Parkinsonism and Related Disorders 63 (2019) 143–148

Contents lists available at ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

Salivary alpha-synuclein in the diagnosis of Parkinson's disease and Progressive Supranuclear Palsy

Giorgio Vivacqua[a,b], Antonio Suppa[a,c], Romina Mancinelli[b], Daniele Belvisi[c], Andrea Fabbrini[a], Matteo Costanzo[a], Alessandra Formica[a,c], Paolo Onori[b], Giovanni Fabbrini[a,c], Alfredo Berardelli[a,c,*]

[a] Department of Human Neurosciences, "Sapienza" University of Rome, V.le delle Università 30, 00185, Rome, Italy
[b] Department of Anatomic, Histologic, Forensic Medicine and Orthopedics Sciences, "Sapienza" University of Rome, via A. Borelli 50, 00161, Rome, Italy
[c] IRCCS NEUROMED, Via Atinense 18, 86077, Pozzilli, IS, Italy

ARTICLE INFO

Keywords:
Alpha-synuclein
Biomarkers
Parkinson's disease
Saliva
Progressive Supranuclear Palsy

ABSTRACT

Introduction: Alpha-synuclein (α-syn) aggregation is the pathological hallmark of Parkinson's Disease (PD). In this study, we measured α-syn total ($\alpha\text{-syn}_{total}$), oligomeric α-syn ($\alpha\text{-syn}_{olig}$) and $\alpha\text{-syn}_{olig}/\alpha\text{-syn}_{total}$ ratio in the saliva of patients affected by PD and in age and sex-matched healthy subjects. We also compared salivary $\alpha\text{-syn}_{total}$ measured in PD with those detected in Progressive Supranuclear Palsy (PSP), in order to assess whether salivary α-syn can be used as a biomarker for PD and for the differential diagnosis between PD and PSP.
Methods: We studied 100 PD patients, 20 patients affected by PSP and 80 age- and sex-matched healthy subjects. ELISA analysis was performed using two commercial ELISA platforms and a specific ELISA assay for α-syn aggregates.
Results: We detected lower $\alpha\text{-syn}_{total}$ and higher $\alpha\text{-syn}_{olig}$ in PD than in healthy subjects. Conversely in PSP salivary $\alpha\text{-syn}_{total}$ concentration was comparable to that measured in healthy subjects. Receiver Operating Characteristic analyses revealed specific cut-off values able to differentiate PD patients from healthy subjects and PSP patients with high sensitivity and specificity. However, there was no significant correlation between clinical and molecular data.
Conclusion: Salivary α-syn detection could be a promising and easily accessible biomarker for PD and for the differential diagnosis between PD and PSP.

1. Introduction

The diagnosis of Parkinson's Disease (PD) is based on clinical criteria, whose accuracy may be limited, particularly in the early stages of the disease or in the differential diagnosis between PD and atypical parkinsonisms. A validated biomarker would strongly help to correlate neurodegeneration with clinical features and to distinguish PD from atypical parkinsonisms.

Alpha-synuclein (α-syn) is a 140-amino acid protein, richly expressed in the central nervous system [1]. In physiological conditions, α-syn is prevalently expressed in a monomeric form ($\alpha\text{-syn}_{mon}$) and it is localized in pre-synaptic vesicles and cellular nuclei [1,2]. PD is characterized by α-syn misfolding, which leads to the formation of α-syn oligomers ($\alpha\text{-syn}_{olig}$) [3], Lewy bodies and Lewy neurites [4]. In PD, $\alpha\text{-syn}_{olig}$, are responsible for α-syn neurotoxicity [3] and neuropathological progression [5].

α-Syn can be detected in different biological fluids, including cerebro-spinal fluid (CSF) and saliva [6–12]. Saliva is particularly easy to collect by means of non-invasive procedures and it is poorly affected by blood contamination [13]. Neuropathological studies demonstrated the presence of α-syn pathology in the small fibers innervating salivary glands [14]. Reduced α-syn total ($\alpha\text{-syn}_{total}$) concentration [9–12] and, more recently, increased salivary $\alpha\text{-syn}_{olig}$ concentration [11,12] has been reported in the saliva of patients with PD. Hence, detection of α-syn in saliva represents a current promising research and clinical challenge.

Abbreviations: (α-syn), Alpha-synuclein; ($\alpha\text{-syn}_{total}$), total alpha-synuclein; ($\alpha\text{-syn}_{olig}$), oligomeric alpha-synuclein; (PD), Parkinson's Disease; (PSP), Progressive Supranuclear Palsy; (HS), healthy subjects; (pLR), positive Likelihood Ratio; (nLR), negative Likelihood Ratio

* Corresponding author. Department of Human Neurosciences, IRCCS Neuromed Institute, Sapienza University of Rome, Viale dell'Università, 30, 00185, Rome, Italy.
E-mail address: alfredo.berardelli@uniroma1.it (A. Berardelli).

https://doi.org/10.1016/j.parkreldis.2019.02.014
Received 21 September 2018; Received in revised form 8 February 2019; Accepted 12 February 2019

In this study, we investigated salivary α-syn$_{total}$ and α-syn$_{olig}$ in a cohort of 100 PD patients and 80 HS using the ELISA methods described previously [11]. Moreover, in a subgroup of 20 PD patients and 20 HS, we further validated the measurement of α-syn$_{olig}$ by comparing two different ELISA methods specifically designed to detect α-syn aggregates [7]. We also compared salivary α-syn$_{total}$ in PD patients and in a cohort of 20 patients with a clinical diagnosis of probable Progressive Supranuclear Palsy (PSP), a neurodegenerative disease clinically presenting with parkinsonism, but pathologically characterized by tau deposition [15]. We calculated sensitivity, specificity, positive and negative likelihood ratio (pLR, nLR) of the diagnostic test and its performance by means of the Receiver Operating Characteristic (ROC) analysis, to differentiate PD patients from HS and from PSP patients. Lastly, we assessed the possible correlations between clinical and molecular data in patients with PD and PSP.

2. Materials and methods

2.1. Participants

A cohort of 112 patients with PD (59 males, 53 females) and 90 age- and sex-matched healthy subjects (HS) (53 males, 37 females) were recruited in 20 consecutive series of outpatient clinical session from November 2016 until May 2017, at the Movement Disorders outpatient clinic of the Department of Human Neurosciences, "Sapienza" University of Rome, Italy. Twenty-two patients with a diagnosis of probable PSP (12 males, 10 females) were also recruited in 5 consecutive series from February 2017 until March 2017 (Supplementary Table 1).

The diagnosis of PD was based on the Queen Square Brain Bank Criteria [16], while the diagnosis of probable PSP was based on the criteria of the Movement Disorders Society (MDS) [17]. A complete neurological examination was performed to each patient. The clinical data collected included: duration and stage of the disease, as scored with the Hoehn &Yahr scale (H&Y); severity of the disease, as assessed with the MDS-Unified Parkinson's Disease Rating Scale (MDS-UPDRS); cognitive impairment, as assessed with the Montreal Cognitive Assessment (MoCA) and the Frontal Assessment Battery (FAB). For patients affected by PSP, disease severity was evaluated by the PSP Rating Scale (PSPRS). Current pharmacological treatment was assessed and calculated for each patient as the L-dopa equivalent daily doses (LEDDs) for each drug [18]. Participants affected by cardiovascular and cerebrovascular diseases, chronic systemic diseases, hematological and solid neoplasms were excluded from the study, as were subjects affected by salivary gland and oral cavity pathologies.

All patients and HS gave their written informed consent to the study, during the first clinical evaluation. The study has previously received ethical approval by the Institutional Review Board of the "Sapienza" University of Rome in accordance to the Declaration of Helsinki.

2.2. Sample collection

Samples collection was performed according to previous studies [9–12]. We collected a minimum quantity of 1 ml of saliva from each subject. At the time of collection, subjects had fasted for 60 min, had not smoked in the preceding 4 h and had not drunk alcohol in the previous 12 h. Saliva was collected by drool into a 50 ml vial, which was immediately placed on ice in order to block proteolytic activity. Samples were then centrifuged for 10 min at 10,000 × *g* at 4 °C. After centrifugation, each sample was treated with a protease inhibitor cocktail (Sigma Aldrich, St. Luis, MO, USA, Cat #P2714), at a concentration of 1:10. Each sample was then aliquoted and stored at −80 °C. Samples storage was performed at −80 °C in accordance with previous studies suggesting the stability of α-syn aggregates at this temperature [19].

2.3. ELISA analysis of samples

α-syn$_{total}$ and α-syn$_{olig}$ were assessed in saliva by enzyme immunoassay (ELISA). Before ELISA analysis, total protein concentration was measured in each salivary sample, through BCA Protein Assay kit (Thermofisher Scientific, UK) and each sample has been normalized in order to ensure that equal amount of total salivary proteins was submitted to the ELISA analysis, thus avoiding any possible bias due to the variation in salivary secretion rate. A final total protein concentration of 4,5 μg/μl was reached in each sample. Normalized salivary samples were further diluted 1:10 for the detection of α-syn$_{total}$ and 1:2 for the detection of α-syn$_{olig}$, respectively, in accordance with the datasheet of the ELISA kits. We used the anti-alpha-Synuclein Quantitative ELISA Kit (SensoLyte 55550) to determine α-syn$_{total}$. To determine α-syn$_{olig}$, we used the Human α-syn sandwich oligomer ELISA Kit (MyBioSource, MBS730762 - $_{olig}$ELISA1). Both ELISA kits were previously validated in saliva in independent cohorts [10,11]. Each sample from both patients and HS was analyzed in triplicate. The concentration of α-syn$_{total}$ and α-syn$_{olig}$ was determined by spectrometric measurement at 450 nm in an appropriate microplate reader (LT 4000, Labtech, Heathfield, UK).

2.4. ELISA analysis using anti α-syn 3D5 home-made monoclonal antibody ($_{olig}$ELISA2)

In order to confirm the presence of α-syn$_{olig}$ in saliva, we randomly selected salivary samples from 20 PD and 20 age- and sex-matched HS and we applied the ELISA method for α-syn aggregates previously described by El Agnaf and colleagues [7]. This ELISA method is based on a conventional sandwich system in which α-syn is captured by a highly specific anti-α-syn monoclonal antibody (mAb) that recognizes the epitope "*D (E)-X-X-V-X-P-D (N)*" on the C-terminal of α-syn (3D5) [2,20]. Capture is followed by the detection of the same mAb using a biotinylated form. Monomeric α-syn cannot emit any signal in this assay because the capture mAb occupies the only antibody binding site available on the protein. Only α-syn aggregates can be both captured and detected because multiple mAb binding sites are available. Briefly, α-syn$_{olig}$ standards were generated by dissolving 1 mg of monomeric α-syn in 500 μl 0.01 M phosphate-buffered saline (PBS) at a concentration of 140 μM (2 mg/ml) and then by incubating the mixture at 37 °C for 48 h with continuous shaking (650 rpm) on a Thermomixer Comfort shaker. α-Syn$_{mon}$ standards were generated by freshly dissolving 1 mg of monomeric α-syn in 500 μl 0.01 M PBS immediately before the ELISA analysis. The ELISA analysis was performed using a plate (96 wells) previously coated with 200 mg/L $NaHCO_3$ buffer containing 1 μg/ml anti-α-syn 3D5 antibody (200 μl each well, incubated at 37 C° for 2 h) and then blocked with 10% BSA (200 μl each well, at 37 °C for 2 h). Saliva samples were diluted 10 times using 0.01 M PBS and 100 μl were added to each well. Thereafter, we used anti α-syn 3D5 biotinylated antibody diluted to 1 μg/ml in blocking buffer (2 h at 37 °C) to detect the aggregates of α-syn previously captured by the same 3D5 antibody coated to the wells. ExtrAvidin Alkaline Phosphatase diluted 1:5000 in blocking buffer, followed by pNPP, was used to visualize the reaction. Optical absorbance was detected at 405 nm.

2.5. Statistical analysis

Statistical analyses were performed using SPSS Statistics software, version 23 (IBM Corporation, Armonk, NY, USA). The normality of the variable distribution was assessed using the Shapiro-Wilk test. The Mann-Whitney *U* test was used to compare salivary concentration levels of α-syn$_{total}$ and α-syn$_{olig}$ as well as the α-syn$_{olig}$/α-syn$_{total}$ ratio in PD patients and HS, and to compare α-syn$_{total}$ concentration in PD and PSP patients. Different ROC analyses were performed to identify the optimal diagnostic cut-off values for salivary α-syn$_{total}$, α-syn$_{olig}$ and the α-syn$_{olig}$/α-syn$_{total}$ ratio in discriminating PD patients from HS, as well as PD patients from PSP patients or PSP patients from HS. Cut-off values

were calculated as the point of the curves with the highest Youden's Index (sensitivity + specificity −1) to maximize the sensitivity and specificity of the diagnostic tests. pLR and nLR were also calculated. The Spearman-Rank correlation coefficient was used to assess any correlation between salivary α-syn and the clinical scores of PD and PSP patients. To further evaluate the possible relationship between salivary α-syn and PD clinical features we used a between-group ANOVA comparing total and oligomeric α-syn in PD patients with MDS-UPDRS scores lower and higher than the median MDS-UPDRS score. The level of statistical significance was set at $p < 0.05$.

3. Results

Detailed clinical scores are reported in Supplementary Table S1. Twelve PD patients and two PSP patients initially enrolled were excluded from the study due to the presence of severe cognitive impairment (MOCA score lower than 18 or FAB score lower than 12) or blood contamination of the salivary sample (hemoglobin values higher than 200 ng/ml) [21]. Ten HS enrolled initially in the study were also excluded for blood contamination [21].

The Mann-Whitney *U* test revealed significantly lower $\alpha\text{-syn}_{total}$ levels in the saliva of patients with PD than in that of HS ($p < 0.05$, Fig. 1A – Table 1). The Mann-Whitney *U* test also showed that $\alpha\text{-syn}_{olig}$ was significantly higher in the saliva of patients with PD than in that of HS ($p < 0.05$, Fig. 1A–Table 1). Accordingly, the $\alpha\text{-syn}_{olig}/\alpha\text{-syn}_{total}$ ratio was significantly higher in patients with PD than in HS ($p < 0.05$, Fig. 1A–Table 1). In a sub-cohort of 20 PD patients and 20 HS, we validated the presence of $\alpha\text{-syn}_{olig}$ detected using the commercial ELISA kit ($_{olig}$ELISA1) with the ELISA platform specifically designed for α-syn aggregates ($_{olig}$ELISA2) [7]. The Mann-Whitney *U* test revealed significantly different concentrations of $\alpha\text{-syn}_{olig}$ in PD patients and HS using both $_{olig}$ELISA1 ($p < 0.05$ – Fig. 1B) and $_{olig}$ELISA2 ($p < 0.05$ – Fig. 1B). The mean concentrations of $\alpha\text{-syn}_{olig}$ were 0.993 ± 1.071 ng/ml in PD patients and 0.289 ± 0.188 ng/ml in HS when the $_{olig}$ELISA1 was used, and 1.18 ± 0.87 ng/ml in PD patients and 0.426 ± 0.353 ng/ml in HS when the $_{olig}$ELISA2 was used. When comparing PD patients and HS, ROC analyses identified optimal diagnostic cut-off values for salivary $\alpha\text{-syn}_{total}$, $\alpha\text{-syn}_{olig}$ and the $\alpha\text{-syn}_{olig}/\alpha\text{-syn}_{total}$ ratio of 8.066 pg/ml (Y.I. = 0.5894), 0.215 ng/ml (Y.I. = 0.4085) and 0.025 (Y.I. = 0.6493), respectively. Using these cut-off values, the sensitivity and the specificity of the molecular tests were 67.44% and 91.04% for $\alpha\text{-syn}_{total}$ (Fig. 1C – Table 1), 56.98% and 83.87% for $\alpha\text{-syn}_{olig}$ (Fig. 1C – Table 1), and 69.77% and 95.16% for the $\alpha\text{-syn}_{olig}/\alpha\text{-syn}_{total}$ ratio (Fig. 1C–Table 1).

The Mann-Whitney *U* test detected significantly higher $\alpha\text{-syn}_{total}$ levels in patients affected by possible PSP than in patients affected by PD ($p < 0.05$, Fig. 2A - Table 1). By contrast, the Mann-Whitney *U* test did not detect any significant difference between the levels of $\alpha\text{-syn}_{total}$ in the saliva of patients with PSP compared to the saliva of HS ($p > 0.05$, Fig. 2A–Table 1). ROC analysis identified an optimal diagnostic cut-off value of 18.058 pg/ml (Y.I. = 1) for salivary $\alpha\text{-syn}_{total}$ in differentiating PD patients from PSP patients. Using these cut-off values, the sensitivity and the specificity of salivary $\alpha\text{-syn}_{total}$ were respectively 100% and 96.51% (Fig. 2B–Table 1).

Differentially to our previous study [11], Spearman-Rank correlation coefficient did not detect any significant correlation between $\alpha\text{-syn}_{total}$, $\alpha\text{-syn}_{olig}$, or the $\alpha\text{-syn}_{olig}/\alpha\text{-syn}_{total}$ ratio and the H&Y, the disease duration or the MDS-UPDRS in PD as well as in PSP patients. Accordingly, the between-group ANOVA did not show any significant effect of the factor "group" when we compared oligomeric (F = 39.23,

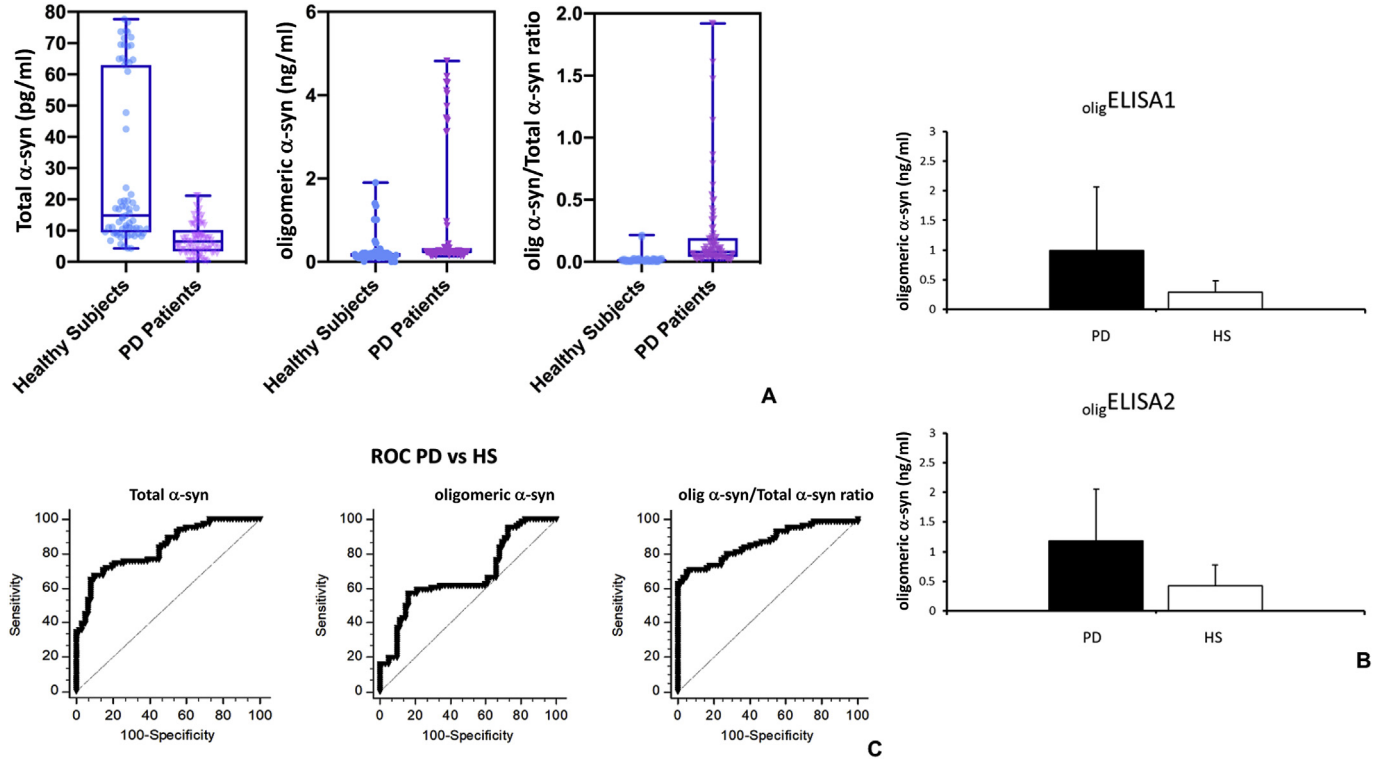

Fig. 1. Salivary α-syn in healthy subjects (HS) and Parkinson's disease (PD) patients. Box and whiskers plots show alpha-synuclein total (Total α-syn), oligomeric alpha-synuclein (oligomeric α-syn) and the oligomeric alpha-synuclein/total alpha-synuclein ratio (olig α-syn/total α-syn ratio) in the saliva of PD patients and HS (panel A). Data points represent the distribution of HS and PD patients for each biochemical parameter measured. In order to confirm the presence of α-syn oligomers in saliva, salivary oligomeric α-syn, was measured comparing two different ELISA methods: $_{olig}$ELISA1 and $_{olig}$ELISA2 (panel B). Each histogram corresponds to the mean concentration of oligomeric a-syn in the saliva of HS and PD patients. Vertical bars denote standard deviation. Receiver Operating Characteristic (ROC) analysis (panel C) showing sensitivity and specificity of salivary Total α-syn, oligomeric α-syn and the olig α-syn/total α-syn ratio used to differentiate patients affected by PD and HS.

Table 1
Salivary α-syn concentrations in PD patients, PSP patients and Healthy Subjects. Performance of the ROC analysis in differentiating PD patients from HS or PSP patients.

	PD patients	PSP patients	HS	Sensitivity PD vs HS	Specificity PD vs HS	Positive Likelihood ratio PD vs HS	Negative Likelihood ratio PD vs HS	Sensitivity PD vs PSP	Specificity PD vs PSP	Positive Likelihood ratio PD vs PSA	Negative Likelihood ratio PD vs PSA
α-syn$_{total}$	7.104 ± 5.122 pg/ml	29.091 ± 18.677 pg/ml	28.444 ± 25.877 pg/ml	67.44%	91.04%	7.53	0.36	100%	96.51%	28.67	< 0.001
α-syn$_{olig}$	0.893 ± 1.949 ng/ml	–	0.217 ± 0.191 ng/ml	56.98%	83.87%	3.53	0.51	–	–	–	–
α-syn$_{olig}$/α-syn$_{total}$	0.235 ± 0.793	–	0.0126 ± 0.0079	69.77%	95.16%	15.58	0.32	–	–	–	–

Salivary concentrations of α-syn$_{total}$ (pg/ml), α-syn$_{olig}$ (ng/ml) and α-syn$_{olig}$/α-syn$_{total}$ ratio, obtained by ELISA analysis, in patients with Parkinson's disease (PD), in healthy subjects (HS) and in patients affected by Progressive Supranuclear Palsy (PSP). Data are expressed as mean ± standard deviation. Test performance of Receiving Operating Characteristic (ROC) analysis in differentiating PD patients from HS or from PSP patients, including: sensitivity, specificity, positive and negative likelihood ratio.

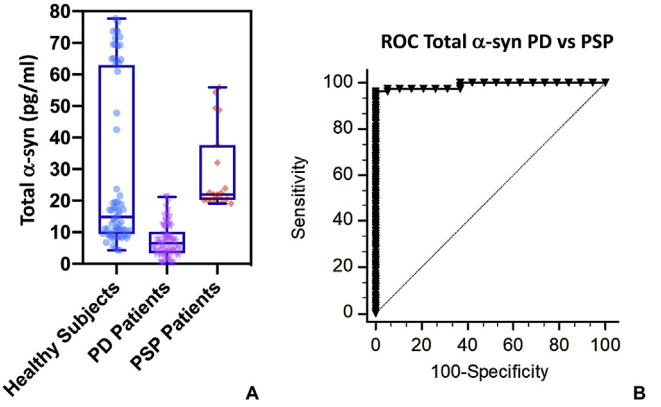

Fig. 2. Salivary alpha-synuclein in patients with Parkinson's disease (PD), Progressive Supranuclear Palsy (PSP) and in healthy subjects (panel A). Each box and whiskers plot shows total alpha-synuclein (Total α-syn) in the saliva of healthy subjects, patients with PD and PSP. Data points represent the distribution of healthy subjects, PD and PSP patients for the concentration of Total α-syn in saliva. Receiving Operating Characteristic (ROC) analysis showing the sensitivity and the specificity of Total α-syn in differentiating PD from PSP (panel B).

p = 0.99) and total α-syn (F = 39.32, p = 0.06) in the group of PD patients with MDS-UPDRS scores lower than the median value with those who had a MDS-UPDRS score higher than the median value.

4. Discussion

In this study, we show that salivary α-syn$_{total}$ is significantly lower in patients with PD than in HS. By contrast, salivary α-syn$_{olig}$, as well as the α-syn$_{olig}$/α-syn$_{total}$ ratio are both significantly higher in PD patients than in HS. The two $_{olig}$ELISA methods used in a subgroup of PD patients and HS confirmed higher salivary concentration of α-syn aggregates in PD patients compared to HS. Furthermore, we report, for the first time, that salivary α-syn$_{total}$ is significantly higher in PSP patients compared with PD patients. Lastly, using the ROC analysis, we calculated the cut-off values able to differentiate PD patients from HS and from PSP patients with highest sensitivity and specificity.

In our cohorts, patients with PD and PSP had varying degrees of disease severity (H&Y stages I to IV in both PD and PSP patients) and disease duration (range 1–23 years in PD patients and 1–4 years in PSP patients). Any patient or HS affected by other systemic diseases was excluded from the study. Blood contamination, which is known to affect α-syn detection [21] was avoided. Salivary samples collection was performed according to previous studies in saliva [10,11]. Temperature of storage was set up at −80 °C, considering that α-syn aggregates are temperature sensitive and in vitro aggregation occurs if samples are stored at −20 °C [19]. Moreover, protease inhibition has been prevented using a specific protease inhibition cocktail. We used two commercial ELISA kits to detect α-syn$_{total}$ and α-syn$_{olig}$ ($_{olig}$ELISA1), previously validated in independent cohorts of PD patients and HS [10,11]. Moreover, to further validate the presence of α-syn aggregates in saliva in a sub-cohort of 20 PD patients and 20 healthy subjects we performed an independent experiment with an ELISA platform, specific for α-syn aggregates ($_{olig}$ELISA2) [7].

In keeping with the results of our previous study [11], the ELISA analysis showed that salivary α-syn$_{total}$ is significantly lower in the saliva of patients affected by PD than in HS. By contrast, when two different ELISA methods were used to measure α-syn$_{olig}$ ($_{olig}$ELISA1 and $_{olig}$ELISA2), they both detected significantly higher concentrations of salivary α-syn$_{olig}$ in PD patients than in HS. Notwithstanding, it is important to note that the determination of α-syn$_{total}$ through ELISA likely underestimates the real total α-syn concentration in saliva. The underestimation of total α-syn is likely due to the antibodies used in the

ELISA assay which are aimed at linear epitopes on the α-syn molecule, thus detecting the unaggregated forms of the protein. The antibodies used may fail to detect aggregated forms of α-syn, such as α-syn_{olig}, suggesting that α-syn_{total} concentration largely estimates unaggregated α-syn_{mon} in saliva.

The present study confirms the significant intra-cohort variability for both α-syn_{total} and α-syn_{olig} concentrations, that we have reported in our previous study, using the same ELISA method [11] and reveals an inter-cohort variability in the concentrations of both α-syn_{total} and α-syn_{olig}. Significant intra-cohort and inter-cohort variability in α-syn_{total} and α-syn_{olig} concentrations in biological fluids has been reported in previous studies on saliva and CSF [6–12] and should be considered a major limitation for the ELISA-detection of α-syn. One possible explanation for this marked variability may be the widespread heterogeneity of α-syn aggregates in PD patients [22]. These different strains of α-syn aggregates have different biological properties [3] and display different tridimensional conformations that may unpredictably affect antigen-antibody binding [3,22]. This hypothesis is supported by the ROC analysis indicating that higher α-syn_{olig} concentrations in saliva is strongly specific to PD patients (specificity: 83.87%), however, the sensitivity to recognize PD patients is lower (56.98%), due to the possible inability of a single antibody to detect the different strains of α-syn aggregates. At the same way, a reduced concentration of α-syn_{total} in saliva is highly specific to PD patients, however, the different strains of α-syn_{olig} may present different aggregation properties on α-syn_{mon} [22], thereby reducing the sensitivity of the salivary α-syn_{total} concentrations (67.44%). Consequently, also the α-syn_{olig}/α-syn_{total} ratio discloses a very high specificity (95.16%), but a lower sensitivity (69.77%) in differentiating PD patients from HS. In accordance with the inter-cohort variability, in our previous study, we found a significant correlation between α-syn_{total} and various PD clinical scores [11]. In the present study, we investigated possible correlations between α-syn and PD clinical features by using two different statistical approaches, but we failed to confirm the presence of any significant association between salivary α-syn and specific clinical features of PD patients. The lack of correlation may reflect the heterogeneity of α-syn oligomers in saliva. We speculate that different molecular sub-classes of α-syn oligomers are related to specific stages of PD and the ELISA method might be unable to detect each of them, owing to different epitope presentation of α-syn aggregates.

The reduced α-syn_{total} and the increased α-syn_{olig} in the saliva of PD patients may be due to α-syn aggregation in the small autonomic fibers innervating salivary glands [14]. The alteration in salivary α-syn in PD may therefore be due to intracellular and axonal aggregation of the protein [14] with a reduced paracrine secretion of α-syn_{mon} and increased secretion of α-syn_{olig} in saliva [11,23]. This hypothesis is supported by a recent immunohistochemical study which detected reduced α-syn and increased phosphorylated α-syn in the minor salivary glands of PD patients compared with HS [24]. The authors reported that phosphorylated α-syn is detectable in neuronal fibers but also in glandular secretive cells, suggesting a possible co-contribution of neuronal fibers and glandular cells in the secretion of α-syn in saliva. Further histopathological investigations are needed to clarify the mechanisms of α-syn secretion in saliva. Furthermore, an altered turnover of α-syn is likely in patients with PD [25], leading to the accumulation of α-syn_{olig} at both the intracellular and extracellular levels.

A very important novel finding of this study is that salivary α-syn_{total} concentration in patients with a clinical diagnosis of probable PSP is significantly higher in comparison to PD patients, being similar to that detected in HS. PSP is a severe neurodegenerative disorder characterized by parkinsonism and additional motor and non-motor signs [26]. In accordance with our data, previous studies have demonstrated that α-syn is significantly reduced in the CSF of PD patients though not in that of PSP patients [27]. Moreover, skin biopsies have shown α-syn pathology in skin fibers of PD patients though not in those of patients affected by PSP [28]. PSP, indeed, is characterized by tau deposition in different regions of the midbrain, basal ganglia and cerebral cortex [26,29] and cross-aggregation between tau and α-syn is likely to be only sporadically present [30].

From the clinical point of view, our ROC analysis detected a moderate sensitivity of salivary α-syn in differentiating PD patients from HS, but detected a very high specificity for both salivary α-syn_{total} and α-syn_{olig} as well as for α-syn_{olig}/α-syn_{total} ratio, suggesting that salivary α-syn could be a useful and easily accessible test to support diagnosis of PD when the clinical diagnosis is uncertain. Moreover, the relatively high values of pLR support the value of our tests in differentiating PD patients from HS. Furthermore, our ROC analyses of salivary α-syn_{total} in differentiating PD from PSP patients, revealed high sensitivity, high specificity and high values of pLR, highlighting the potential application of salivary α-syn_{otal} in the differential diagnosis between PD and PSP.

The small size of the cohort of PSP patients, the lack of pathological confirmation for the diagnosis of PSP and the fact that the study has been conducted with a monocentric design represent – together with intra-cohort and inter-cohort variability of the ELISA measurements – some important limitations of this study. However, detection of α-syn in saliva could represent a new promising and less invasive method for the molecular diagnosis of PD and for the differentiation of PD from atypical parkinsonisms, such as PSP. The variable molecular and aggregation properties of α-syn require further investigations in order to understand the biodynamic of α-syn aggregation in saliva and to design a validated biological platform able to specifically detect the different subtypes of α-syn aggregates, thus improving the reproducibility and the clinical application of salivary α-syn.

In conclusion, in the present study we have demonstrated the presence of α-syn aggregates in saliva using two different biological methods in a new large cohort of PD patients and healthy subjects. We also report that salivary α-syn can differentiate PSP patients from PD patients. The results suggest the possible application of salivary α-syn as new biomarker in patients with PD. The present results need to be confirmed in future multicenter longitudinal studies including large cohorts of patients and healthy controls.

Authors' contribution

GV: designed of the work; performed ELISA analysis and statistical analysis; writing the first draft of the work; **AS**: designed of the work; performed statistical analysis and revised critically the first version of the draft; **RM**: performed validation experiments and pre-analytical assessments of the salivary samples; **DB**: performed processing of clinical data of PD and PSP patients; contributed to the critical revision of the first version of the draft; **AFb; MC and AFr:** performed PD and PSP patients enrolment and clinical evaluation; **PO:** performed validation of ELISA data and revised draft critically for methodological content; **GF:** revised draft critically for clinical aspects; **AB**: designed of the work; revised draft critically for important intellectual content; coordinated the research group.

Acknowledgements

We thank Dr. Lewis Baker for the English language editing. We thank Prof. Shun Yu from the Capital Medical University of Beijing (China) for providing 3D5 anti a-synuclein monoclonal antibody.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2019.02.014.

References

[1] J.T. Bendor, T.P. Logan, R.H. Edwards, The function of α-synuclein, Neuron 79

(2013) 1044–1066.
[2] G. Vivacqua, A. Casini, R. Vaccaro, F. Fornai, S. Yu, L. D'Este, Different sub-cellular localization of alpha-synuclein in the C57BL\6J mouse's central nervous system by two novel monoclonal antibodies, J. Chem. Neuroanat. 41 (2011) 97–110.
[3] H.L. Roberts, D.R. Brown, Seeking a mechanism for the toxicity of oligomeric α-synuclein, Biomolecules 5 (2015) 282–305.
[4] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, Alpha-synuclein in Lewy bodies, Nature 388 (1997) 839–840.
[5] H. McCann, H. Cartwright, G.M. Halliday, Neuropathology of α-synuclein propagation and braak hypothesis, Mov. Disord. 31 (2016) 152–160.
[6] T. Tokuda, M.M. Qureshi, M.T. Ardah, S. Varghese, S.A. Shehab, T. Kasai, N. Ishigami, A. Tamaoka, M. Nakagawa, O.M. El-Agnaf, Detection of elevated levels of α-synuclein oligomers in CSF from patients with Parkinson disease, Neurology 75 (2010) 1766–1772.
[7] O.M. El-Agnaf, S.A. Salem, K.E. Paleologou, M.D. Curran, M.J. Gibson, J.A. Court, M.G. Schlossmacher, D. Allsop, Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease, FASEB J. 20 (2006) 419–425.
[8] I. Devic, H. Hwang, J.S. Edgar, K. Izutsu, R. Presland, C. Pan, D.R. Goodlett, Y. Wang, J. Armaly, V. Tumas, C.P. Zabetian, J.B. Leverenz, M. Shi, J. Zhang, Salivary α-synuclein and DJ-1: potential biomarkers for Parkinson's disease, Brain 134 (2011) e178.
[9] O. Hansson, S. Hall, A. Ohrfelt, H. Zetterberg, K. Blennow, L. Minthon, K. Nägga, E. Londos, S. Varghese, N.K. Majbour, A. Al-Hayani, O.M. El-Agnaf, Levels of cerebrospinal fluid α-synuclein oligomers are increased in Parkinson's disease with dementia and dementia with Lewy bodies compared to Alzheimer's disease, Alzheimer's Res. Ther. 6 (2014) 25.
[10] M.S.M. Al-Nimer, S.F. Mshatat, H.I. Abdulla, Saliva α-synuclein and A high extinction coefficient protein: a novel approach in assessment biomarkers of Parkinson's disease, N. Am. J. Med. Sci. 6 (2014) 633–637.
[11] G. Vivacqua, A. Latorre, A. Suppa, M. Nardi, S. Pietracupa, R. Mancinelli, G. Fabbrini, C. Colosimo, E. Gaudio, A. Berardelli, Abnormal salivary total and oligomeric alpha-synuclein in Parkinson's disease, PLoS One 11 (2016) e0151156.
[12] W. Kang, W. Chen, Q. Yang, L. Zhang, L. Zhang, X. Wang, F. Dong, Y. Zhao, S. Chen, T.J. Quinn, J. Zhang, S. Chen, J. Liu, Salivary total α-synuclein, oligomeric α-synuclein and SNCA variants in Parkinson's disease patients, Sci. Rep. 6 (2016) 28143.
[13] Z. Hong, M. Shi, K.A. Chung, J.F. Quinn, E.R. Peskind, D. Galasko, J. Jankovic, C.P. Zabetian, J.B. Leverenz, G. Baird, T.J. Montine, A.M. Hancock, H. Hwang, C. Pan, J. Bradner, U.J. Kang, P.H. Jensen, J. Zhang, DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease, Brain 133 (2010) 713–726.
[14] K. Del Tredici, C.H. Hawkes, E. Ghebremedhin, H. Braak, Lewy pathology in the submandibular gland of individuals with incidental Lewy body disease and sporadic Parkinson's disease, Acta Neuropathol. 119 (2010) 703–713.
[15] M.P. Frosch, Tau aggregates: where, when, why and what consequences? Neuropathol. Appl. Neurobiol. 43 (2017) 371–372.
[16] R.B. Postuma, D. Berg, M. Stern, W. Poewe, C.W. Olanow, W. Oertel, J. Obeso, K. Marek, I. Litvan, A.E. Lang, G. Halliday, C.G. Goetz, T. Gasser, B. Dubois, P. Chan, B.R. Bloem, C.H. Adler, G. Deuschl, MDS clinical diagnostic criteria for Parkinson's disease, Mov. Disord. 30 (2015) 1591–1601.
[17] G.U. Höglinger, G. Respondek, M. Stamelou, C. Kurz, K.A. Josephs, A.E. Lang, B. Mollenhauer, U. Müller, C. Nilsson, J.L. Whitwell, T. Arzberger, E. Englund, E. Gelpi, A. Giese, D.J. Irwin, W.G. Meissner, A. Pantelyat, A. Rajput, J.C. van Swieten, C. Troakes, A. Antonini, K.P. Bhatia, Y. Bordelon, Y. Compta, J.C. Corvol, C. Colosimo, D.W. Dickson, R. Dodel, L. Ferguson, M. Grossman, J. Kassubek, F. Krismer, J. Levin, S. Lorenzl, H.R. Morris, P. Nestor, W.H. Oertel, W. Poewe, G. Rabinovici, J.B. Rowe, G.D. Schellenberg, K. Seppi, T. van Eimeren, G.K. Wenning, A.L. Boxer, L.I. Golbe, I. Litvan, Movement Disorder Society-endorsed PSP Study Group, Clinical diagnosis of progressive supranuclear palsy: the movement disorder society criteria, Mov. Disord. 32 (2017) 853–864.
[18] C.L. Tomlinson, R. Stowe, S. Patel, C. Rick, R. Gray, C.E. Clarke, Systematic review of levodopa dose equivalency reporting in Parkinson's disease, Mov. Disord. 25 (2010) 2649–2653.
[19] L.A. Volpicelli-Daley, K.C. Luk, V.M.-Y. Lee, Addition of exogenous α-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous α-synuclein to Lewy body and Lewy neurite-like aggregates, Nat. Protoc. 9 (2014) 2135–2146.
[20] S. Yu, X. Li, G. Liu, J. Han, C. Zhang, Y. Li, S. Xu, C. Liu, Y. Gao, H. Yang, K. Uéda, P. Chan, Extensive nuclear localization of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody, Neuroscience 145 (2007) 539–555.
[21] D.-P. Hong, S. Han, A.L. Fink, V.N. Uversky, Characterization of the non-fibrillarα-synuclein oligomers, Protein Pept. Lett. 18 (2011) 230–240.
[22] C. Peng, R.J. Gathagan, V.M.-Y. Lee, Distinct α-Synuclein strains and implications for heterogeneity among α-Synucleinopathies, Neurobiol. Dis. 109 (2018) 209–218.
[23] S.A. Grathwohl, J.A. Steiner, M. Britschgi, P. Brundin, Mind the gut: secretion of α-synuclein by enteric neurons, J. Neurochem. 125 (2013) 487–490.
[24] R. Carletti, F. Campo, M. Fusconi, C. Pellicano, M. De Vincentiis, F.E. Pontieri, C.R. Di Gioia, Phosphorylated α-synuclein immunoreactivity in nerve fibers from minor salivary glands in Parkinson's disease, Park. Relat. Disord. 38 (2017) 99–101.
[25] M. Xilouri, O.R. Brekk, L. Stefanis, α-Synuclein and protein degradation systems: a reciprocal relationship, Mol. Neurobiol. 47 (2013) 537–551.
[26] A.L. Boxer, J.T. Yu, L.I. Golbe, I. Litvan, A.E. Lang, G.U. Höglinger, Advances in progressive supranuclear palsy: new diagnostic criteria, biomarkers, and therapeutic approaches, Lancet Neurol. 16 (2017) 552–563.
[27] N.K. Magdalinou, R.W. Paterson, J.M. Schott, N.C. Fox, C. Mummery, K. Blennow, K. Bhatia, H.R. Morris, P. Giunti, T.T. Warner, R. de Silva, A.J. Lees, H. Zetterberg, A panel of nine cerebrospinal fluid biomarkers may identify patients with atypical parkinsonian syndromes, J. Neurol. Neurosurg. Psychiatry 86 (2015) 1240–1247.
[28] I. Rodríguez-Leyva, E.G. Chi-Ahumada, J. Carrizales, M. Rodríguez-Violante, S. Velázquez-Osuna, V. Medina-Mier, M.G. Martel-Gallegos, S. Zarazúa, L. Enríquez-Macías, A. Castro, A.L. Calderón-Garcidueñas, M.E. Jiménez-Capdeville, Parkinson disease and progressive supranuclear palsy: protein expression in skin, Ann.Clin. Transl. Neurol 3 (2016) 191–199.
[29] T. Guo, W. Noble, D.P. Hanger, Roles of tau protein in health and disease, Acta Neuropathol. 133 (2017) 665–704.
[30] S. Moussaud, D.R. Jones, E.L. Moussaud-Lamodière, M. Delenclos, O.A. Ross, P.J. McLean, Alpha-synuclein and tau: teammates in neurodegeneration? Mol. Neurodegener. 9 (2014) 43.