

# Saliva $\alpha$ -Synuclein and A High Extinction Coefficient Protein: A Novel Approach in Assessment Biomarkers of Parkinson's Disease

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## Abstract

**Background:** The pathological hallmark of Parkinson's disease (PD) is the appearance of intracytoplasmic inclusions known as Lewy bodies in which its principal component is  $\alpha$ -synuclein. **Aim:** This study aimed to determine salivary  $\alpha$ -synuclein and the extinction coefficient of the saliva protein as biomarkers of PD. **Materials and Methods:** This observational study was done in Department of Pharmacology, College of Medicine in cooperation with Department of Oral Medicine, College of Dentistry at Al-Mustansiriya University in Baghdad, Iraq from September 2013 to March 2014. A total number of 20 PD patients and 20 healthy subjects were enrolled in the study. Unstimulated saliva obtained from each participant obtained for determination of salivary flow rate, saliva protein and  $\alpha$ -synuclein using enzyme linked immune sorbent assay (ELISA) technique. **Results:** Total saliva protein and uncontaminated protein with nucleic acids are significantly higher in PD compared with healthy subjects. The mean extinction coefficient of that protein is  $27.25 \text{ M.cm}^{-1}$  which significantly ( $P < 0.001$ ) less than corresponding value of healthy subjects ( $33.48 \text{ M.cm}^{-1}$ ). Saliva  $\alpha$ -synuclein level is significantly less in PD ( $65 \pm 52.2 \text{ pg/ml}$ ) than healthy subjects ( $314.01 \pm 435.9 \text{ pg/ml}$ ). **Conclusions:** We conclude that saliva  $\alpha$ -synuclein serves as a biomarker for PD if its level compared with healthy subjects, and a specific protein with extinction coefficient  $27.25 \text{ M.cm}^{-1}$  is detected in saliva of Parkinson's patients.

**Keywords:** Parkinson's disease, saliva,  $\alpha$ -synuclein

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## Introduction

Parkinson's disease (PD) is a chronic progressive neurodegenerative condition that recognized as a movement disorder. The physical signs of PD are resting tremor, rigidity on passive movement, akinesia (bradykinesia and hypokinesia), and postural instability.<sup>[1]</sup> The pathological hallmark of PD is degeneration of dopaminergic neurons that located in the substantia nigra pars compacta in the brain and the appearance of intracytoplasmic inclusions known as Lewy bodies.<sup>[2]</sup>

The principal component of Lewy bodies is  $\alpha$ -synuclein that expressed in the neocortex, hippocampus, substantia nigra, thalamus, and cerebellum.<sup>[3]</sup>  $\alpha$ -synuclein is a small (140 amino acid; 14 kDa) highly acidic, heat stable protein that is soluble and natively "unfolded." It tended to polymerize into fibrils, and to accumulate in pathologic inclusions, such as Lewy bodies, Lewy neuritis, and glial cytoplasmic inclusions.<sup>[4-6]</sup>  $\alpha$ -synuclein filaments are observed in a spectrum of neurodegenerative diseases termed "synucleinopathies" and predominantly expressed in neurons of the central nervous system (CNS), where it localized to presynaptic terminals in close proximity to synaptic vesicles. A low level of  $\alpha$ -synuclein in the cerebrospinal fluid is considered as a good biomarker for diagnosis of PD.<sup>[7]</sup>

In PD, hyposalivorrhea is an early autonomic manifestation and a significant reduction of both basal ( $0.0964 \pm 0.08 \text{ ml/min}$ ) and stimulated

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(0.263  $\pm$  0.213 ml/min) saliva flow rate observed in patients presented with motor symptoms for less than one year.<sup>[8]</sup>

Devic *et al.*<sup>[9]</sup> demonstrated in the supernatant of the whole saliva anti- $\alpha$ -Synuclein antibody as a distinct band at 15 kDa, and the anti-DJ-1 antibody protein as a two bands around 25 kDa. The rationale of doing this study based on the evidence of the presence of  $\alpha$ -synuclein in the autonomic nerve fibers that supplied the salivary glands and the involvement of salivary glands by synucleinopathy in the early stage of PD.<sup>[10,11]</sup> Add to this, the saliva is free from blood contamination that make it the ideal biofluid for detection this marker.

Therefore, the estimation of saliva  $\alpha$ -synuclein level can substitute its determination in the cerebrospinal fluid. The aims of this study are to use the saliva as a biological fluid in determination the  $\alpha$ -synuclein as a biomarker for PD and to identify the extinction coefficient of the saliva protein in patients with PD by using a simple procedure.

## Materials and Methods

This study was done in Department of Pharmacology, College of Medicine in cooperation with Department of Oral Medicine, College of Dentistry, Al-Mustansiriya University in Baghdad, Iraq from September 2013 to April 2014. The study was approved by the Institutional Scientific Committee and a consent form was obtained from each participant enrolled in study. The patients were allocated from Baghdad Teaching Hospital and the Public Clinics. Patients who eligible for this study were cases of PD treated with anti-Parkinson's medications, both genders and their ages ranged from 40-75 years. The criteria of inclusion were PD (primary, familial, and sporadic) of different duration of illnesses, treated with dopamine agonists and/or anticholinergic agents.

Healthy subjects of both genders were also included in the study served as control. The criteria of exclusion included: Severely debilitated patients and patients aged more than 80 or less than 15 years. A total number of 20 subjects who appeared healthy and 20 PD patients (Group II) were enrolled in the study. Specific information that related to the demographic characteristics and illness obtained from each patient. The Brain Bank Diagnostic Criteria for Parkinson's disease that established by United Kingdom Parkinson's Disease Society was used. Dental health care was assessed using (Decayed, Missing, and Filled teeth) DMF-T index. The degree of caries restoration (%) was calculated: Ratio of filled teeth (F-T) to the carious (D-T) plus filled teeth (F-T)  $(F-T / (D-T + F-T) \times 100)$ . The problem of drooling was assessed using the Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS) scale.<sup>[12]</sup>

The scale composed of 5 rates according to the drooling severity. These rates are: Normal (no problems); Slight (have too much saliva, but do not drool); Mild (have some drooling during sleep, but none when awake); Moderate (have some drooling when awake, but usually do not need tissues or a handkerchief); and Severe (have so much drooling that regularly need to use tissues or a handkerchief to protect clothes).

From each participant the un-stimulated (basal) saliva was collected. The participants asked to refrain from eating or drinking 2 hours prior to collection. The subject/patient rinsed their mouth with tap water then the saliva samples collected into disposable containers. Then centrifuged (3000 rpm) for 10 min. the supernatants were separated from samples and the volume of each saliva sample was measured, then samples of saliva were kept in Eppendorf's tube and frozen at ( $-20^{\circ}\text{C}$ ) until use for biochemical analysis. The saliva flow rate is estimated by dividing the total collected saliva volume (ml) by the collection time (min) that was measured during sample collection. Total saliva protein was determined using Bradford's method.<sup>[13]</sup> The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The total protein concentration in saliva was calculated from the regression equation of best-fit line of human serum albumin standard curve (Absorbance =  $[0.022 \times \text{concentration of albumin } (\mu\text{g})] + 0.046$ ). Protein contaminated with nucleic acids absorbed the light at wavelength 280 nm and it absorb much strongly at wavelength 205 nm when it is free from nucleic acids. The absorbance of a known volume (50 $\mu\text{l}$ ) of saliva diluted with 4 milliliters distil water was measured at wavelengths 205 nm, 260 and 280 nm for each subject and patient. The concentration of uncontaminated protein was determined by using the following equations:<sup>[14]</sup>

The concentration of Uncontaminated protein ( $\mu\text{g/ml}$ ) =  $31 \times \text{absorbance at } 205 \text{ nm}$  Saliva  $\alpha$ -synuclein is quantified using sandwich ELISA (Sensolyte Anti-alpha-Synuclein Quantitative ELISA (AnaSpec, Inc., USA). The wells of microtitre plate are pre-coated with anti- $\alpha$  Synuclein monoclonal antibodies, blocked and stabilized for long term storage. The amount of  $\alpha$ -synuclein is quantified using rabbit polyclonal  $\alpha$ -synuclein specific antibodies directly conjugated to horseradish peroxidase (HRP) are used to detect captured  $\alpha$ -synuclein. The sensitivity of this test is 5 pg/ml of  $\alpha$ -synuclein and saliva  $\alpha$ -synuclein level was calculated by using the regression equation of the standard curve using different concentration (0-500 pg/ml) (The regression equation of the standard curve was: Absorbance at  $\lambda 450 \text{ nm} = 0.168 + (\text{concentration} \times 0.004)$ .

## Statistical analysis

The results were expressed as number, percent, and whenever possible as mean  $\pm$  standard deviation (SD) using Excel 2007. The data were analyzed using two-tailed unpaired student's t test, differences between percentages, confidence interval at 95% and simple correlation test taking  $P \leq 0.05$  as the lowest limit of significance.

## Results

Table 1 shows the characteristics of the subjects and patients enrolled in the study. There are no significant differences between two groups regarding the gender distribution and the mean of age. The median of duration

**Table 1: Characteristics of the healthy subjects and Parkinson's patients**

Characteristics	Healthy subjects	Parkinson's patients
Gender (male: female)	18:2	16:4
Age (year)	65.4 $\pm$ 8.2 (64)	64.4 $\pm$ 10.6 (66)
Duration of disease (Year)	—	6.55 $\pm$ 6.83 (4)
Family history of disease	—	6
Smoking		
Current	10	4*
Ex-smoker	4	3
Clinical features		
Static tremor	—	20
Rigidity	—	14
Gait disturbances	—	15
Slurred speech	—	10
Movement abnormalities		
Dyskinesia	—	9
Akathisia	—	5
Bradykinesia	—	14
Medications		
Levodopa	—	15
Bromocriptine	—	0
Amantidine	—	3
Anticholinergics (e. g. benhexol, procyclidine)	—	12
Evidence of autonomic dysfunction		
Orthostatic dizziness	—	15
Dry mouth	—	13
Sweating	—	12
Difficulties in swallowing	—	8
Constipation	—	14
Urinary problems		
Nocturia	—	10
Urgency	—	1
Frequency	—	6

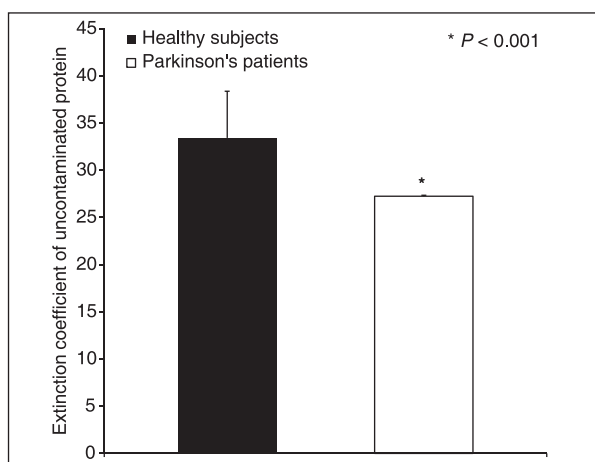
The results expressed as number, mean  $\pm$  standard deviation (SD) (median),

\* $P < 0.05$  compared with healthy subjects

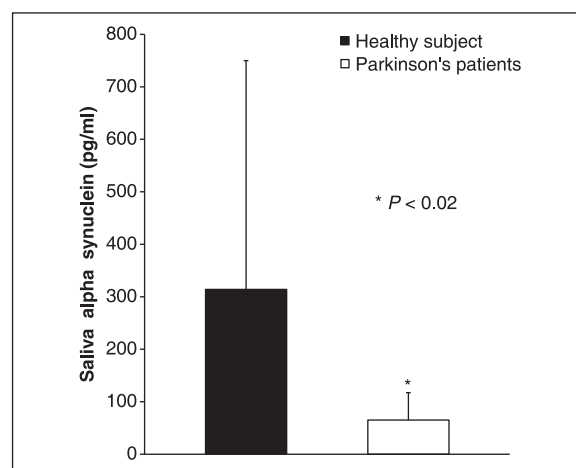
of PD is four years and 30% of cases have family history of PD. Current smoking reported in 20% of PD compared with 50% in healthy subjects; the difference reached significant ( $P < 0.05$ ) level. The clinical features of PD observed in all patients despite of their treatment. Static tremor observed in 100%, rigidity in 75%, rigidity and bradykinesia in 70% of patients [Table 1]. The majority of cases are treated with a combination of levodopa and carbidopa (75%) and anticholinergic agents (70%) [Table 1]. No patient received bromocriptine or other drugs that related to dopamine-2 receptor agonists. Dysfunction of autonomic nervous system observed in PD in term of orthostatic dizziness (75%), dry mouth (65%) difficulties in swallowing (60%) and constipation (70%), whereas the urinary system disturbed in a lower percents [Table 1]. The symptom of drooling reported in 80% of cases with a variable presentation according to the severity of this symptom [Table 2]. Examination of the teeth revealed that PD have a non-significant high means of decay and missing teeth compared with healthy subjects [Table 2]. The mean number of filled teeth in Parkinson's patients is non-significantly less than corresponding mean of health subjects [Table 2]. The overall score of decay, missing and filling teeth is non-significantly higher in PD compared with healthy subjects. Caries restoration up to 50% is observed in 16 healthy subjects (80%) compared to 19 Parkinson's patient (90%) that does not reach to significant level [Table 2].

The overall un-stimulated saliva volume over ten minutes was less than 1 ml in 18 patients, whereas the saliva volume in healthy subjects ranged between 2.5-3.5 ml.

The total saliva protein, determined by Bradford's method is significantly ( $P < 0.001$ ) higher in PD than corresponding value of healthy subjects (689.1  $\pm$  442.7 versus 180.3  $\pm$  137.7  $\mu$ g/ml, respectively). Direct detection of uncontaminated protein with nucleic acid at  $\lambda$ 205 nm ultraviolet (UV)-spectrophotometer shows significant ( $P < 0.002$ ) high level in PD compared with healthy subjects (77.8  $\pm$  37.9 versus 46.7  $\pm$  16.0  $\mu$ g, respectively). The extinction coefficient of uncontaminated protein that detected directly by UV spectrophotometer at  $\lambda$  205nm in PD ranged between 27.08–27.40 M.cm<sup>-1</sup> with a mean  $\pm$  SD 27.25  $\pm$  0.086 M.cm<sup>-1</sup> which significantly ( $P < 0.001$ ) less than corresponding values of healthy subjects (range: 27.42-50.72 M.cm<sup>-1</sup>, mean  $\pm$  SD: 33.48  $\pm$  4.94 M.cm<sup>-1</sup>) [Figure 1]. Saliva  $\alpha$ -synuclein level is significantly ( $P < 0.02$ ) less in PD (65  $\pm$  52.2 pg/ml) than healthy subjects (314.01  $\pm$  435.9 pg/ml) and its mean level is about 20.7% of the mean of healthy subjects [Figure 2]. There is no significant correlation ( $r = 0$ ) between the total saliva protein and saliva  $\alpha$ -synuclein level in PD whereas an inverse non-significant correlation ( $r = -0.158$ ) observed in healthy subjects.



**Figure 1:** Extinction coefficient of saliva uncontaminated protein in healthy subjects and Parkinson's patients



**Figure 2:** Saliva  $\alpha$ -synuclein levels in Parkinson's patients and healthy subjects

**Table 2: Assessment of dental health using Decay, Missing, and filled (DMF) scoring and drooling**

Variables	Healthy subjects	Parkinson's patients
DMF scoring		
Decay	3.2±1.44 (3)	5.6±5.6 (4)
Missing	13.35±6.52 (11.5)	16.05±10.2 (14.5)
Filled	2.45±2.19 (3)	1.95±2.52 (1)
Total	19.05±4.96 (19.5)	23.6±8.18 (25)
Caries restoration (%)		
0	7	9
1-25	0	3
26-50	9	6
51-75	2	1
76-100	1	1
Drooling severity		
No problem	—	4 (20)
Slight	—	3 (15)
Mild	—	7 (35)
Moderate	—	1 (5)
Severe	—	5 (25)

The results expressed as number (%) and mean  $\pm$  standard deviation (SD) (median)

## Discussion

The results of this study show significant low saliva  $\alpha$ -synuclein level in Parkinson's patients. The significant high saliva protein level in Parkinson's patients characterized by extinction coefficient of 27.4 M.cm<sup>-1</sup> at  $\lambda$  205 nm. In respect to the characteristics of subjects and patients enrolled in this study, there is no significant difference in age between healthy subjects and PD patients. Therefore, the effect of ageing on the saliva determinants does not bias the results obtained in this study. Family history of PD reported in one third of the

cases in this study. This observation in agreement with others who found that most cases of neurodegenerative disease are not explained by Mendelian inheritance of known genetic variants, but instead are thought to have a complex etiology with numerous genetic and environmental factors contributing to susceptibility.<sup>[15]</sup> One significant characteristic of Parkinson's patients is low frequency of smoking. Chang *et al.* (2014) mentioned in their review that cigarette smoking associated with reduced Parkinson's pathology in post-mortem brains.<sup>[16]</sup> Autonomic nervous system dysfunction is reported in all patients enrolled in this study and presented in different organs and systems. This finding is in agreement with Perez-Lloret *et al.*<sup>[17]</sup> who highlighted different medications to treat sialorrhea, constipation, orthostatic hypotension, urinary incontinence, sexual dysfunction. The total small volume of saliva that obtained from PD patient may be attributed to ageing, the disease itself and to the adverse effects of medications.<sup>[18]</sup> Drooling is reported in 16 out of 20 patients in which this finding is in agreement with other studies.<sup>[19]</sup>

Hyposalivation played a role in alteration the buffer capacity of saliva leading to increase dental caries around the neck of the teeth and this explains the non-significant high DMF scoring in this study.<sup>[20,21]</sup> The significant high salivary protein in PD was previously reported and it did not influenced by the medications that prescribed to the patients. Significant high level of uncontaminated protein with nucleic acid run in parallel with significant increase in total protein and it expressed unique extinction coefficient of 27.25 M.cm<sup>-1</sup>. Salivary  $\alpha$ -synuclein level is significantly less in PD patients than healthy and its mean level is about 20.7% of the mean of healthy subjects. Hong *et al.*<sup>[7]</sup> demonstrated, in a large cohort study included patients with PD and controls, a low level of  $\alpha$ -synuclein in cerebrospinal fluid of Parkinson's patients. Several groups have examined

serum/plasma concentrations of  $\alpha$ -synuclein as potential biomarkers of Parkinson's disease but the major drawback in assessing serum/plasma  $\alpha$ -synuclein levels is the fact that 95% of total blood  $\alpha$ -synuclein derived from red blood cells. Therefore, the measurement of  $\alpha$ -synuclein in saliva provides an advantages over other biological fluids because the possibility of blood contamination can be avoided and the sample of saliva be readily obtained. In one pioneer work, Wang *et al.*<sup>[22]</sup> demonstrated circular dichroism spectra of the soluble tetramer  $\alpha$ -synuclein construct exhibit negative bands at 222 nm and 208 nm, and a positive band at 193 nm. Limitations of the study included small sample size and variations in duration of disease.

## Conclusion

We conclude that saliva  $\alpha$ -synuclein serves as a biomarker for PD if its level compared with healthy subjects, and a specific protein with extinction coefficient 27.5 M.cm<sup>-1</sup> is detected in saliva of Parkinson's patients.

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