Oligomeric α -synuclein is increased in basal tears of Parkinson's patients

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Aim: Secretion of proteins into basal tears of Parkinson's disease (PD) patients may be altered by changes in nerve function. **Materials & methods:** Oligomeric α -Syn_{Oligo} and total α -Syn_{Total}, CCL-2, DJ-1, LF and MMP-9 were measured in basal tears from 93 PD patients and 82 age- and sex-equivalent healthy controls. **Results:** α -Syn_{Total} was decreased ($p = 0.0043$), whereas α -Syn_{Oligo} ($p < 0.0001$) and the ratio of α -Syn_{Oligo}/ α -Syn_{Total} ($p < 0.0001$) were increased in basal tears from PD patients compared with healthy controls. Area under receiver-operating curves of α -Syn_{Oligo} and α -Syn_{Oligo}/ α -Syn_{Total} contents were 0.70 (95% confidence limits: 0.621–0.774) and 0.72 (95% confidence limits: 0.642–0.792). **Conclusion:** PD patient basal tears may contain biomarkers that can be assayed noninvasively and inexpensively.

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Parkinson's disease (PD) affects approximately 1% of the population and is associated with loss of dopaminergic neurons [1] and accumulation of Lewy bodies in the *Substantia nigra* [2]. Lewy bodies are also found throughout the CNS and peripheral nervous system and in other organ systems in PD patients. These bodies are formed of aggregates of monomeric α -synuclein that assemble first into oligomers (α -Syn_{Oligo}), then fibrils. While implicated normally in neurotransmitter release and membrane remodeling [3], mutations and polymorphisms in the α -synuclein protein are associated with increased susceptibility to PD [4].

Gastrointestinal (GI) tract dysfunction in PD can occur prior to manifestation of classic motor deficits [5], and cases of Lewy body pathology in the myenteric plexus may precede a clinical diagnosis of PD by many years [6]. The GI tract is innervated by parasympathetic cholinergic neurons that may be particularly sensitive to the pathologic changes responsible for PD dopaminergic neuronal dysfunction.

The lacrimal gland is similarly responsive to stimulation by cholinergic neurons [7], and other neural inputs including adrenergic and dopaminergic pathways [8], stimulating the secretion of proteins into tears. PD-dependent alterations in cholinergic stimulation and/or in the trophic effects of cholinergic stimulation on lacrimal gland acinar cells function may lead to a characteristic tear protein profile in PD patients that can be measured cost-effectively and noninvasively. There is significant interest in the identification of biomarkers for PD that can be easily measured and provide indications of active disease prior to the manifestation of debilitating motor symptoms, when significant neuronal degeneration has already occurred [9].

Here, we investigated whether tear proteins in basal (or 'unstimulated' tears, obtained with topical anesthesia) in PD patients differ from those from healthy controls (HC). Proteins chosen for analysis included: total α -synuclein (α -Syn_{Total}), oligomeric α -synuclein (α -Syn_{Oligo}), CCL-2 and DJ-1/PARK-7. α -synuclein is the main abnormal CNS protein in the PD brain: specifically, we utilized an analysis of its relative abundance in the free (total) and oligomeric forms, which has been studied in other bodily fluids including cerebrospinal fluid (CSF), plasma and saliva [10–12]. CCL-2 is a chemokine that plays a crucial role in activation of inflammatory cells such as monocytes

and microglia and has been implicated as an effector of PD progression [13], possibly through the proinflammatory cytokine, TNF- α and other downstream effectors [14]. CCL-2 levels are reported to be elevated in CSF samples from PD patients [15]. DJ-1 is the second most commonly mutated protein in inherited forms of PD, and its loss of function is associated with PD pathogenesis [16]. DJ-1 may function as a redox-sensitive chaperone and function in preventing aggregation of α -synuclein into oligomers [17].

Two additional commonly studied tear proteins were included: MMP9 and LF. MMP9 is a protease elevated in patients with dry eye disease [18] and inflammatory ocular surface conditions [19], while LF is an antimicrobial protein produced by the tear-producing lacrimal gland [20] and is a marker for measuring its normal function.

Materials & methods

Standard protocol approvals, registrations & patient consents

All procedures were approved by the University of Southern California (USC's) Health Sciences Campus Institutional Review Board and performed in accordance with the Declaration of Helsinki. All subjects gave written informed consent for research and were provided with copies of their informed consents. The study is registered at clinical trials.gov with the identifier NCT03037463.

Study enrollment & clinical assessment

93 nondemented patients with a diagnosis of PD established for at least 6 months were recruited from the USC Movement Disorders Clinic in the Department of Neurology at the Keck School of Medicine of USC between May 2017 to May 2018. The diagnosis was made according to the UK Parkinson's Disease Society Brain Bank Diagnostic Criteria, based on clinical exam, the presence of asymmetric rigidity and bradykinesia with or without rest tremor and with positive response to medication [21]. 82 HC individuals were recruited in parallel, primarily from companions accompanying PD patients to the clinic, and USC employees. The initial estimate of study sample size was determined from published comparisons of average salivary α -synuclein levels between PD patients and HC [22,23], and our data comparing average LF levels in patients with Sjögren's syndrome, other autoimmune diseases and autoimmune-independent dry eye diseases [24], utilizing a two-sided α -level of 0.05 with 80% power. From these studies, the average effect size (mean difference between groups, divided by SD) was 0.76, yielding an initial sample size of 29 subjects per group (total sample size: 58). An intermediate evaluation of our initial data at the 50% recruitment point utilizing actual data values for our proteins of interest in PD and HC groups revealed possible sex differences and established a final recruitment target of at least 80 individuals in each group comprising approximately equal numbers of males and females. Recruitment of HC sought to represent the sex and age distribution of the PD sample. PD patients and HC were excluded from the study if they had an active eye infection, any major eye surgery in the last 3 months, or were currently taking amantadine or anticholinergic medication. Subjects wearing contact lenses participated in the study if they were able to remove contact lenses 15 min prior to the Schirmer's test measurement. The Montreal Cognitive Assessment (MoCA) was given to all subjects. Demented PD patients were excluded. PD patients and HC were also given an ocular surface disease index survey (OSDI) to assess symptoms related to dry eye and their effects on vision.

In addition to the MoCA and OSDI assessments, additional clinical assessments of PD patients included administration of the Unified Parkinson's Disease Rating Scale (UPDRS), Hoehn and Yahr scale and the Parkinson's Disease Questionnaire-39 (PDQ-39). PD patient demographics, clinical assessment measures and the HC demographics are shown in Table 1.

Tear sample collection & preparation

Fluid was removed from the conjunctival fornix. Subjects' eyes were anesthetized by one drop of 0.5% proparacaine hydrochloride (Bauch & Lomb, NY, USA) to minimize stimulated tear secretion during collection, thereby representing the collection of basal tears. Prior to collection, excess fluid was carefully removed with a cotton swab and a Schirmer's strip was gently placed on the lower lid margin of each eye. The strips were removed after 5 min and the length of wetting (in millimeters) was recorded. This test is referred to as an anesthetized Schirmer's test. The strips collected from both eyes were pooled in microcentrifuge tubes and stored at -80°C . Tear samples were analyzed within 2 weeks of sample collection, at which time minimal degradation of proteins of interest occurred (see Results section for details). For elution of tear proteins from Schirmer's strips, 280 μl of phosphate-buffered saline containing protease inhibitor cocktail was added to the tubes, which were centrifuged at $10,000 \times g$ for 10 s in an Eppendorf minispin centrifuge (Hamburg, Germany).

Table 1. Demographic and clinical characteristics of Parkinson's disease patients and healthy controls.

Characteristic	Healthy controls (n = 82)	PD patients (n = 93)	p-value
Age (years)			
Median (IQR)	69 (60–75)	71 (64.5–76)	0.08 [†]
Sex, n (%)			
			0.30 ^{‡,§}
Men	43 (52)	56 (60)	0.19 [‡]
Women	39 (48)	37 (40)	0.81 [‡]
Ethnicity, n (%)			
			0.36 ^{‡,¶}
Hispanic	11 (13)	10 (11)	
Race, n (%)			
White	62 (76)	63 (68)	
Asian	8 (10)	5 (5)	
Black	1 (1)	1 (1)	
Other	11 (13)	24 (26)	
MoCA			
n	78	90	
Median (IQR)	27.00 (25–29)	26.00 (24–28)	0.0013 [†]
UPDRS I/II			
n	NA	87	
Median (IQR)	NA	12 (7–17)	
UPDRS III			
n	NA	87	
Median (IQR)	NA	28 (21–34)	
PDQ-39			
n	NA	90	
Median (IQR)	NA	26 (11–44.25)	
Hoehn and Yahr scale			
n	NA	93	
Median (IQR)	NA	2 (2–2)	
OSDI			
n	79	88	
Median (IQR)	2.27 (0.0–6.81)	4.36 (0–12.22)	0.20 [†]
Years with PD			
n	NA	93	
Median (IQR)	NA	4.15 (2.21–6.19)	

[†]p-values by Wilcoxon rank sum test for continuous variables.

[‡] χ^2 test for categorical variables.

[§]Represents difference in sex distribution between groups.

[¶]Represents difference in ethnicity between groups.

IQR: Interquartile range; MoCA: Montreal cognitive assessment; NA: Not available; OSDI: Ocular surface disease index; PD: Parkinson's disease; PDQ-39: Parkinson's Disease Questionnaire-39; UPDRS: Unified Parkinson's disease rating scale.

Measurement of total protein concentration

Total protein concentration in tear samples was measured using the bicinchoninic acid assay (ThermoFisher Scientific, NY, USA). Bovine serum albumin was used to produce a standard curve, and the assay was performed according to the manufacturer's instruction.

Tear protein quantification using single & multiplex ELISA

LF and α -Syn^{Oligo} in eluted tears were measured using the lactoferrin (HLF2) human ELISA Kit (ab108882, Abcam, MA, USA) and the human α -synuclein oligomeric ELISA kit (MBS043824, MyBiosource.com, CA, USA), respectively, according to the manufacturers' protocols. Samples were analyzed using a Spectramax iD3 Multi-Mode Microplate Reader (Molecular Devices, CA, USA). CCL-2, DJ-1, MMP9 and α -Syn^{Total} were quantified using a Human Magnetic Luminex Assay, a bead-based multiplex ELISA kit (LXSAHM; R&D Systems, MN, USA).

Figure 1. Stability of measured proteins at -80°C in tears from Parkinson's disease patients and healthy controls. The stability of the indicated tear proteins against proteolytic degradation was tested in tear samples stored 'dry' on Schirmer's strips (e.g., without eluting proteins with elution buffer). After storing the strips for 1 and 2 weeks at -80°C, all specific proteins of interest were incubated with elution buffer and assayed with the respective ELISA assay. Total protein (BCA) was measured with the BCA protein assay. Data are represented as percentages of the concentration measured on day 0 (mean ± standard error mean), $n = 5$ for all proteins at 1 week and $n = 8$ for all proteins at 2 weeks. BCA: Bicinchoninic acid.

Samples were incubated at room temperature for 4 h on an orbital shaker at 75 rpm before performing multiplex ELISA. Data from the multiplex ELISA were acquired on a validated and calibrated Bio-Plex Suspension Array 200 system (MAPTM Technology, TX, USA) and analyzed using Bio-Plex Manager 6.1 software. Individual protein values from each ELISA assay were normalized to total protein concentration of the tears for comparison across samples.

Statistical analysis

Demographic comparisons between PD and HC participants used Wilcoxon rank sum and χ^2 test. As most putative biomarkers were highly skewed, measures of central tendency and variability used medians and interquartile range (IQR). Group comparisons (PD/HC, sex) on biomarkers were made with nonparametric Wilcoxon rank sum tests. A separate logistic regression was run for each biomarker, with study group (PD vs HC) as the dependent variable; a receiver-operating characteristic (ROC) curve with area under the ROC curve and 95% confidence limits were calculated. As PD and HC groups were equivalent on major demographic variables, these group comparisons were not adjusted for possible demographic confounders. Due to sex differences in some biomarker levels, PD/HC comparisons were also completed in sex subgroups. Biomarker data were available on all recruited participants.

Results

Study sample characteristics

575 subjects were screened for eligibility, 89 declined participation (usually due to time restrictions or sensitive eyes) and 311 were excluded based on study criteria. Of those subjects excluded, more than 90% were either on amantadine (~30% of the excluded population), had recent eye surgery or an eye condition or failed to meet other defined study criteria. As summarized in Table 1, 82 HC and 93 diagnosed, nondemented PD patients, males and females, participated in the study, with median (IQR) ages of 69 (60–75) and 71 (64.5–76), respectively ($p = 0.08$). Both groups included slightly more men than women ($p = 0.30$), while the ethnicity of both groups was primarily white ($p = 0.36$). Median MoCA scores were 27 (25–29) and 26 (24–28) in HC and PD patients, respectively; these averages were significantly different at $p = 0.0013$, although neither mean indicated significant cognitive impairment [25]. The median Hoehn and Yahr score for PD patients enrolled in the study was 2, suggestive of mild-to-moderate motor impairment [26]. OSDI values (a subjective index of patient dry eye discomfort, with scores ranging from 0 to 100 points) fell in the normal range (0–12) for both groups, relative to values indicative of mild (13–31), moderate (23–32) or severe (33–100) eye discomfort [27], with no differences between groups ($p = 0.20$).

The presence of CCL-2, DJ-1, LF, MMP9, α -Syn_{Total} and α -Syn_{Oligo} were confirmed in HC control tears, while estimates of the concentration ranges of each enabled determination of the optimal formats for measurement to maximize utilization of the tear sample, for example, single ELISA (LF, α -Syn_{Oligo}) versus multiplex ELISA (CCL-2, DJ-1, MMP9, α -Syn_{Total}). The most abundant of these proteins per mg of total tear protein was LF, while the least abundant was CCL-2. The stability of CCL-2, DJ-1, LF, MMP9, α -Syn_{Total}, α -Syn_{Oligo} and total protein on Schirmer's strips collected under topical anesthesia (i.e., basal tears) from PD patients and HC was measured in the same samples on the day of collection (day 0) and after 1 or 2 weeks of storage at -80°C. The stability of each marker, relative to the amount on day 0, was assessed at 1 and 2 weeks, with no significant loss of signal detected for any protein of interest (Figure 1). Therefore, all samples were stored dry on Schirmer's strips at -80°C until

Figure 2. α -Synuclein and receiver-operating characteristic curves in basal tears of Parkinson's disease and healthy control in the total population. (A) $\alpha\text{-Syn}_{\text{Total}}$ is decreased in tears of PD patients compared with HC; (B) $\alpha\text{-Syn}_{\text{Oligo}}$ is increased in tears of PD patients compared with HC and (C) the ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ is increased in tears of PD patients ($n = 93$) versus HC ($n = 82$). Protein of interest values were normalized to total tear protein and transformed to a natural log scale. The transverse line indicates the median, boxes the 25th–75th percentile and the whiskers the 5th and 95th percentiles, while the additional points are outliers. Wilcoxon rank sum test was used to analyze the difference between groups. (D) Area under the receiver-operating characteristic (ROC) curve for $\alpha\text{-Syn}_{\text{Total}}$ levels in tears of PD patients versus tears of HC. (E) Area under the ROC for $\alpha\text{-Syn}_{\text{Oligo}}$ levels in PD patient tears versus HC. (F) Area under the ROC for ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ in PD patients versus to HC.

$\alpha\text{-Syn}_{\text{Oligo}}$: Oligomeric α -synuclein; $\alpha\text{-Syn}_{\text{Total}}$: Total α -synuclein; AUC: Area under the curve; HC: Healthy control; PD: Parkinson's disease.

analysis was performed within 2 weeks of collection.

$\alpha\text{-Syn}_{\text{Total}}$ was decreased, whereas $\alpha\text{-Syn}_{\text{Oligo}}$ & the ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ were increased in basal tears from all PD patients when compared with all HCs

$\alpha\text{-Syn}_{\text{Total}}$ levels are thought to reflect levels of monomeric α -synuclein, since epitopes for other forms of the protein in this assay may be masked as α -synuclein assembles into oligomers [10]. $\alpha\text{-Syn}_{\text{Total}}$ was significantly decreased in tears of PD patients compared with HC subjects ($p = 0.004$; Figure 2A & Table 2). In contrast, $\alpha\text{-Syn}_{\text{Oligo}}$ levels in tears were significantly elevated in PD patients relative to HC subjects ($p < 0.0001$; Figure 2B & Table 2). The ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$, determined within each patient prior to averaging, was significantly higher in PD patients compared with ratios in HC ($p < 0.0001$; Figure 2C & Table 2). All concentrations shown are presented as values normalized to the total tear protein in the sample. The area under the ROC curve (95% CI), a measure of the sensitivity and specificity for putative tear biomarkers in patients compared with HC, revealed values of 0.63 (0.542–0.708; $p = 0.045$; Figure 2D & Table 2) for $\alpha\text{-Syn}_{\text{Total}}$, 0.70 (0.621–0.774; $p < 0.0001$;

Table 2. Tear protein comparisons and area under the receiver-operating characteristic in Parkinson's disease patients versus healthy control.

Biomarker	Healthy control (n = 82); median (IQR)	PD patients (n = 93); median (IQR)	p-value [†]	AUROC (95% CI)	p-value [‡]
Total protein concentration (mg/ml)	2.61 (1.85–4.00)	3.30 (1.92–4.62)	0.32	0.544 (0.458–0.630)	0.32
CCL-2 (pg/mg)	108.38 (63.26–231.51)	129.56 (58.26–372.23)	0.36	0.540 (0.454–0.626)	0.28
DJ-1 (ng/mg)	73.80 (48.6–116.46)	91.18 (62.46–143.55)	0.054	0.585 (0.500–0.670)	0.38
MMP-9 (ng/mg)	12.32 (3.94–30.94)	15.03 (7.29–34.14)	0.24	0.552 (0.466–0.638)	0.30
Lactoferrin (μg/mg)	156.54 (71.48–300.53)	158.38 (87.86–287.07)	0.93	0.504 (0.417–0.591)	0.89
α-synuclein:					
– Total (pg/mg)	361.16 (234.31–731.39)	254.54 (151.05–479.69)	0.004	0.625 (0.542–0.708)	0.045
– Oligomeric (ng/mg)	0.44 (0.05–1.17)	1.68 (0.32–3.82)	<0.0001	0.698 (0.621–0.774)	<0.0001
– Oligomeric/total	0.96 (0.16–3.76)	4.40 (1.27–24.70)	<0.0001	0.717 (0.642–0.792)	0.001
Schirmer's (mm) [§]	19.0 (12.0–30.0)	18.0 (12.0–28.0)	0.99	0.499 (0.413–0.585)	0.75

[†]p-value was measured by Wilcoxon rank sum test.
[‡]Logistic regression p-value.
[§]Schirmer's values are the sums of wetting length in mm of strips from both eyes.
AUROC: Area under the receiver-operating characteristic curve; HC: Healthy control; IQR: Interquartile range; PD: Parkinson's disease.

Figure 2E & Table 2) for α -Syn_{Oligo} and 0.72 (0.642–0.792; $p < 0.001$; Figure 2F & Table 2) for the ratio of α -Syn_{Oligo}/ α -Syn_{Total}.

CCL-2, DJ-1, LF, MMP9 & total protein values in basal tears showed no changes in PD patients relative to HC, & basal tear flow was unaffected by PD

Table 2 shows that the concentrations of CCL-2, DJ-1, LF and MMP9 in all PD patient basal tears were not statistically significantly different relative to HC basal tears. DJ-1 was slightly elevated in PD patients and approached statistical significance ($p = 0.054$). The total protein concentration of the PD patient basal tears also did not significantly differ from that of HC, nor did basal tear flow, measured in mm of wetting of the Schirmer's strip summed from the right and left eyes (Table 2), although Schirmer's values in both populations tended to the dry side (<10 mm/eye is considered an indicator of dry eye disease) [28], which was expected based on the average age of the study population.

α -Syn_{Total} & α -Syn_{Oligo} values differ in male & female PD patients

Sex differences in PD patients and HC populations were also evaluated with respect to the levels of these basal tear proteins. In Table 2, we show that α -Syn_{Oligo} is significantly elevated in the total PD population (male and female) relative to all HCs. Analysis of levels by sex reveals that α -Syn_{Oligo} levels were significantly elevated in male relative to female PD patients ($p = 0.008$), contributing also to a significantly elevated ratio of α -Syn_{Oligo}/ α -Syn_{Total} in male versus female PD patients ($p = 0.017$; Table 3). No other significant sex differences were seen in individual basal tear proteins of interest, total protein concentration or other measures such as Schirmer's test values in PD or HC groups (Table 3).

α -Syn_{Oligo} & α -Syn_{Oligo}/ α -Syn_{Total} changes are more strongly correlated with disease in male PD patients, while DJ-1 is decreased in basal tears only in male PD patients

Further PD to HC comparisons by sex were prompted by the significant increase in α -Syn_{Oligo} noted in male versus female PD patients in Table 3. As shown in Table 4, the group difference of the medians for α -Syn_{Total}, α -Syn_{Oligo} and the α -Syn_{Oligo}/ α -Syn_{Total} ratio between PD patients and HC is stronger in males than in females. For α -Syn_{Total}, the group difference (HC to PD) is 130.1 for males and 64.16 for females, in comparison with 106.62 for the total population (males and females). For α -Syn_{Oligo}, the group difference (PD to HC) was 1.76 for males and 0.41 for females, compared with 1.24 in the total population (males and females). Similarly, the group difference for the α -Syn_{Oligo}/ α -Syn_{Total} in males was 4.52, 1.95 in females and 3.44 in the total population (males and females).

DJ-1 was significantly elevated in tears of male PD patients relative to tears of male HC ($p = 0.01$, Table 4); no differences in DJ-1 were seen in tears of female PD patients relative to female HC ($p = 0.99$).

Table 3. Tear protein comparisons between males and females in healthy control and Parkinson's disease patients.

Biomarker	Healthy controls			PD patients		
	Female HC (n = 39); median (IQR)	Male HC (n = 43); median (IQR)	p-value [†]	Female (n = 37); median (IQR)	Male (n = 56); median (IQR)	p-value [†]
Total protein concentration (mg/ml)	2.17 (1.80–3.67)	3.05 (2.03–4.10)	0.085	3.44 (2.00–4.79)	3.23 (1.74–4.34)	0.80
CCL-2 (pg/mg)	104.87 (68.96–226.10)	111.90 (39.51–344.48)	0.84	90.44 (49.04–283.67)	139.85 (76.44–396.51)	0.12
DJ-1 (ng/mg)	89.98 (59.51–125.48)	66.29 (44.15–113.32)	0.11	83.06 (55.66–184.23)	99.25 (69.64–132.73)	0.71
MMP-9 (ng/mg)	11.31 (2.50–25.68)	13.54 (5.86–38.21)	0.11	11.54 (3.77–30.94)	16.50 (10.40–34.64)	0.15
Lactoferrin (μg/mg)	170.90 (55.79–343.05)	154.23 (85.85–299.81)	0.66	157.17 (67.29–222.24)	160.93 (92.82–319.30)	0.39
α -synuclein:						
– Total (pg/mg)	361.30 (272.70–762.55)	361.03 (191.12–622.28)	0.45	297.14 (153.19–479.69)	230.92 (142.28–460.65)	0.74
– Oligomeric (ng/mg)	0.35 (0.05–0.99)	0.53 (0.05–1.88)	0.32	0.76 (0.05–2.19)	2.29 (0.73–4.62)	0.008
– Oligomeric/total	0.88 (0.16–2.66)	1.41 (0.15–5.82)	0.25	2.83 (0.42–9.72)	5.93 (3.08–27.57)	0.017
Schirmer's (mm) [‡]	17.0 (12.0–26.0)	20.0 (11.0–32.0)	0.61	13.0 (9.0–29.0)	19.0 (14.0–26.5)	0.19

[†]p-value by Wilcoxon rank sum test.
[‡]Schirmer's values are the sums of wetting length in mm of strips from both eyes.
 HC: Healthy control; IQR: Interquartile range; PD: Parkinson's disease; ROC: Receiver-operating characteristic.

Table 4. Tear protein comparisons in male and female Parkinson's disease patients versus healthy control.

Biomarker	Males			Females		
	Male HC (n = 43); median (IQR)	Male PD (n = 56); median (IQR)	p-value [†]	Female HC (n = 39); median (IQR)	Female PD (n = 37); median (IQR)	p-value [†]
Total protein concentration (mg/ml)	3.05 (2.03–4.10)	3.23 (1.74–4.34)	0.85	2.17 (1.80–3.67)	3.44 (2.00–4.79)	0.12
CCL-2 (pg/mg)	111.90 (39.51–344.48)	139.85 (76.44–393.51)	0.17	104.87 (68.96–226.10)	90.44 (49.04–283.67)	0.63
DJ-1 (ng/mg)	66.29 (44.15–113.32)	99.25 (69.64–132.73)	0.01	89.98 (59.51–125.48)	83.06 (55.66–184.23)	0.99
MMP-9 (ng/mg)	13.54 (5.86–38.21)	16.50 (10.40–34.64)	0.48	11.31 (2.50–25.68)	11.54 (3.77–30.94)	0.50
Lactoferrin (μg/mg)	154.23 (85.85–299.81)	160.93 (92.82–319.30)	0.77	170.90 (55.79–343.05)	157.17 (67.29–222.24)	0.70
α -synuclein:						
– Total (pg/mg)	361.03 (191.12–622.28)	230.92 (142.28–460.65)	0.05	361.30 (272.70–762.55)	297.14 (153.19–479.69)	0.07
– Oligomeric (ng/mg)	0.53 (0.05–1.88)	2.29 (0.73–4.62)	<0.0001	0.35 (0.05–0.99)	0.76 (0.05–2.19)	0.051
– Oligomeric/total	1.41 (0.15–5.82)	5.93 (3.08–27.57)	<0.0001	0.88 (0.16–2.66)	2.83 (0.42–9.72)	0.01
Schirmer's (mm) [‡]	20.0 (11.0–32.0)	19.0 (15.0–26.5)	0.86	17.0 (12.0–26.0)	13.0 (9.0–29.0)	0.46

[†]p-value by Wilcoxon rank sum test.
[‡]Schirmer's values are the sums of wetting length in mm of strips from both eyes.
 HC: Healthy control; IQR: Interquartile range; PD: Parkinson's disease; ROC: Receiver-operating characteristic.

Analysis of area under the ROC values for male and female PD patients relative to their respective controls reveals that these sex-specific changes contribute to stronger associations for α -Syn_{Oligo} and α -Syn_{Oligo}/ α -Syn_{Total} ratios in males (Figure 3) relative to females (not shown), and relative to the total population.

Discussion

Here, we report on the use of a routine clinical and noninvasive ocular test, an anesthetized Schirmer's test, to collect basal tear fluid for measurement of a panel of proteins of interest from the collected tears utilizing single and multiplex ELISA assays. We find a significant difference in PD patient α -Syn_{Oligo} and α -Syn_{Total} levels relative to HC, and in their ratios (α -Syn_{Oligo}/ α -Syn_{Total}), such that these values may be able to distinguish PD patient tears from those of HC in a mixed population of male and female PD patients. These proteins may, therefore, represent potential biomarkers for PD. Our study is the first report of the identification of α -Syn in basal tears, in both its monomeric and oligomeric forms, and represents one of the first reports validating the use of tears as a source of putative PD biomarkers.

Due to the noninvasive nature of tear collection and the relatively simple composition of tears in contrast to serum and plasma, interest in the use of tears for biomarker discovery and for diagnostic purposes has been increasing [29]. The utilization of tear biomarkers is best studied in ocular surface diseases such as dry eye [30] and

Figure 3. α -Synuclein in basal tears and receiver-operating characteristic curves in male Parkinson's disease and healthy control. **(A)** $\alpha\text{-Syn}_{\text{Oligo}}$ is increased in tears of male PD patients compared with HC and **(B)** the ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ is increased in tears of male PD patients ($n = 56$) versus male HC ($n = 43$). Protein of interest values were normalized to total tear protein and transformed to a natural log scale. The transverse line indicates the median, boxes the 25th–75th percentile and the whiskers the 5th and 95th percentiles, while the additional points are outliers. Wilcoxon rank sum test was used to analyze the difference between groups. **(C)** Area under the receiver-operating characteristic for $\alpha\text{-Syn}_{\text{Oligo}}$ levels in male PD patient tears versus male HC. **(D)** Area under the receiver-operating characteristic for ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ in male PD patients versus male HC. $\alpha\text{-Syn}_{\text{Oligo}}$: Oligomeric α -synuclein; $\alpha\text{-Syn}_{\text{Total}}$: Total α -synuclein; HC: Healthy control; PD: Parkinson's disease.

Sjögren's syndrome [24]. Two studies have analyzed specific tear protein changes in PD, one reporting increased TNF- α in tears of PD patients relative to controls [31]. However, as TNF- α is also increased in tears of dry eye patients [30], this increase may be due to the dry eye previously reported in PD [32], and any observed changes in TNF- α may not necessarily be unique to the PD population. A second recent study reported the tear fluid proteome in PD patients when compared with neurologically healthy controls in a small cohort, reporting possible biomarkers that need to be validated in a larger cohort [33].

However, previous studies have explored the utility of diverse proteins, including $\alpha\text{-Syn}_{\text{Oligo}}$ and $\alpha\text{-Syn}_{\text{Total}}$, as potential biomarkers of PD in other tissues and biofluids including serum, plasma, CSF, saliva, skin and salivary gland [10–12,34,35]. In CSF, skin and salivary gland, biomarker identification efforts are complicated by invasive and/or complex collection techniques. CSF collection has been contaminated with blood products, while blood and serum are biochemically complex mixtures.

Saliva, like tear fluid, is secreted via innervated exocrine glands. Salivary $\alpha\text{-Syn}_{\text{Total}}$ levels also have been reported to be decreased in PD patients [22,23]. Moreover, $\alpha\text{-Syn}_{\text{Oligo}}$ levels and the ratio of $\alpha\text{-Syn}_{\text{Oligo}}/\alpha\text{-Syn}_{\text{Total}}$ in saliva were increased in PD patients when compared with HC in a larger study cohort [10], similar to our findings. However, in that study the decreased $\alpha\text{-Syn}_{\text{Total}}$ levels in saliva in their total PD population (males and females) was sixfold, a stronger effect compared with the approximately 30% decrease that we observed in tears from our total PD patient sample. On the other hand, our strongest tear biomarker, $\alpha\text{-Syn}_{\text{Oligo}}$, which was increased fourfold in our total PD sample, was only twofold increase in PD patient saliva. A similar ELISA assay was utilized in both studies for measurement of $\alpha\text{-Syn}_{\text{Oligo}}$. Similar to our study, salivary levels of DJ-1 (PARK-7) have also been evaluated. However, its reported levels and its use as a biomarker have been inconsistent across several studies [23,36–38]. The consistency of DJ-1 in tears serving as a biomarker remains to be evaluated further.

Tears and saliva are not identical mucosal fluids. Saliva is produced by multiple glands (parotid, submandibular, sublingual) with varied composition since some glands produce a primarily serous (proteinaceous and fluid) output while others produce primarily mucous output. The lacrimal gland, a histologically homogeneous serous gland, produces a primarily proteinaceous output. Tears are also not complicated by the complexity of oral bacteria in saliva associated with oral hygiene, although tears do possess proteolytic activity, similar to saliva. In addition, the total protein concentration of saliva is approximately tenfold lower than that in tears [39,40]. Altogether these properties suggest that tears may be a viable alternative to saliva as a biofluid source.

We found marked differences in the levels of DJ-1, α -Syn_{Total} and α -Syn_{Oligo} in male and female PD patients relative to their respective HC. Several studies have shown that PD is 1.5- to 2-times more prevalent in males [41]. Males are much younger at the time of diagnosis [42] and have more frequent disease symptoms than females over the disease duration [43]. Some differences between males versus females in PD-related gene and protein expression levels in plasma, neurons and urine have been reported in previous studies [44–47]. In fact, in our study, basal tear α -Syn_{Oligo} was more strongly correlated with PD in male than in female patients. DJ-1 was only significantly correlated with disease in male PD patients, but not in our total PD population nor in female PD patients.

These sex differences may be due to the documented protective role of sex hormones against neurodegeneration [43]. Sex hormones can also affect the synthesis and secretion of tear proteins from the lacrimal gland that are involved in mucosal defense, such as secretory component and secretory IgA [48]. Interestingly, lacrimal gland dysfunction, such as dry eye and loss of aqueous tear production, disproportionately affects women [49], particularly after menopause. While we saw no significant difference in tear flow nor in tear protein content between any of the groups, it is possible that a selective loss in specific lacrimal gland secretory capacity in females with aging, which may be retained in males, can lead to a reduced ability to secrete pathological PD proteins that accumulate in disease. Conversely, these proteins may be present in higher abundance in male PD patients due to a sex-specific feature of PD.

A limitation of our study is that it was conducted at a single-site academic tertiary referral center. This may result in the representation of a more motivated or educated group of patients than seen in the general population. The PD patient group studied here may also have a different socioeconomic status than the US PD population in general. There is also a limited representation of more severe PD cases in our cohort including those with Hoehn and Yahr scores of III, IV and V, so a more comprehensive correlation of α -Syn_{Oligo} with disease severity could not be performed. Therefore, it still remains to be determined whether levels of these biomarkers increase with increasing severity of disease and/or decrease with the severity of disease.

As clinical trials are now underway to evaluate potential therapies to decrease the burden of α -synuclein in the brain of PD patients, a true biomarker to follow levels prior to, during and following these interventions would be extremely useful. Abnormally high level of α -Syn_{Oligo} in biological fluids such as tears may ideally also precede clinical manifestation of the disease and in this way could be used in a diagnostic fashion in asymptomatic patients. Comparing and/or differentiating results to populations of patients with atypical Parkinsonian syndromes such as multiple system atrophy and progressive supranuclear palsy, would additionally be of interest.

While we recruited healthy controls for equivalence on age and gender to our PD case sample, residual unmeasured confounders related to biomarker levels differing between PD and HC may be present. While there are no obvious selection biases involving sample selection related to biomarker levels in either PD or HC, HCs were a sample of convenience rather than a random sample from the general population.

Conclusion

This study supports the potential for the use of basal tears as a source of potential biomarkers of PD, enabling relatively rapid, inexpensive and noninvasive detection of proteins of interest that correlate with disease status.

Future perspective

We anticipate expanding our study to multiple sites to include greater diversity in our PD and HC patients, both ethnically and socioeconomically. We will also include patients with PD of greater and lesser disease severity, and those with other related conditions including synucleinopathies and tauopathies. While tears appear a viable and possibly preferable source of α -Syn_{Oligo} to saliva, improving the sensitivity of detection would enable us to understand how levels of α -Syn_{Oligo} normally vary in HC, since they were below the current threshold of detection in many HC individuals in the ELISA assay. We will explore alternative assays for measurement of this protein

form. Finally, the origin of the sex-specific differences in the tear film will be investigated from the perspective of patient hormonal status.

Summary points

- Tears can be collected atraumatically from Parkinson's disease (PD) patients and contain proteins implicated in the etiology of PD.
- Tear proteins can be analyzed simply in an ELISA format.
- Total α -synuclein (α -Syn_{Total}) is decreased, whereas oligomeric α -synuclein (α -Syn_{Oligo}) and the ratio of α -Syn_{Oligo}/ α -Syn_{Total} are increased in basal (anesthetized) tears from PD patients compared with tears of healthy controls.
- α -Syn_{Oligo} increases are more robust in basal tears in male PD patients relative to basal tears of male healthy controls, versus in basal tears of female PD patients relative to basal tears of female healthy controls.
- DJ-1 is increased only in basal tears of male PD patients relative to basal tears of male healthy controls.

Author contributions

SF Hamm-Alvarez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. SF Hamm-Alvarez, CT Okamoto, SR Janga, MC Edman and MF Lew contributed to the study conception and design. SR Janga, D Freire, D Feigenbaum, M Shah, R Ghanshani and MF Lew acquired the data. SF Hamm-Alvarez, CT Okamoto, SR Janga, D Feigenbaum, MC Edman, D Freire, M Shah, WJ Mack and MF Lew performed the data analysis and interpretation. Statistical analysis is performed by WJ Mack. Manuscript draft preparation is done by SF Hamm-Alvarez, CT Okamoto, SR Janga, MC Edman and MF Lew. SF Hamm-Alvarez, CT Okamoto, SR Janga, D Feigenbaum, MC Edman, D Freire, M Shah, R Ghanshani, WJ Mack and MF Lew revised final manuscript for scientific content.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All procedures were approved by the University of Southern California (USC's) Health Sciences Campus Institutional Review Board and performed in accordance with the Declaration of Helsinki. All subjects gave written informed consent for research and were provided with copies of their informed consents. The study is registered at clinicaltrials.gov with the identifier NCT03037463.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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