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Elevated salivary protein in Parkinson's disease and salivary DJ-1 as a potential marker of disease severity

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Abstract

Introduction: There is an urgent need to identify robust biomarkers for Parkinson's disease (PD).

Previous studies have shown changes in composition and secretion of saliva in patients with PD, including an increase in salivary DJ-1 concentration. Autonomic dysfunction is a known feature of PD and could contribute to abnormal saliva gland function.

Methods In this pilot cross-sectional study, characterisation of the saliva of 16 patients with PD and 22 age-matched controls was performed. Salivary DJ-1 concentration was measured with quantitative immunoblotting; total protein concentration with a BCA assay and spectrophotometry; amylase with an amylase activity assay; albumin with an ELISA and mucin concentration with periodic-acid Schiff staining of SDS-gels.

Results: Patient saliva showed an increase in both total protein concentration (8.4 vs 5.0 mg/ml, $p=0.0002$) and DJ-1 concentration (0.84 vs 0.42 $\mu\text{g/ml}$, $p=0.001$), but there was no difference in salivary DJ-1 after adjusting for total protein concentration. In patients, adjusted DJ-1 levels correlated with disease severity measured with the MDS-Unified Parkinson's Disease Rating Scale ($p=0.019$). Patient saliva had elevated concentrations of amylase (127 vs 64 units/ml, $p=0.0005$) and albumin (110 vs 41 $\mu\text{g/ml}$, $p=0.0003$) but not mucins.

Conclusions: This study suggests that the saliva of patients with PD is different in composition to that of healthy age-matched controls, supporting the notion that saliva may be a good candidate for biomarker discovery in PD. The specific differences suggest that major salivary glands and gingival crevice fluid may both be sources of additional DJ-1 and protein in patient saliva.

Keywords: Parkinson's disease; biomarkers; diagnostics; autonomic function; DJ-1

Introduction

There is an unmet need for biomarkers of Parkinson's disease (PD), to help achieve earlier diagnosis, improve the accuracy of diagnosis and to monitor disease progression and response to neuroprotective treatment. Saliva may be an ideal fluid for the discovery of biomarkers of PD because collection is inexpensive and non-invasive. Intra-neuronal inclusions of aggregated alpha-synuclein protein, termed Lewy pathology, are the pathological hallmark of PD and have been found in the salivary glands as well as in multiple sites of the autonomic nervous system involved in control of saliva production in patients with PD [1]. The function of salivary glands may be affected in PD, manifesting as hyposalivation and abnormal saliva composition [2]. DJ-1 is a protein involved in diseases associated with aggregated alpha-synuclein and has previously been examined as a potential biomarker of PD in CSF, blood, and saliva [3]. In this cross-sectional study, we examined saliva from a group of patients with PD and age-matched controls in order to identify abnormalities in saliva gland function among patients, as well as to determine whether changes in the levels of specific proteins, such as DJ-1, may be useful biomarkers of disease.

Methods

Participants

The study was approved by Queen Square Ethics Committee as part of a larger project examining risk factors for PD (PREDICT-PD, ethics reference 10/H0716/85 [4]). Written informed consent was obtained from all participants. Unstimulated whole-mouth saliva was collected from patients with PD in the movement disorders clinics at The National Hospital for Neurology and Neurosurgery, Queen Square, London, UK. Patients fulfilled Queen Square Brain Bank criteria for PD [5]. Patients were examined by two trained clinicians (AJN and TTW) using the motor section (part 3) of the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS), while 'ON' medication. Anticholinergic burden (ACB) score and levodopa equivalent dose were calculated for

each patient (see supplementary methods). Control saliva was collected from age-matched healthy volunteers from a care home in London. Assuming a large effect size (Cohen's $d = 0.8$), it was calculated that 25 patients and 25 controls would be needed to achieve a statistical power of 80%. Saliva was collected using the 'passive drool' method: participants were instructed to tilt their head forward, allow saliva to collect in the front of the mouth and drain into a sterile 20 ml tube for 5 min. Salivary flow rate was calculated by dividing the volume of saliva produced by the collection time.

Measurement of total protein, DJ-1, mucins, amylase and albumin concentrations

Salivary protein concentration was measured by absorbance at 280 nm, using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Cramlington, UK). DJ-1 concentration was assessed by quantitative western blotting. Samples were gel electrophoresed and probed with affinity purified anti-DJ-1 goat IgG, followed by secondary antibodies and chemiluminescent substrate. Band intensities were compared to a standard curve produced by loading different concentrations of a DJ-1 standard on each blot. Salivary mucin concentrations were assessed using periodic acid-Schiff staining of glycoproteins on gels. Concentrations are reported as relative to the staining intensity on a selected control sample. Salivary amylase concentrations were assessed using an amylase activity assay and salivary albumin concentrations were assessed using an ELISA.

Statistical analysis

Wilcoxon tests were used to compare total protein and mucin concentrations between the patient and control groups. T-tests were used to compare DJ-1, amylase and albumin concentrations between groups. Pearson's product moment was used to assess correlations between total protein, DJ-1, amylase and albumin concentrations; Spearman's rank was used to assess the correlation between mucins and total protein, mucins and DJ-1 and DJ-1 and UPDRS.

Results

Saliva was collected from 16 patients and 22 controls (Table S1). All patients and none of the controls were taking dopaminergic medication and 8 of 16 patients were taking medications with anticholinergic effects according to the ACB scale (trihexyphenidyl and amantadine). There was no significant difference in salivary flow rate between the patient and control groups (Fig. 1a) and there were no effects of age, gender or pharmacotherapy (anticholinergic burden score, levodopa equivalent dose or time since last levodopa dose) on flow rate (Fig S1).

Total protein concentration

Patients with PD had higher total salivary protein concentration than controls (8.4 vs 5.0 mg/ml, $p=0.0002$) (Fig 1b). Among our volunteers a salivary protein concentration of 7.3 mg/ml or higher differentiated patients from controls with a sensitivity of 68.8% and specificity of 86.4%. There were no effects of age, gender or pharmacotherapy on total protein concentration (Fig S2).

DJ-1

Patients with PD had higher salivary concentrations of DJ-1 than controls (0.84 vs 0.42 $\mu\text{g/ml}$, $p=0.001$) but after adjusting for total protein concentration, there was no longer any difference between the two groups (Fig 1c). Among patients, the adjusted value for salivary DJ-1 concentration was positively correlated with UPDRS ($\rho=0.6$, $p=0.019$) (Fig. 1d) but did not correlate with disease duration (Fig S3). There were no effects of age, gender or pharmacotherapy on DJ-1 concentration (Fig S4).

Mucins, amylase and albumin

There was no significant difference in mucin concentration between patient and control saliva (Fig 2a). Amylase concentration was higher in the patient than in the control saliva (127 vs 64 units/ml,

p=0.0005) (Fig 2b) and was positively correlated with both total protein concentration ($r=0.57$, $p<0.001$) (Fig 2d) and with DJ-1 concentration ($r=0.43$, $p=0.013$) (Fig 2e). Albumin concentration was higher in patient than in control saliva (110.1 vs 47.1 $\mu\text{g/ml}$, $p=0.0003$) (Fig 2c) and was positively correlated with both total protein concentration ($r=0.59$, $p=0.0001$) (Fig 2f) and with DJ-1 concentration ($r=0.69$, $p<0.0001$) (Fig 2g). There were no effects of age, gender or pharmacotherapy on concentrations of mucins (Fig S5), amylase (Fig S6) or albumin (Fig S7). Salivary protein, amylase and albumin concentrations showed no correlation with UPDRS (see fig S8).

Discussion

In this cross-sectional study, significant differences were observed between patients and controls in the total salivary protein concentration and in the concentration of specific salivary proteins, including DJ-1, amylase and albumin. To our knowledge, this is the first time that salivary DJ-1 has been shown to be correlated with UPDRS score. This observation is supported by recent findings that salivary DJ-1 is correlated with both putaminal uptake of $^{99\text{m}}\text{Tc}$ -TRODAT (an imaging marker of disease severity) and Hoehn and Yahr stage [6] and suggests that salivary DJ-1 may be valuable as a marker of disease severity. However, our lack of longitudinal data limits inferences about DJ-1's utility as a marker of disease progression, especially given the failure to identify an increase in DJ-1 in patients vs controls or a correlation between DJ-1 and disease duration.

Previous studies have demonstrated reduced salivary flow rates among patients with PD [2], although we did not observe any differences in salivary flow rate between patients and controls. Despite being a simple measurement, total salivary protein concentration was able to correctly classify patients and controls in our study group with a sensitivity of 69% and specificity of 86%. The increase in salivary protein concentration may be a result of autonomic dysfunction, which is a recognised feature of PD [7]. Reflex salivary protein secretion is dependent upon dual activation by parasympathetic and sympathetic nerves which together provide an enhanced secretion [8], and

such signalling is modified by the action of central nerves on the salivary centres in the brain. Lewy pathology has been shown within the salivary glands themselves as well as several sites in the autonomic nervous system involved in the control of saliva gland function [1]. Given that autonomic disturbances are thought to often precede the motor symptoms of PD [7], salivary gland dysfunction might be useful as an early biomarker of disease (although further studies would be needed to confirm this).

We sought to determine the source of the additional protein and DJ-1 in the saliva of patients with PD by using concentrations of amylase, albumin and mucins as markers of different salivary protein sources. Amylase is the most abundant protein in saliva and is primarily secreted by the parotid glands and lesser amounts from the submandibular and sublingual salivary glands [9]; therefore, an increase in salivary amylase in patients with PD might suggest that the additional protein in patient saliva is coming from these glands. Salivary amylase was higher among patients with PD than controls (Fig 2b), and was positively correlated with total protein concentration (Fig 2c), suggesting that over-secretion of protein by the submandibular, sublingual and parotid glands may account for the higher protein concentration in the saliva of patients with PD.

Ultrafiltration of serum is believed to be the main mechanism through which albumin enters the oral cavity and its salivary concentration has therefore been proposed to reflect compromised mucosal integrity and periodontal disease [10]. Increases in salivary albumin have also been shown in patients with a variety of medical conditions and with advancing age [10]. Several studies have shown patients with PD to have worse oral health compared with controls [11] and therefore the increases in salivary protein and DJ-1 concentration among patients with PD reported here may be due to worse oral health, reduced mucosal integrity and an increased leakage of serum proteins into the saliva, rather than as a direct result of the Parkinson's disease process itself.

Salivary mucins are primarily produced by minor mucous salivary glands [9] and therefore an increase in salivary mucins in patients with PD might suggest that the additional protein in their saliva is derived from these glands. However, no difference was observed between the salivary mucin concentration in patient and control saliva (Fig 2a) and salivary mucin concentration was not correlated with either total protein concentration or DJ-1 concentration, suggesting that the additional DJ-1 and total protein in the saliva of patients with PD was not coming from the minor salivary glands. This fits with recent findings that minor salivary glands are relatively spared by Lewy pathology in PD subjects [12].

We acknowledge that the present study has several limitations. Firstly, the sample size was relatively modest. Secondly, patients were taking different doses of dopaminergic and anticholinergic medication at the time of collection and both are known to affect saliva production [2].

Furthermore, the time since last levodopa dose varied between participants. However, we did not observe significant effects of anticholinergic burden score, levodopa equivalent dose or the time since last levodopa dose on either salivary flow rate or salivary concentration of DJ-1, mucins, amylase or albumin (Figs S1-S2, S4-S7, Table S2). Thirdly, follow-up and serial sampling would be required to confirm the ability of DJ-1 to act as a marker of disease progression. Fourthly, the correlation between total protein and DJ-1 concentration with amylase and albumin concentration does not definitively prove that the source of the increases in total protein and DJ-1 are the major salivary glands. Perhaps major saliva gland secretions and fluid transudate from gingival crevices are increased, yet the additional DJ-1 comes from other sources, such as dental plaque or oral bacteria or is due to greater levels of inflammation in the oral cavity. Further studies, perhaps the analysis of the saliva from isolated glands or of fluid collected from the gingival crevice, would be required to confirm the source of the additional proteins. Lastly, examination of the oral cavity was not performed making it difficult to assess whether periodontal disease was the cause of increased salivary albumin in the patient group.

In conclusion, the present study found an increase in the total protein and DJ-1 concentration in the saliva of patients with PD, which appears to be due to increased secretion by the major salivary glands. Reduced mucosal integrity, worse oral health and increased exudation from the gingival crevice may also contribute to the additional protein and DJ-1 in patient saliva. The differences between the saliva of patients with PD and controls supports the notion that saliva may be an ideal fluid for further biomarker studies.

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Author contributions: JMM, GBP, GG and AJN conceived the project. TW and AJN recruited subjects. TW and AJN performed clinical assessment. JMM performed experiments. JMM, GBP and AJN analysed the data. JMM, GBP and AJN drafted the manuscript. TW and GG critically reviewed the manuscript. GBP and AJN supervised the project.

Competing interests: The authors declare no competing interests.

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Figure legends:

Figure 1. Salivary flow rates and concentrations of total protein and DJ-1 in patients with Parkinson's disease and controls. a) Saliva flow rates. b) Total protein concentration in saliva of patients and controls. c) Salivary concentration of DJ-1 among patients and controls both before and after adjusting for total protein concentration. d) Correlation between UPDRS and salivary DJ-1

concentration normalised by total protein concentration, among the patient group. Best fit line fitted with linear regression.

Figure 2. Salivary concentrations of mucins, amylase and albumin in patients with Parkinson's disease and controls. Concentrations of mucins (a), amylase (b) and albumin (c) in the saliva of patients with PD and controls. Correlations between amylase concentration and total protein concentration (d), amylase concentration and DJ-1 concentration (e), albumin concentration and total protein concentration (f) and albumin concentration and DJ-1 concentration (g) . Best fit lines fitted with linear regression