extravesicles (NDEs) can thus be isolated from blood and analyzed in their content.

α-Synuclein was shown to be increased in NDEs from PD patients (Shi et al., 2014), however, to the best of our knowledge, although oligomeric α-Syn is augmented in CSF of PD patients (Eusebi et al., 2017), no data are available on oligomeric α-Syn in NDEs from these patients.

We measured the concentrations of oligomeric α-Syn, and of the SNARE complex components SNAP-25, VAMP-2 and syntaxyn-1 in NDEs isolated from blood of PD patients and healthy controls (HC) in order to identify any difference in their concentrations and to investigate possible relationships between these proteins and the progression of PD.

2. Materials and methods

2.1. Study design and participants

Participants’ recruitment took place at the Neurology Unit of IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy. From September 2019 to February 2020 a total of 32 PD patients were consecutively recruited and screened by movement disorder specialists. All patients were diagnosed according to the Movement Disorder Society (MDS) Clinical Diagnostic Criteria for PD (Postuma et al., 2015). All patients were enrolled in a rehabilitation program. Forty age- and gender-matched healthy subjects (HC) were included in the study as controls. The individuals in the control group did not exhibit any signs or symptoms indicative of neurological disease. Exclusion criteria included vascular parkinsonism; familiar forms of parkinsonism; brain tumor; drug-induced parkinsonism; other known or suspected causes of parkinsonism (e.g. metabolic, etc.) or any suggestive features of a diagnosis of atypical parkinsonism; dementia defined using Movement Disorder Society criteria (Dubois et al., 2007); severe speech problems and poor general health; severe systemic diseases, such as anemia, hepatitis, heart failure, pulmonary disorders, and chronic renal failure; concomitant neurologic and/or psychiatric diseases.

Demographic and clinical variables including age, gender, disease duration, PD severity based on the Modified Hoehn and Yahr (H&Y) stage and MDS-Unified Parkinson’s Disease Rating Scale-III (MDS-UPDRS-III) scores (Goetz et al., 2008), as well as dopaminergic and non-dopaminergic anti-parkinsonian therapy were collected for each patient. We classified PD patients by MDS-UPDRS score into the manifesting tremor-dominant (TD) and the postural instability/gait difficulty (PIGD) groups (Stebbins et al., 2013). Levodopa equivalent daily dose (LEDD) was calculated for each patient (Tomlinson et al., 2010). Cognition was evaluated via the Montreal Cognitive Assessment (MoCA) (Santangelo et al., 2015). Subjects’ demographic and clinical data are summarized in Table 1. The Ethical Committee of Fondazione Don Carlo Gnocchi approved the study and all the participants gave informed consent.

2.2. Sample collection and storage

Six ml of whole blood were collected following standard procedures using a serum separator tube (SST II Advance, BD Vacutainer®). Samples were allowed to clot for 30 min at room temperature and then centrifuged for 10 min at 1500g. After centrifugation samples were aliquoted and stored at –80 °C until use.

2.3. NDEs isolation from serum

Total exosomes were isolated from 500 μl of serum with ExoQuick® (System Biosciences, LCC, USA) according to manufacturer’s instructions. NDEs were isolated as previously described (Mustapic et al., 2017) by immunocapture with biotinylated L1CAM (CD171) (eBioSG3, eBioscience®) antibody, L1CAM, a neuronal surface marker, was selected as target for immunocapture due to its high and relatively specific expression in neural tissue and because previous research demonstrated its high expression on exosomes derived from cultured neurons (Fauré et al., 2006).

2.4. NDEs characterization

NDEs were characterized as recommended by MISEV (Lotvall et al., 2014; Thery et al., 2018) from intact particles by nanoparticle tracking analysis (NTA) with a NanoSight LM10 instrument (Malvern, Worcestershire, UK) and corresponding software version NTA 2.3. NDEs were pre-diluted in PBS. Three videos of 30 s were acquired per sample. The mean sizes were calculated by integrating the data from three records. For TEM imaging, NDEs suspensions were fixed for 1 h with 1% glutaraldehyde solution at room temperature. Five μl of suspension was adsorbed on 200 mesh thin-film carbon coated TEM grids for 5 min and excess was blotted on filter paper. TEM grids were then washed with Milli-Q water (Merck-Millipore, Germany) and negatively stained with 1% Uranyl Acetate aqueous solution at 30 min. TEM micrographs were acquired by JEOL JEM 2100Plus Transmission Electron Microscope (JEOL, Japan) operating with an acceleration voltage of 200 kV, and equipped with an 8 megapixel Gatan (Gatan, USA) Rio Complementary Metal-Oxide-Superconductor (CMOS) camera. Remaining NDEs were lysed

| Population characteristics | PD patients | HC subjects | p |
|-----------------------------|-------------|-------------|---|
| N | 32 | 40 | 0.378 |
| Age at enrollment (years), mean ± SD | 69.47 ± 8.56 | 67.45 ± 7.64 | 0.294 |
| Age at onset (years), mean ± SD | 63.19 ± 8.79 | 6.28 ± 3.63 | n.a. |
| Disease duration (years), mean ± SD | 24.23 ± 2.48 | n.a. |
| MoCA, mean (SD) | 28.52 ± 13.16 | n.a. |
| Modified H&Y, median (range) | 2 (1–3) | n.a. |
| LEDD (mg/die), mean ± SD | 507.3 ± 270.0 | n.a. |
| n.a. = not applicable; n.c. = not calculated; SD = standard deviation; MDS-UPDRS Part III = Movement Disorders Society Unified Parkinson’s disease Rating Scale; Modified H&Y = Modified Hoehn and Yahr scale; LEDD = levodopa equivalent daily dose; MoCA = Montreal Cognitive Assessment. |
with M-PER™ reagent (ThermoFisher scientific, USA) according to manufacturer’s instructions, adding a cocktail of protease and phosphatase inhibitors. Protein concentrations were determined with a Qubit™ fluorometer (ThermoFisher scientific, USA). Lysates were tested for the expression of specific exosomal and neural markers using a commercial kit based on western blotting (Exo-check Exosome Antibody Array (Neuro), SBI, USA). It consists of 14 pre-printed spots including 5 antibodies for exosomal associated markers (CD9, CD63, CD81: tetraspanins, ICAM1: intercellular adhesion molecule 1 and TSG101: tumor susceptibility 101 protein), 6 neuronal associated markers (L1CAM: L1 cell adhesion molecule, NCAM1: neural cell adhesion molecule, ENO2: enolase 2, MAPT: microtubule associated protein tau, GRIA1: glutamate ionotropic receptor AMPA type subunit 1 and PLP1: proteolipid protein 1) and 3 controls (one positive assay control, one negative control and CANX: calnexin, as cellular contamination control). Fifty µg of protein lysates from isolated NDEs were used and manufacturer’s instructions were followed. The membranes were developed with Clarity Max Western ECL Substrate (Bio-Rad, USA) and imaged by ChemiDoc™ Gel Imaging System (Bio-Rad, USA).

2.5. Western blot analyses

2.5.1. α-Synuclein

For Western blot analysis of α-Synuclein, 40 µg of proteins extracted from NDEs or serum were treated at 50 ºC for 30 min, separated by SDS-PAGE using Mini PROTEAN® TGX™ precast gels (Bio-Rad, USA), and transferred to PVDF (polyvinylidene difluoride) membrane with Trans-Blot® instrument (Bio-Rad, USA). The membrane was fixed in 0.4% PFA for 30 min and then blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The membrane was then then incubated with an anti-α-Synuclein monoclonal antibody (Syn 211) (1:1000, Invitrogen AHB0261, ThermoFisher Scientific, USA) at 4 ºC overnight, followed by incubation with a secondary anti mouse HRP-conjugated antibody for 1 h (1:20000, Cat number HAF018, R&D, USA) at room temperature. The membrane was developed with Clarity Max Western ECL Substrate (Bio-Rad, USA) and imaged by ChemiDoc™ Gel Imaging System (Bio-Rad, USA).

2.5.2. Transferrin

For Western blot analysis of Transferrin, Human Apo-Transferrin protein (3188-AT, R&D systems, USA) was included in the reaction as positive control. 40 µg of proteins extracted both from NDEs and serum were boiled at 95 ºC for 10 min, separated by SDS-PAGE using Mini PROTEAN® TGX™ precast gels (Bio-Rad, USA), and transferred to PVDF (polyvinylidene difluoride) membrane with Trans-Blot® instrument (Bio-Rad, USA). The membrane was blocked (5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20), then incubated with a primary goat polyclonal antibody against Transferrin (1:1000; Goat Anti-Transferrin Antibody (polyvinylidene difluoride) membrane was then incubated with an anti goat HRP-conjugated antibody for 1 h 45 min at room temperature. The membrane was developed with Clarity Max Western ECL Substrate (Bio-Rad, USA) and imaged by ChemiDoc™ Gel Imaging System (Bio-Rad, USA).

2.6. Quantification of biomarkers by ELISA

Oligomeric α-Syn, total α-Syn, SNAP-25, VAMP-2 and STX-1A dosages were measured in protein extracts from NDEs by sandwich enzyme-linked immunosorbent assay (ELISA). The following assays were used: Human Alpha Synuclein oligomer (SNCOs) ELISA kit (cat n°: MBS730762, MyBioSource, USA), Human α-Synuclein ELISA kit (cat n°: KHB0061, Invitrogen, ThermoFisher Scientific, USA), Human SNAP-25 ELISA kit (cat n°: LS-F17747, LifeSpan Biosciences, Inc., USA), Human Vesicle Associated Membrane Protein 2 (VAMP-2) ELISA kit (cat n°: MBS062679, MyBioSource, USA) and Human Syntaxin 1A, Brain (STX-1A) ELISA kit (cat n°: MBS076045, MyBioSource, USA) according to the manufacturer instructions. For SNAP-25 quantification a 1:10 dilution of NDEs protein extracts was used, undiluted NDEs protein extracts were used to measure oligomeric α-Syn and VAMP-2 and syntaxin-1. Standard curves and samples were run in duplicate.

2.7. Statistical analysis

Differences in demographic variables between PD and HC were evaluated with Chi-square and student’s t-test. Kolmogorov-Smirnov test showed that oligo α-Syn, total α-Syn, STX-1A and VAMP-2 concentration data were normally distributed in the cohort. One-way ANOVA tests were performed to compare oligo α-Syn, STX-1A and VAMP-2 concentration between groups. Logistic regression was applied considering the condition of illness as dependent variable and NDEs oligo α-Syn, STX-1A, VAMP-2 concentrations and gender as covariates. Correlations between variables were determined by Pearson correlation coefficient r with 95% CI. The discriminatory ability of NDEs oligo α-Syn, STX-1A, VAMP-2 levels and oligo α-Syn/STX-1A, oligo α-Syn/ VAMP-2 ratios to distinguish between HC and PD patients was presented by using receiver operating characteristic (ROC) analyses with calculation of the area under the curve (AUC), sensitivity and specificity as well as 95% confidence interval (CI). Linear regression was applied in order to verify the relation between oligo α-Syn and disease duration using sex as covariate. Based on H&Y score, PD patients were split into two groups using 2.5 value of H&Y score as cut-off value, then a Student’s t-test was applied in order to find differences in the means of oligo α-Syn concentrations between the two groups of patients with different disease severity: “mild” < 2.5 and “moderate” ≥ 2.5 (Lee et al., 2016). The statistical power of the sample size was 99.2% and was calculated based on difference of the means of oligo α-Syn concentrations in PD and HC subjects (https://www.openepi.com). In all cases differences were considered statistically significant when p < 0.05. MedCalc® software (MedCalc®, 14.10.2, Belgium) and SPSS software (v.27, IBM, USA) were used for all statistical analyses.

3. Results

3.1. Epidemiologic characterisation

A total of 32 PD patients met the inclusion criteria and were recruited. Mean age was 69.47 ± 8.56 years with a mean disease duration of 6.12 ± 3.63 years and mean total LEDD of 507.3 ± 270.0. At the baseline evaluation, the MDS-UPDRS Part III mean score and Modified Hoehn-Yahr median (range) score were, respectively, 28.52 ± 13.16 and 2 (1–3). Of this cohort, 5 patients met the criteria for TD (16%), 23 met the criteria for PIGD (72%), and 4 were classified as indeterminate (12%) based on the MDS-UPDRS definitions (Stebbins et al., 2013). There was no difference in age or gender distribution between PD and HC (p > 0.05). In the PD group, there was no significant difference in age, gender, MDS-UPDRS III scores between patients with TD and patients with PIGD (p > 0.05). Demographic and clinical data are summarized in Table 1.

3.2. NDEs characterisation

NTA analyses were performed to measure the size of NDEs in PD and HC (mean: PD = 116 ± 0.3 nm, SD = 76 ± 0.8 nm; HC = 97.6 ± 0.2 nm, SD = 53.6 ± 1.3 nm) as well as their quality. In Fig. 1A results from one representative sample and a frame of the video recorded during NTA analysis are presented. TEM imaging showed cup-shaped structures of representative sample and a frame of the video recorded during NTA.
EVs confirming the neural origin of the vesicles. In Fig. 1D Western blot results show that Transferrin was present in serum of PD patients but not in NDEs lysates of the same patients. The experiment was performed in order to exclude serum proteins contaminations in NDEs extracts.

3.3. Western blot analysis of \( \alpha \)-Syn in NDEs

Western blot analysis of \( \alpha \)-Synuclein in NDEs of PD patients revealed quantitative and qualitative differences compared to HC. Bands of different \( \alpha \)-Syn oligomeric species are visible and, in particular, a band of about 120–130 KDa compatible with \( \alpha \)-Synuclein octamer is present only in PD patients. No bands corresponding to \( \alpha \)-Syn monomer were detectable (Fig. 2).

3.4. Oligomeric \( \alpha \)-Syn, total \( \alpha \)-Syn and SNARE complex proteins in NDEs of PD patients and HC

Protein extracts from NDEs isolated from both PD patients and healthy controls were analyzed to determine their content of oligomeric \( \alpha \)-Syn, total \( \alpha \)-Syn, SNAP-25, VAMP-2 and STX-1A. The concentration of oligomeric \( \alpha \)-Syn was significantly augmented in in NDEs of PD patients (mean: 0.573 ng/ml, SD = 0.154 ng/ml) compared to HC (mean: 0.389 ng/ml, SD = 0.148 ng/ml) \((p < 0.001)\). On the contrary, VAMP-2 was significantly reduced in NDEs from PD patients (mean: 4.379 ng/ml, SD = 1.739) compared to HC (mean: 6.298 ng/ml, SD = 1.778 ng/ml) \((p < 0.001)\). Similarly, STX-1A was significantly reduced in NDEs from PD patients (mean: 2.214 ng/ml, SD = 0.492) compared to HC (mean: 2.769 ng/ml, SD = 0.522) \((p < 0.001)\) (Fig. 3). In contrast with these results, no differences were observed when SNAP-25 concentration was analyzed in NDEs of PD patients (mean: 175.658 ng/ml, SD = 115.381) and HC (mean: 208.637 ng/ml, SD = 130.157) \((p = 0.27)\).

A logistic regression was performed next using the backward method, considering the presence/absence of a PD diagnosis as the dependent variable, and NDEs oligo-\( \alpha \)-Syn, STX-1A and VAMP2 concentrations and gender as covariates. The model confirmed the presence of strong correlations between NDEs oligo-\( \alpha \)-Syn concentration and PD \((p = 0.001)\) as well as between STX-1A and PD \((p = 0.0009)\) (overall

Fig. 1. Characterization of neural derived extravesicles (NDEs). A. Representative nanoparticle tracking analysis (NTA) of NDEs from a PD patient and a HC. NanoSight LM10 instrument. B. Transmission electron microscopy (TEM) micrograph of NDEs. Cup-shaped structures, with 30–100 nm size were identified as being exosomes. Scale bar: 100 nm. C. Western blot on NDEs lysates. Exosomal associated markers (CD9, CD63, CD81: tetraspanins, ICAM1: intercellular adhesion molecule 1 and TSG101: tumor susceptibility 101 protein), neuronal associated markers (L1CAM: L1 cell adhesion molecule, NCAM1: neural cell adhesion molecule, ENO2: enolase 2, MAPT: microtubule associated protein tau, GRIA1: glutamate ionotropic receptor AMPA type subunit 1 and PLP1: proteolipid protein 1) and controls (positive assay control, negative control and CANX: calnexin, as cellular contamination control). D. Western blot analysis of Transferrin. Lane 1: protein marker (11–190 KDa #13953 Cell Signaling Technology®; USA); lanes 2,3: serum protein extracts from 2 different PD patients; lanes 4,5: NDEs protein extracts from the same 2 PD patients; lane 6: recombinant Apo-transferrin protein.

Fig. 2. Western blot analysis of \( \alpha \)-Synuclein aggregation in NDEs. Lane 1: protein marker (11–190 KDa #13953 Cell Signaling Technology®, USA); lanes 2,3: PD patients NDEs extracts; lanes 4,5: HC subjects NDEs extracts.
model fit: $\chi^2 = 38.222; \ p < 0.0001$). The model has the power to correctly classify 79.17% of cases. Finally, total $\alpha$-Syn was augmented in HC (6.564 ng/ml, SD = 6.143 ng/ml) compared to PD patients (3.503 ng/ml, SD = 3.960 ng/ml) ($p = 0.02$), and total $\alpha$-Syn/oligo $\alpha$-Syn ratio was higher in HC (18.6) than in PD patients (6.96) ($p = 0.006$).

3.5. Correlation between markers

The possible correlation between the biomarkers was then TESTED. A statistically significant negative correlation between oligo $\alpha$-Syn concentration and both STX-1A and VAMP2 SNARE proteins was observed. In particular, the correlation between oligomeric $\alpha$-Syn levels and STX-1A levels showed a $r = -0.280, p = 0.02$, 95%CI:0.480 to 0.051. The negative correlation between oligomeric $\alpha$-Syn levels and VAMP2 levels resulted stronger: $r = -0.334, p = 0.004$, 95%CI:0.525 to 0.111. A strong positive correlation was found instead between STX-1A and VAMP-2 levels: $r = 0.916, p < 0.0001$, 95%CI:0.868 to 0.947 (Fig. 4). Finally, no correlation was seen between oligomeric $\alpha$-Syn levels and SNAP-25 levels (data not shown).

3.6. ROC curve analyses

ROC analyses were performed next in order to assess the diagnostic value of NDEs oligomeric $\alpha$-Syn, STX-1A and VAMP-2 concentration toward the diagnosis of PD separately. Results indicated that the performance of ROC analysis was “good” for NDEs oligo $\alpha$-Syn (AUC = 0.824, sensitivity = 78.1%, specificity = 75.0%, cut off value = 0.496 ng/ml; $p < 0.0001$) and VAMP2 (AUC = 0.780, sensitivity = 81.3%, specificity = 72.5%, cut off value = 4.609 ng/ml; $p < 0.0001$). ROC curve analysis for total $\alpha$-Syn/oligo $\alpha$-Syn ratio resulted “fair” (AUC = 0.712, sensitivity = 90.6%, specificity = 55.3%, cut off value ≤14.132; $p < 0.00$) (Fig. 4).

ROC curve analyses were also applied to NDEs oligo $\alpha$-Syn/STX-1A and oligo $\alpha$-Syn/VAMP-2 ratios that resulted even stronger in the discrimination between PD and HC: AUC = 0.871 sensitivity = 85.7%, specificity = 82.5%, cut off value ≥0.195; $p < 0.0001$ and AUC = 0.876 sensitivity = 75.0%, specificity = 92.5%, cut off value ≥0.119 ng/ml; $p < 0.0001$, respectively (Fig. 5).

3.7. Correlation with clinical variables

Possible correlations between NDEs proteins and different clinical variables were subsequently analyzed in PD patients. Results showed the presence of a positive correlation between oligo $\alpha$-Syn concentrations and disease duration ($r = 0.483, p = 0.005$, 95%CI:0.162 to 0.712) (Fig. 6). A linear regression using oligo $\alpha$-Syn as dependent variable and disease duration and gender as covariates confirmed the significant association between oligo $\alpha$-Syn and disease duration ($p = 0.005$). No significant correlations were found between NDEs STX-1A, VAMP-2, SNAP-25 concentrations and disease duration.

Possible relationships between oligo $\alpha$-Syn, STX-1A, VAMP-2 and SNAP-25 concentrations and the progression of PD were finally analyzed by splitting the cohort of patients into two groups using an H&Y score value of 2.5 as cut-off. Twenty-two PD patients had a H&Y score < 2.5. A Student t-test was then applied to compare the means of oligo $\alpha$-Syn, STX-1A, VAMP-2 and SNAP-25 concentrations in the two groups. Oligo $\alpha$-Syn concentration was significantly higher in the H&Y score ≥ 2.5
group (0.678 ± 0.179 ng/ml) compared to the H&Y score < 2.5 group (0.525 ± 0.127 ng/ml) (p = 0.03, t = 2.23). No associations were found between other NDEs biomarkers and the remaining clinical variables: age at onset, UPDRS score, TD and PIGD forms, LEDD, MoCA score.

4. Discussion and conclusions

The identification of blood-based biomarkers which could combine lack of invasiveness, reliability, the possibility of repeated acquisitions, and low costs is a necessity in neurodegenerative conditions and in particular in Parkinson’s disease, in which diagnostic criteria are actually based on the analysis of proteins in the CSF and on clinical and imaging analyses. The development of techniques allowing the isolation of neural derived extravesicles (NDEs) from peripheral blood has opened interesting opportunities, as NDEs and their contents could be used as biomarkers in the diagnosis and the prognosis of a number of diseases. Currently, there are inconsistencies in studies assessing blood α-Syn. This could be due both to the heterogeneity of the techniques used for the different forms of α-Syn measurement (western-blot, ELISA, Luminex assay or mass spectometry) and to contamination due, e.g., to hemolysis or to presence of platelets (Atik et al., 2016). The use of NDEs as source of measurable α-Syn is a way to overcome some of these concerns. In this study, we performed an in-depth analysis of NDEs in patients suffering from Parkinson’s disease and found that the concentration of the toxic oligomeric form of α-Syn is significantly increased in NDEs and, inversely, total α-Syn is decreased; importantly the amount of oligomeric form of α-Syn in NDEs correlated with disease duration and clinical severity. Results also showed that lower concentrations of STX-1A and VAMP-2, key proteins in the SNARE complex, which mediates the neurotransmitter release of synaptic vesicles by neurons, were detected as well in NDEs of the same patients. Notably, a negative correlation was observed between oligomeric α-Syn and both STX-1A and VAMP-2 concentrations in NDEs. In other words, when the oligomeric form of α-Syn increased the pre-synaptic proteins STX-1A and VAMP-2 decreased. The likely diagnostic importance of these results is underlined by results of ROC curve analyses that indicated the discriminatory ability of NDEs oligomeric α-Syn, STX-1A, VAMP-2 concentrations and oligo α-Syn/STX-1A and oligo α-Syn/VAMP-2 ratios to distinguish between PD patients and HC.

It has been long known that α-Syn accumulates within Lewy bodies (LB) in brains of PD patients (Spillantini et al., 1997; Lang and Lonzano, 1998 a,b) and that such accumulation plays a pivotal role in the disease. α-Syn forms oligomeric or fibrillar structures which are a continuum of species ranging from unstable low-molecular weight particles to stable fibrils composed of more than 15 monomers with seeding capacity (Pieri et al., 2016). Different types of oligomers were found to have a negative impact on neuronal excitability (Kaufmann et al., 2016) and the emerging notion is that α-Syn oligomer (protefibril), rather than α-Syn fibril, is neurotoxic (Winner et al., 2011; Forloni et al., 2016). α-Syn aggregates cause cytotoxicity by affecting many cellular mechanisms, and results in synaptic dysfunction by interfering with the axonal transport of synaptic proteins, inhibiting synaptic vesicle reclusterling, and SNARE-mediated vesicle docking (Scott et al., 2010; Nemani et al., 2010). Thus, synaptic accumulation of oligomers and aggregated α-Syn, and its interaction with components of the SNARE complex, could explain the synaptic dysfunction observed in PD. In support of this hypothesis, studies in vitro have demonstrated that large α-Syn oligomers block SNARE-mediated vesicle docking as a consequence of their binding to the N-terminal domain of VAMP-2 (Choi et al., 2013). Moreover, post mortem studies investigating levels of SNARE complex components
in PD, in PD with dementia, and in DLB (Dementia with Lewy body) showed a misregulation of these proteins (Kramer and Schulz-Schaeffer, 2007; Dijkstra et al., 2015; Bereczki et al., 2018). Thus, when LB purified from postmortem DLB brain tissues were analyzed by mass spectrometry, more than 20% of the proteins found in LB were found to be proteins involved in synaptic vesicle fusion (SNAP-25, VAMP-2 and STX-1A) (McCormack et al., 2019).

Results herein showing that the concentrations of oligomeric α-Syn and those of STX-1A and VAMP-2 are inversely correlated in NDEs of PD patients are in accordance with the observation that α-Syn oligomers can sequester VAMP-2 and, probably, other synaptic fusion proteins, resulting in synaptic dysfunction (Choi et al., 2018). Thus, whereas normally the C-terminal α-Syn monomer binds to the N-terminal VAMP-2 resulting in physiological synaptic function (Burré et al., 2010), the short rod configuration of α-Syn aggregates inhibit the lipid mixing process of SNARE complex by interacting with the N-terminal domain of VAMP-2 (Choi et al., 2013). Moreover, spherical shaped α-Syn aggregates interact with VAMP-2 via C-terminal domain of SNARE motif (Choi et al., 2018).

In a very recent work, Niu et al. measured total α-Syn in plasma NDEs of PD patients and observed that α-Syn is increased in PD patients compared to healthy controls and its concentration correlates with clinical scores (Niu et al., 2020). To the best of our knowledge, though, there is only one previous study in which oligomeric α-Syn was measured in serum NDEs. That study was conducted on PD patients, essential tremor (ET) patients and HC. In contrast with our results, lower oligomeric α-Syn levels were observed in NDEs from PD patients (Si et al., 2019); differences in the methods used as well as in patient selection might justify this discrepancy. Oligomeric α-Syn and oligomeric α-Syn/total α-Syn ratio were also measured in salivary EVs and were observed to be augmented in PD patients (Cao et al., 2018); the salivary EVs population is nevertheless very heterogeneous, and only few of those EVs are of CNS origin.

Notably, many studies have previously investigated CSF α-Synuclein. Thus, meta analyses showed that the CSF levels of total α-Synuclein are decreased in patients with PD (Sako et al., 2014; Gao et al., 2015; Eusebi et al., 2017) whereas oligomeric α-Synuclein is increased in these same patients (Eusebi et al., 2017). These observations are in agreement with
Fig. 6. Pearson’s correlation between NDEs oligomeric α-Synuclein concentration and disease duration in PD patients ($p = 0.005$).

the results of our analyses on total and oligomeric α-Syn levels in NDEs. Decreased total α-Syn may be an indirect index of changes in the balance between its secretion, solubility and state of aggregation in the brain, reflecting changes in its turnover. If this is the case, this observation confirms the importance and the validity of peripheral NDEs as easily accessible biomarkers that reflects what is happening in the central nervous system.

Western blot analyses of α-Synuclein in NDEs extracts revealed its state of aggregation. In particular, the observation that a band compatible with α-Syn octamer could be observed in PD patients alone is of particular interest and will have to be investigated in depth, especially in regard to the ability of α-Syn octamers to form annular pore-like structures that could increase cell permeability and calcium influx (Tosatto et al., 2012).

In conclusion, we must underline that there is the possibility that a minimum percentage of NDEs isolated from serum could be secreted by cells of peripheral nervous system, but it is also to be said that, in PD, synuclein pathology has been reported even in sympathetic ganglia and autonomic nerves (Sprecher and Derkinderen, 2012). Although the sample size of this study was calculated to reach statistical power, further studies in larger cohorts will be needed to shape an ideal panel of NDEs-based biomarkers that could be useful in PD diagnosis and prognosis and, possibly become a tool to monitor the effects of rehabilitation programs and novel therapies in longitudinal studies.

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Declaration of Competing Interest
None.

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