

LETTER TO THE EDITOR

Salivary α -synuclein and DJ-1: potential biomarkers for Parkinson's disease

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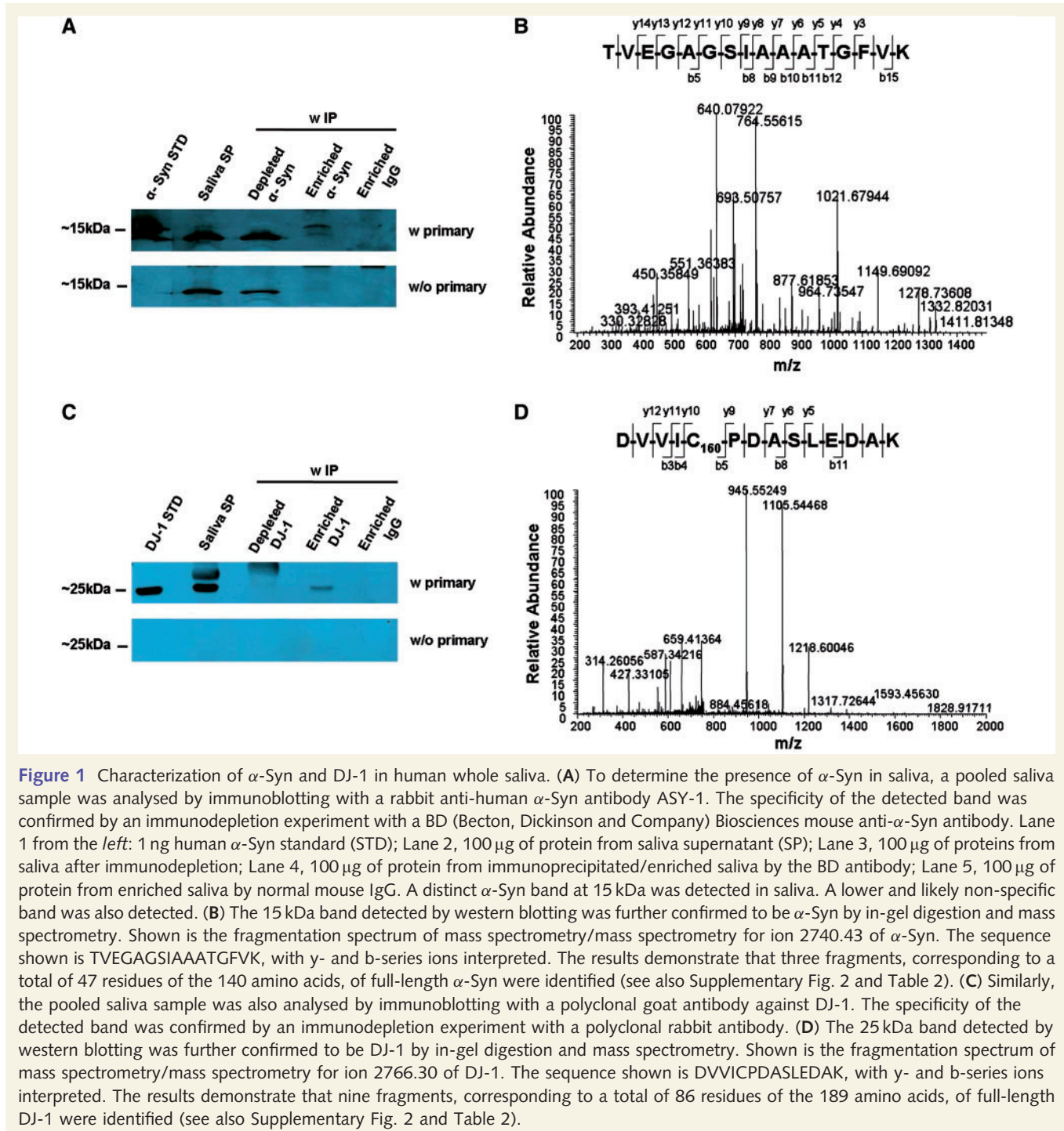
Sir, Parkinson's disease is a neurodegenerative disorder belonging to a group of heterogeneous diseases characterized by a progressive and relatively selective loss of anatomically or physiologically related neuronal systems (Lang and Lozano, 1998; Silvers and Som, 1998). The identification of Parkinson's disease specific biomarkers, particularly at early stages, is critical for Parkinson's disease diagnosis, monitoring disease progression and patient management as well as the development of therapeutic interventions. Thus far, the proteins α -synuclein (α -Syn) and DJ-1 have been tested rigorously in Parkinson's disease. In our recent study published in *Brain* (Hong *et al.*, 2010), where a large cohort of patients with Parkinson's disease and controls were included, we provided evidence that α -Syn, along with DJ-1, decreases in Parkinson's disease CSF, providing high sensitivity and specificity for Parkinson's disease diagnosis. However, even though CSF is close to the main site of pathology in Parkinson's disease and other neurodegenerative disorders in the CNS, it cannot be readily obtained in most clinical settings (Shi *et al.*, 2010). To address this

issue, several groups have examined serum/plasma concentrations of α -Syn and DJ-1 as potential biomarkers of Parkinson's disease. Unfortunately, a major drawback in assessing serum/plasma α -Syn and DJ-1 levels is the fact that >95% of total blood α -Syn and DJ-1 are derived from red blood cells. After controlling for several major variables, we concluded in a recent investigation that, unlike CSF, these two markers in plasma are unable to differentiate patients with Parkinson's disease from controls (Shi *et al.*, 2010). Of note, blood contamination of human CSF is also a major problem when assessing levels of α -Syn and DJ-1 in CSF (Hong *et al.*, 2010; Shi *et al.*, 2010).

In an effort to look for other potential sources of clinically accessible samples for Parkinson's disease diagnosis and monitoring of disease progression, as well as to address the difficulties in dealing with CSF α -Syn and DJ-1, in this follow-up investigation we asked whether these two proteins are present in human saliva and examined their potential as Parkinson's disease biomarkers. The research was also motivated by the following recent findings:

(i) the human submandibular gland produces 70% and 63% of the total resting and stimulated salivary volume, respectively (Cook, 1994); and (ii) the submandibular gland has recently been shown to be involved by synucleinopathy in the early stages of Parkinson's disease (Beach *et al.*, 2010; Cersosimo *et al.*, 2010; Del Tredici *et al.*, 2010). Thus saliva, typically free of blood contamination, seems to be another ideal biofluid to study potential biomarkers for Parkinson's disease diagnosis and progression.

To investigate whether α -Syn and DJ-1 are present in human saliva, and if so, whether their concentrations are different in Parkinson's disease versus controls, saliva samples were collected from patients with Parkinson's disease ($n = 24$; M/F = 17/7; average age = 63.5) as well as healthy control subjects ($n = 25$; M/F = 11/14; average age = 58.0) in a rested, unstimulated state. Detailed characterization of these subjects and methods for characterization of salivary α -Syn and DJ-1 can be found in the online Supplementary Material.



In the initial analysis, whole saliva (collected from several controls) was separated into a cellular component and a supernatant, which was then probed with α -Syn and DJ-1 antibodies, respectively. The results, shown in Fig. 1, demonstrated that the anti- α -Syn antibody detected a distinct band at 15 kDa (Fig. 1A) and the anti-DJ-1 antibody detected two bands around 25 kDa (Fig. 1C), which correspond to α -Syn and DJ-1 standards, respectively. Comparing the banding patterns when equal fractions of the saliva supernatant and pelleted cellular component were loaded (Supplementary Fig. 1A and B); it appears that there was slightly more α -Syn in the cellular component than in the supernatant, but clearly more DJ-1 in the supernatant than in the cellular component.

To further validate the identity of the putative α -Syn and DJ-1 bands, two experimental approaches were taken. First, the saliva supernatant was incubated with either α -Syn or DJ-1 antibodies mounted on Santa Cruz ExactaCruz matrix beads. This was followed by detecting α -Syn and DJ-1 with another pair of antibodies, in both the resulting supernatant and proteins associated with the beads. The distinct α -Syn band was almost depleted from the supernatant after pre-absorption of the protein to the beads by a different anti- α -Syn antibody (Fig. 1A). The lower DJ-1 band was also largely depleted while the upper band was much reduced (Fig. 1C). These results indicate that the western bands very likely represent the α -Syn and DJ-1 proteins. To substantiate this argument, the bands corresponding to α -Syn and DJ-1 were cut out and analysed by mass spectrometry after an in-gel digestion. The α -Syn band and the lower DJ-1 band were further confirmed to be α -Syn (Fig. 1B) and DJ-1 (Fig. 1D), respectively. The amino acid coverage was 34% for α -Syn and 45% for DJ-1 (Supplementary Fig. 2 and Supplementary Table 2). The upper DJ-1 band was not confirmed by mass spectrometry, suggesting that it might be a highly modified isoform that is difficult to detect at normal mass spectrometry settings. It is also possible that the upper DJ-1 band is non-specific; however, the band was not detectable when the primary antibody was omitted.

Next, α -Syn and DJ-1 were quantified in patients with Parkinson's disease and controls using our established Luminex assay with modifications to achieve the best assay accuracy and minimal matrix effect in saliva, i.e. with the spike recovery rate close to 100% (see details in the online Supplementary Material). Differences in α -Syn and DJ-1 levels between patients and controls did not reach significance, regardless of whether or not protein concentrations were normalized. However, there was a clear trend for α -Syn to decrease and DJ-1 to increase, respectively, in patients with Parkinson's disease compared to controls (Fig. 2A and C). Furthermore, visual inspection of a plot of α -Syn level versus UPDRS (Unified Parkinson's Disease Rating Scale) motor scores in patients with Parkinson's disease suggested a negative trend between α -Syn level and severity of motor symptoms, though the effect did not reach significance ($P > 0.05$) (Fig. 2B). However, no correlation was observed between total DJ-1 levels and UPDRS motor scores (Fig. 2D).

Identification of α -Syn and DJ-1 in human saliva, two proteins that are critically involved in both familial and sporadic Parkinson's disease, suggests that saliva could be a potentially important diagnostic sample source for Parkinson's disease. The significance of

this discovery is 2-fold. First, salivary glands are linked to the CNS and have been found to be involved in Parkinson's disease at relatively early stages of the disease (Del Tredici *et al.*, 2010). More specifically, it appears that the submandibular gland is directly linked to the central nervous system through the peripheral nervous system, including both the parasympathetic and sympathetic arms of the autonomic nervous system (Silvers and Som, 1998; Blessing, 2004). Parasympathetic innervation to the submandibular glands is provided by the superior salivatory nucleus via the chorda tympani of the VIIth cranial nerve, a branch of the facial nerve that synapses in the submandibular ganglion after which it follows the lingual branch of the sensory mandibular root of the trigeminal nerve V₃ and leaves this nerve as it approaches the gland. Direct sympathetic innervation of the salivary glands takes place via preganglionic nerves located in the intermediolateral nucleus of spinal cord segments T1–T3, which synapse in the superior cervical ganglion after which it follows the external and internal carotid plexus. In the production and flow of saliva, both sympathetic and parasympathetic nerves cooperate with each other. These nerves enable the aforementioned salivary glands to form saliva quickly and empty it into the mouth. In sporadic Parkinson's disease, there is a substantial amount of evidence demonstrating the appearance of Lewy pathology in the autonomic nervous system and spinal cord prior to neuronal loss in the substantia nigra pars compacta (Iwanaga *et al.*, 1999; Bloch *et al.*, 2006; Braak *et al.*, 2007; Fumimura *et al.*, 2007; Orimo *et al.*, 2007, 2008; Fujishiro *et al.*, 2008; Probst *et al.*, 2008; Miki *et al.*, 2009). In addition, incidental Lewy body disease, considered a preclinical form of Parkinson's disease by most investigators, has routinely been characterized by the presence of Lewy bodies and Lewy neurites located in the autonomic nervous system. Moreover, in a recent study, phosphorylated α -Syn was investigated with an immunohistochemical method in different body sites, including the submandibular gland, revealing that in patients with Parkinson's disease, phosphorylated α -Syn is found at a high frequency throughout the gastrointestinal system, with the highest frequencies located in the lower oesophagus and the submandibular gland (Beach *et al.*, 2010).

The sources of salivary α -Syn and DJ-1 are currently unknown. Given that α -Syn can be actively secreted by neurons (Lee *et al.*, 2005), it is possible that the nerves innervating salivary glands release α -Syn into the saliva. However, it is also possible that α -Syn is derived from the CSF and/or plasma by yet-to-be defined mechanisms or from the cellular component of the saliva (Supplementary Fig. 1A). Similarly, DJ-1 could come from any of the sources discussed for α -Syn, although it is clear that DJ-1 levels in the supernatant are higher than in the cellular component of the saliva (Supplementary Fig. 1B), meaning that it is more likely that salivary DJ-1 is secreted by the nerves or exchanged from other body fluids. It is critical in future investigations to explore the precise sources and contributions of salivary α -Syn and DJ-1 not only for understanding the fundamental mechanisms involved in the transportation of these two proteins, but also for controlling potential variables when salivary α -Syn and DJ-1 are used for Parkinson's disease biomarkers.

Besides providing the relevance of using saliva as a biomarker discovery source for Parkinson's disease, these findings also

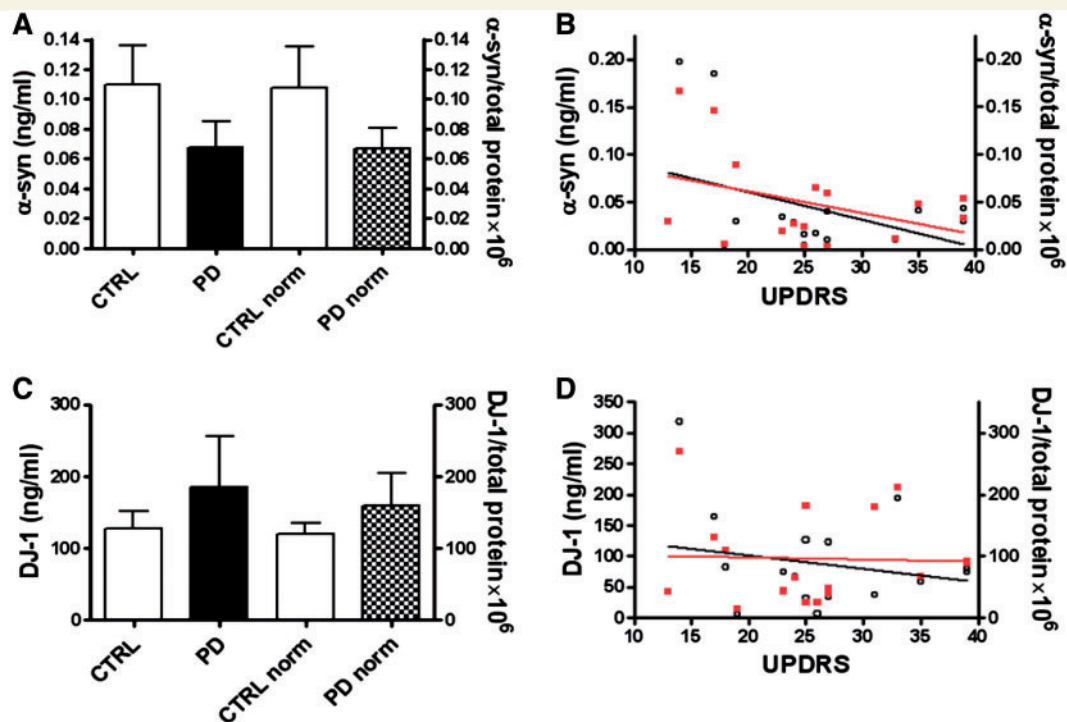


Figure 2 Salivary α -Syn and DJ-1 levels in patients with Parkinson's disease and healthy controls. α -Syn and DJ-1 protein levels in saliva were measured by Luminex in healthy controls (CTRL) and patients with Parkinson's disease (PD). The α -Syn levels (A) tended to decrease while the DJ-1 levels (C) tended to increase in the Parkinson's disease compared with the control group, whether normalized to the saliva total protein level or not. In a subset of the Parkinson's disease subjects with known UPDRS motor scores, the correlation between the UPDRS scores and salivary α -Syn (B) or DJ-1 (D) levels was also examined. α -Syn levels appeared to be negatively correlated with severity of motor symptoms, whether normalized (red rectangle) or not (black circle), while DJ-1 levels did not correlate well with UPDRS motor scores. UPDRS = Unified Parkinson's Disease Rating Scale.

suggest that α -Syn pathology within sympathetic and/or parasympathetic innervations of salivary glands could contribute to the phenomenon of dry mouth in Parkinson's disease. Indeed, it has been demonstrated that although most patients with Parkinson's disease develop sialorrhea, the production of saliva is actually reduced in these patients (Rajput and Rozdilsky, 1976; Edwards *et al.*, 1992; Johnston *et al.*, 1995; Bagheri *et al.*, 1999; Tumilasci *et al.*, 2006). To fully address this issue, a large cohort of patients with Parkinson's disease is needed to investigate whether salivary levels of α -Syn and DJ-1 are correlated with the production of saliva as well as whether it is influenced by disease severity. The latter issue is obviously important, given that our preliminary data already suggested that α -Syn levels tended to correlate with UPDRS motor scores in addition to the trend of lower α -Syn levels in Parkinson's disease, as compared to controls. In contrast, salivary DJ-1 levels did not correlate with UPDRS motor scores, (Fig. 2D). In addition, the ratio of α -Syn to DJ-1 levels did not enhance the performance of either biomarker (data not shown). However, we acknowledge that the results of our pilot investigation are far from conclusive. An analysis of much larger cohorts of patients with Parkinson's disease and healthy and diseased controls, including those with other parkinsonian disorders, is now needed. Other issues to be considered in future studies are age and gender as well as drug effects on salivary α -Syn and DJ-1

levels [please refer to our CSF investigations for more details (Hong *et al.*, 2010)].

Utilization of saliva in biomarker discoveries has several advantages over other biofluids. For example, when compared to CSF or serum/plasma, human saliva is more readily available, and is easier and less invasive to collect in sufficient quantities for various DNA, microRNA, protein and metabolite analyses. Saliva is also free of blood contamination, which could confound the evaluation of relevant protein concentrations, including α -Syn and DJ-1 as demonstrated recently (Hong *et al.*, 2010; Shi *et al.*, 2010). Moreover, the collection of saliva, unlike other body fluids, requires only a small amount of training and is painless to the participant. Finally, since saliva is readily accessible and its collection poses minimal risk to the patient, it is possible to screen the general population to identify patients with pre-clinical Parkinson's disease, thereby greatly increasing neuroprotective opportunities. Also, with such easy access to samples, salivary biomarkers with comparable or better performance than α -Syn and DJ-1 could allow robust monitoring of disease progression or treatment effects (Aguirre *et al.*, 1993).

In conclusion, in this study, we were able to successfully identify two of the most important Parkinson's disease related proteins— α -Syn and DJ-1—in human saliva. Additionally, based on the findings in this preliminary study, it is possible that α -Syn and DJ-1

could be potential diagnostic markers of Parkinson's disease, as we observed that α -Syn levels tended to decrease while DJ-1 levels tended to increase in Parkinson's disease. Preliminary results also suggest that α -Syn might correlate with severity of motor symptoms in Parkinson's disease. Our results now provide the impetus for undertaking much larger studies on the potential utility of α -Syn and DJ-1 as Parkinson's disease biomarkers.

Acknowledgements

We thank Dr W. Michael Caudle for his helpful discussion and critical reading. We also deeply appreciate those who have donated their saliva for our study.

Funding

National Institutes of Health grants (AG033398, NS057567, NS065070, E5004696, DE17634, NS062684); NINDS Institutional Centre Core Grant-Neuroproteomics P30 (NS055088) as well as grants from the Cheng-Mei Shaw Endowment.

Supplementary material

Supplementary material is available at *Brain* online.

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