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Original Articles

Salivary ATP13A2 is a potential marker of therapy-induced motor complications and is expressed by inclusions in submandibulary glands in Parkinson´s disease

Emilio Fernández-Espejo [a,b], Ana L. Gavito [b,c], Juan Suárez [b,c,d], Eduardo Tolosa [e,f,g], Dolores Vilas [h], Iban Aldecoa [i,j], Joan Berenguer [k], Antonio Córdoba-Fernández [l], Fátima Damas-Hermoso [m], Fernando Rodríguez de Fonseca [b,c,*]

[a] *Instituto de Estudios Campogibraltareños (IECG), 11027 Algeciras, Cádiz, Spain*
[b] *Red Andaluza de Investigación Clínica y Traslacional en Neurología (Neuro-RECA), Laboratorio de Medicina Regenerativa, Hospital Regional Universitario, 29010 Málaga, Spain*
[c] *Unidad de Gestión Clínica de Salud Mental, Instituto de Investigación Biomédica de Málaga, Hospital Regional Universitario de Málaga, 29010 Málaga, Spain*
[d] *Departamento de Anatomía Humana, Medicina Legal e Historia de la Ciencia, Universidad de Málaga, 29071 Málaga, Spain*
[e] *Unidad de Parkinson y movimientos anormales, Servicio de Neurología, Hospital Clínic, 08036 Barcelona, Spain*
[f] *Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, 28031 Madrid, Spain*
[g] *Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, 08036 Barcelona, Spain*
[h] *Servicio de Neurología, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Barcelona*
[i] *Departamento de Anatomía Patológica, Centro de Diagnóstico Biomédico, Hospital Clinic de Barcelona, Universitat de Barcelona, 08036 Barcelona, Spain*
[j] *Banco de Tejidos Neurológicos del Biobanco, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain*
[k] *Servicio de Radiología, Hospital Clínic, 08036 Barcelona, Spain*
[l] *Departamento de Podología, Universidad de Sevilla, 41009 Sevilla, Spain*
[m] *Servicio de Neurología, Hospital Universitario de Valme, 41014 Sevilla, Spain*

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ABSTRACT

Background: ATP13A2 holds promise as biomarker for Parkinsońs disease (PD). No study has examined how salivary ATP13A2 is related to motor features in idiopathic PD.
Methods: Salivary ATP13A2 concentration was evaluated with ELISA, and statistical correlations of ATP13A2 level with PD parameters were examined. The dose intensity of the dopaminergic medication regimen was expressed as levodopa equivalent daily dose (LEDD). ATP13A2 expression on histological sections of submandibular glands was evaluated using immunohistochemistry.
Results: Salivary ATP13A2 was undetectable in many subjects (28 % of patients, 43.7 % of controls). However, all the patients with motor complications ($n = 28$) showed quantifiable levels of ATP13A2, that positively correlated with MDS-UPDRS (total, parts III and IV), and LEDD ($p < 0.05$). Dyskinetic patients showed the highest LEDD values ($p < 0.05$). The histological study revealed: a) ATP13A2 staining was very intense in duct cells and vascular endothelium, and b) two patterns of ATP13A2-positive deposits are observed: rounded inclusions of 10–20 μm in diameter located in the interlobular tissue of the patients, and amorphous aggregates inside duct lumen in controls and patients.
Conclusions: The sensitivity of the ELISA assay was a major limitation for quantifying ATP13A2. However, salivary ATP13A2 was detected in all patients with motor complications, where a direct relationship among ATP13A2 concentration, levodopa equivalent daily dose, and MDS-UPDRS was found. Therefore, salivary ATP13A2 might be a reliable index of therapy-induced motor complications. ATP13A2 was expressed by rounded inclusions in the submandibulary gland of patients. This is the first description of ATP13A2-positive inclusions outside the nervous system.

Abbreviations: ATP13A2, ATPase Cation Transporting 13A2.

* Corresponding author at: Instituto de Investigación Biomédica de Málaga, 29010 Málaga, Spain.
E-mail address: fernando.rodriguez@ibima.eu (F. Rodríguez de Fonseca).

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1. Introduction

Parkinson's disease (PD) is characterized by progressive degeneration of dopaminergic neurons of the substantia nigra. Many factors such as neuroinflammation, redox balance, mitochondria bioenergetics, autophagy, exosomal α-synuclein release, and aging are involved in its pathogenesis [1–5]. The enzyme ATP13A2 (ATPase Cation Transporting 13A2) participates in most of these mechanisms [6–11]. In addition, ATP13A2 mutation is involved in monogenic PD, underlying an autosomal recessive form of early-onset parkinsonism or Kufor-Rakeb syndrome, and ATP13A2 is noted in Lewy inclusions in the brain of patients with idiopathic PD [6,8,10,11,12]. Therefore, the ATPase enzyme holds promise as biomarker for diagnosis of idiopathic Parkinson's disease.

Recently, we examined the association between serum and cerebrospinal fluid (CSF) ATP13A2 levels with clinical features in PD patients [13]. The findings revealed that ATP13A2 concentration in serum, but not in the CSF, positively correlated with the dose intensity of the dopaminergic medication, expressed as levodopa equivalent daily dose (LEDD) [13]. These findings suggest that serum ATP13A2 content might be an index of the intensity of dopaminergic medication in patients with idiopathic PD.

Salivary ATP13A2 content and its expression in salivary glands in patients with idiopathic PD is unknown. Saliva is an interesting biofluid that is being studied in PD because of its easy accessibility, and the occurrence of Lewy pathology in salivary glands is well known [14–20]. To date, the salivary ATP13A2 levels and its correlation with clinical features in idiopathic PD has not been assessed, nor the presence of ATP13A2-expressing inclusions in salivary glands. The objectives of this study are: (a) to study the concentration and histological expression of ATP13A2 in the saliva and submandibulary glands in patients with idiopathic PD and control subjects, and (b) to explore the relationship of salivary ATP13A2 level with clinical features of the disease. For histological analysis, the submandibulary gland was selected because it is the most active salivary gland [16].

2. Patients and methods

2.1. Participants, and demographic and clinical information

Patients were enrolled at Hospital Valme and Hospital Macarena (Sevilla, Spain) and were diagnosed of PD by expert physicians. Patients fulfilled the Movement Disorders Society criteria of PD [14,21], and they presented a reliable loss of dopamine-transporter binding signal on basal ganglia as evaluated with dopamine transporter single-photon emission computed tomography (DAT-SPECT) [22]. All SPECT scans were performed, quantitatively analyzed, and visually assessed by expert physicians at the Service of Nuclear Medicine [22]. Patients had an age-at-PD onset from 45 to 75 years, and those subjects with atypical deficits, Deep Brain Stimulation (DBS), or first-degree relatives with PD were discarded. Control participants were recruited from volunteers, and they were group-matched by age and sex to PD subjects. Controls were excluded if they had a first-degree family member with a neurological disorder.

Exclusion criteria of all participants included liver, renal, hematological, and cardiovascular dysfunctions, as well as malabsorption, morbid obesity, autoimmune diseases, acquired immunodeficiency syndrome, cancer, and infectious conditions because lysosomal biomarkers can be affected in these diseases [23]. Morbid obesity was diagnosed when BMI was higher than 35 kg/m^2. In addition, all participants were non-alcohol drinkers, non-smokers, and non-coffee drinkers, according to established criteria [13,24,25].

Standard demographic information was obtained from patients, including age, gender, body mass index, and years of education. Clinical data included the modified Hoehn and Yahr staging, the International Parkinson and Movement Disorder Society-Sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS), the modified Schwab-England scale, age of onset of PD, disease duration, and dopaminergic treatment. Regarding the medication regimen, patients were treated not only with levodopa, but also with dopamine agonists and supportive medication that enhance dopaminergic effect. For this reason, the antiparkinsonian medication regimen was expressed as levodopa equivalent daily dose or LEDD (mg), following an established formula [26,27].

2.2. Saliva collection and ELISA analysis

Three ml of saliva were collected in 5-ml polypropylene tubes (Eurotube DeltaLab, Barcelona, Spain). Saliva was centrifuged at 2500 rpm during 10 min, and then the liquid portion was immediately frozen at −80 °C in 0.5-ml aliquots. Hemoglobin concentration in a fresh 0.5-ml saliva aliquot was quantified as recommended [7], and those saliva samples with hemoglobin concentration higher than 1200 mg/ml were discarded. Saliva aliquots were unfrozen and sonicated with homogenizing solution (150 mM NaCl, 50 mM HEPES, 1 mM phenylmethylsulfonil fluoride, 0.6 μm leupeptin, 1 % Triton X-100, pH 7.4). ATP13A2 content was evaluated with a commercially available Enzyme-linked Immunosorbent Assay (ELISA) kit (Human ATP13A2 ELISA kit, Sandwich ELISA, Catalog #LS-F10891, Lifespans Biosciences Inc., USA), following manufacturer's instructions. Sensitivity of the ELISA kit was ~ 230 pg/ml. Each sample was analyzed in duplicate without diluting the saliva.

2.3. Immunohistochemical study of the submandibulary gland

Histological slides containing 5-μm sections of human submandibular gland tissue were obtained from the IDIBAPS Biobank (Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona). Submandibular gland tissue had been obtained through transcutaneous core needle biopsy with ultrasound guidance in patients with Parkinson's disease (n = 6) and healthy controls (n = 6), as explained elsewhere [17]. Histological sections were deparaffinized and then stained against ATP13A2 alone, or in combination with Dehalogenase type 1 or DEHAL1 (also known as Iodotyrosine deiodinase or IYD). This latter enzyme was chosen because it is selectively expressed by excretory duct cells, not by secretory acinar cells (Dr. Fernández-Espejo, personal observation). The antibodies used were as follows: mouse monoclonal antibody to ATP13A2 (ATP13A2 (4B7) Antibody, sc-293367, Santa Cruz Biotechnology, Inc.), and IYD polyclonal antibody (ThermoFisher Scientific, cat. #PA5-63757, produced in rabbit). Sections were incubated in the primary antibody, diluted 1:100, for 24 h at 4 °C. The next day the sections were incubated in the respective secondary antibody for 90 min: biotinylated goat anti-mouse IgG (1:500; cat. no. B7264, Sigma) or biotinylated donkey anti-rabbit IgG (1:500; cat. no. RPN1004, Amersham, Little Chalfont, England). The sections were then incubated in ExtrAvidin peroxidase (Sigma) diluted 1:2000, in darkness at room temperature for 1 h. Finally, immunoreactivity was revealed with 0.05 % diaminobenzidine (DAB; Sigma) diluted in 0.1 M phosphate-buffered saline (PBS) or DAB and 0.05 % nickel ammonium sulfate diluted in PBS. Peroxidase reaction was activated after the addition of 0.03 % H_2O_2.

All sections were reviewed by researchers blinded to the clinical information (E.F.E. and J.S.). Sections with positive immunoreactivity were visualized using a standard optical microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) coupled to the NIS-Elements Imaging Software 3.00 (Nikon). We screened 4–6 serial sections per subject with ATP13A2 immunostaining. The intensity of immunoreactive ATP13A2 staining within different cell regions of the submandibular gland was assessed in contiguous tissue sections according to a five-point rating scale: no expression (0), mild (1), moderate (2), intense (3), and very intense (4). The submandibular gland is an exocrine organ that contain secretory acinar cells arranged in lobules. These lobules are separated by interlobular connective tissue that contain excretory ducts, blood vessels, adipocytes, and autonomic nerves supplying the gland.

Finally, the presence of ATP-positive aggregates was also analyzed, and the density degree of immunoreactive inclusions was assessed according to a five-point rating scale: not detectable (0), mild (1), moderate (2), frequent (3), and very frequent (4).

2.4. Statistics

Data are given as mean ± standard deviation of the mean or SD. Two groups were compared using the Student's *t* test (parametric distribution), or the Mann-Whitney *U* test (non-parametric distribution). Dichotomous variables were compared with the χ^2 test. Correlations between two dependent variables were carried out with the Pearson's test. Data normalization was verified using the Shapiro-Wilk test. Correlation was adjusted for age, gender, BMI, and education. Data were analyzed with Minitab19 statistical package (AddLink Software Cientifico, Spain).

3. Results

3.1. Demographic and clinical parameters

Sixty-eight patients with idiopathic PD and 36 controls were enrolled at Hospital Valme and Hospital Macarena, but eight saliva samples (4 patients, 4 controls) were discarded due to high hemoglobin concentration or technical problems. Hence, the final number was 64 patients and 32 controls. First, we verified that basic demographic features were not found to be different between controls and patients with idiopathic PD. Demographic and clinical parameters of the patients and controls are shown in Table 1.

3.2. Salivary ATP13A2

Salivary ATP13A2 was undetectable in 18 out of 64 patients (28 %), and in 14 out of 32 control participants (43.7 %). The detection threshold of the ELISA assay (~230 pg/ml) was a limitation for quantifying ATP13A2 levels in the saliva.

In the cohort of patients, a significant correlation was found between salivary ATP13A2 level and MDRS-UPDRS part-IV score ($r = 0.344$, $p = 0.0053$), a scale which allows evaluating motor complications in PD patients that include motor fluctuations and dyskinesias. In addition, all the patients with motor complications (n = 28) showed quantifiable levels of salivary ATP13A2 (Table 1, Fig. 1). For these reasons, the group of patients with motor complications was further studied. Main clinical parameters of PD patients with and without motor complications are shown in Table 1. Individual values of salivary ATP13A2 content in patients with motor complications and controls are illustrated in Fig. 1.

After comparing the subgroup of patients with motor complications with control participants with quantifiable ATP13A2 levels (n = 18), ATP13A2 concentration was found to be significantly higher in PD patients with motor complications relative to controls ($t = 5.9245$, $p < 0.0001$), as shown in Table 1. Salivary ATP13A2 content in patients with motor complications significantly correlated with MDS-UPDRS part-IV ($r = 0.566$, $p = 0.0016$), MDS-UPDRS part-III ($r = 0.388$, $p = 0.0413$), total MDS-UPDRS ($r = 0.391$, $p = 0.0396$), and LEDD ($r = 0.384$, $p = 0.0436$), as shown in Fig. 1. Total MDS-UPDRS (parts I-III) is a scale that allows evaluating motor deficits and non-motor experiences of daily living. MDS-UPDRS part-III is the rating scale for motor deficits. LEDD also correlated with MDS-UPDRS part IV (all patients, $r = 0.643$, $p < 0.001$; patients with motor complications, $r = 0.538$, $p = 0.0031$). Statistical correlations of PD patients with motor complications are shown in Supplementary Table 1. Scores of disease severity, disease duration, and LEDD were significantly higher in patients with motor complications relative to patients without motor complications (Hoehn-Yahr, $t = 4.002$, $p = 0.0002$; MDS-UPDRS part-III, $t = 11.4647$, $p < 0.0001$; total MDS-UPDRS, $t = 5.946$, $p < 0.0001$; disease duration, $t = 2.7328$, $p = 0.0082$; LEDD, $t = 4.0827$, $p = 0.0001$, Table 1).

Table 1
Demographic and clinical parameters, as well as salivary ATP13A2 concentration in PD patients and control subjects.

	All patients (n = 64)	Patients without motor complications (n = 36)	Patients with motor complications (n = 28)	Controls (n = 32)
Demographic and clinical parameters				
Age (years)	65.5 ± 11.7	65 ± 12	66.5 ± 11	61.4 ± 10
Gender, male n (%)	33 (52)	19 (53)	14 (50)	18 (56)
Body mass index	24.3 ± 2.2	24.2 ± 2.1	24.4 ± 2.3	25.6 ± 2.6
Education (years)	18.1 ± 2.4	17.9 ± 2.5	18.2 ± 2.2	17.8 ± 1
Levodopa equivalent daily dose (mg)	682 ± 644	427 ± 520	1008 ± 618 **	
Disease duration (years)	13.3 ± 6.7	11.5 ± 5	15.6 ± 7 **	
Age at PD onset (years)	55.6 ± 11	56.5 ± 12	54.5 ± 9	
Hoehn-Yahr stage	2.2 ± 0.9	1.8 ± 0.7	2.6 ± 0.9 **	
Modified Schwab-England	81.8 ± 24	86 ± 20	76.8 ± 28	
MDS-UPDRS part III (on)	20.7 ± 15	13.1 ± 10	49 ± 15 ***	
Total MDS-UPDRS (on)	34.6 ± 26	21 ± 14	52 ± 27 ***	
MDS-UPDRS part IV	1.7 ± 2.6	0	3.7 ± 2.9	
ATP13A2 content (pg/ml)	nd (n = 18)	nd (n = 18)	983 ± 361 ##	nd (n = 14) 426 ± 209 (n = 18)
Patients with motor complications (without or with dyskinesias)				
	Without dyskinesias (n = 17)	With dyskinesias (n = 11)		
ATP13A2 content (pg/ml)	959 ± 370	1001 ± 304		
Levodopa equivalent daily dose (mg)	762 ± 394	1168 ± 699 §		
MDS-UPDRS part III (on)	30.5 ± 18	29.5 ± 13		
Total MDS-UPDRS (on)	53.1 ± 31	50.5 ± 25		
MDS-UPDRS part IV	1.7 ± 0.9	5.1 ± 3 §§		

Mean ± SD, ** $p < 0.01$, *** $p < 0.001$ vs patients without motor complications; ## $p < 0.0001$ versus controls with quantifiable ATP13A2 levels; § $p < 0.05$, §§ $p < 0.01$ versus patients without dyskinesias. Statistical comparisons were carried out with the χ^2 test (dichotomous variables) or the Student's *t*-test or the Mann-Whitney *U* test (quantitative variables). Abbrev.: ATP13A2, ATPase Cation Transporting 13A2; nd, nondetectable; MDS-UPDRS, International Parkinson and Movement Disorder Society-Sponsored revision of the Unified Parkinson's Disease Rating Scale; PD, Parkinson's disease.

To further studying patients with motor complications, this group was divided into patients with motor fluctuations and without dyskinesias (n = 17), and patients with motor fluctuations and dyskinesias (n = 11). LEDD and MDS-UPDRS part-IV score were found to be

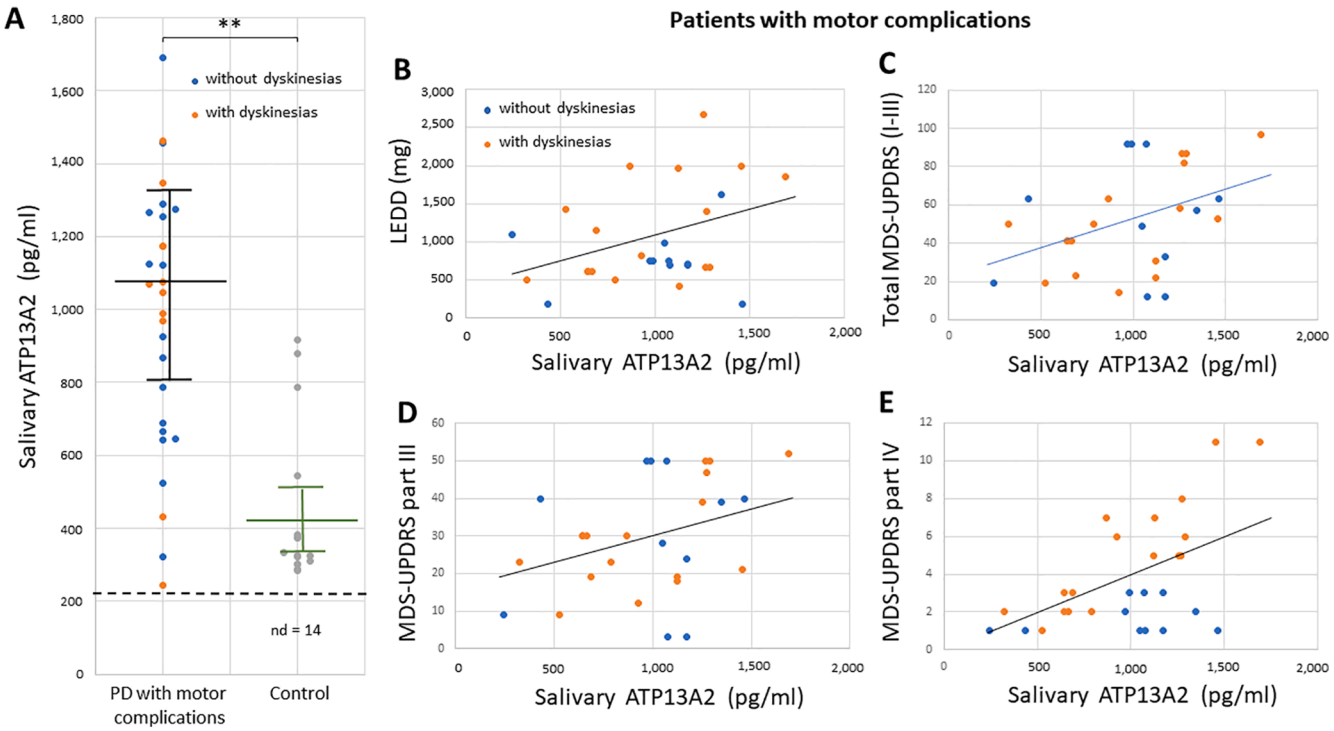

Fig. 1. (A) Individual concentration of ATP13A2 in the saliva in patients with motor complications (n = 28) and controls with detectable ATP13A2 levels (n = 18); and correlation of salivary ATP13A2 concentration with (B) levodopa equivalent daily dose, (C) total MDRS-UPDRS, (D) MDRS-UPDRS part-III, and (E) MDRS-UPDRS part-IV in patients with motor complications. Patients with dyskinesias (n = 17) and patients without dyskinesias (n = 11) are represented in orange and blue, respectively. Correlation lines are those of the group of patients with motor complications as a whole. Mean ± SD, ** $p < 0.001$ vs controls (green). Abbrev.: ATP13A2, ATPase Cation Transporting 13A2; LEDD, levodopa equivalent daily dose; nd, nondetectable (n).

significantly higher in patients with dyskinesias relative to patients without dyskinesias (LEDD, $t = 2.0678$, $p = 0.0487$; MDS-UPDRS part-IV, $t = 4.4154$, $p = 0.0002$). Main clinical parameters of both subgroups are shown in Table 1.

3.3. *ATP13A2 immunohistochemical expression in the submandibular gland*

The immunohistochemical study of the submandibulary gland revealed that ATP13A2 is expressed by most regions of the organ. A very intense ATP13A2 staining was observed in glandular duct cells (apical region and villi) and vascular endothelial cells, in both the patients and controls (see Fig. 2). Acinar cells and red blood cells showed diffuse and moderate ATP13A2 staining (Fig. 2). Mild intensity of ATP13A2 signal was observed in the interlobular connective tissue in the patients and controls, and adipocytes scarcely expressed ATP13A2. Duct cells showed a cytoplasmic gradation of immunoreactivity because the basal region of the cell presented less intense ATP13A2 expression than the apical region (Fig. 2).

Regarding ATP13A2-expressing inclusions, two patterns of deposits were observed: lumenal aggregates in duct cells, and rounded deposits in the interlobular tissue. First, in both patients and controls, deposits with amorphous shape and fibrils were observed inside lumen of gland ducts. These lumenal inclusions are found in 5/6 IPD patients and 4/6 controls. Second, the interlobular connective tissue of the patients, but not controls, contained aggregates of spheroid/ovoid shape (Fig. 2). Spheroid or ovoid shape might result from a different viewing angle. These rounded inclusions had a diameter of 10–20 μm and were found in 5/6 IPD patients. The intensity of ATP13A2 staining in regions of the submandibulary gland along with density degree of ATP13A2-expressing inclusions in patients and controls are shown in Table 2. There were no significant differences in age (patients with IPD, 66.8 ± 8 years; control participants, 63.5 ± 10 years), and gender between the patients and controls.

4. Discussion

The sensitivity of the ELISA assay was a major limitation for quantifying ATP13A2 levels in the saliva because this molecule was below the detection threshold in many subjects. However, all the patients with motor complications (motor fluctuations and dyskinesias) showed quantifiable levels of ATP13A2. In these patients, MDS-UPDRS and LEDD correlated with salivary ATP13A2 content. In other words, patients with motor fluctuations and dyskinesias were treated with a high-intensity dopaminergic regimen, as measured with LEDD, and presented high salivary ATP13A2 content in comparison with controls with quantifiable levels.

Motor fluctuations are variable motor responses to medication, and dyskinesias are involuntary random movements. Motor complications are attributed to the interaction of intense dopaminergic therapy, advanced nigrostriatal cell loss, aging and genetic factors [2,28,29]. A role of the pharmacological factor in the development of motor complications was corroborated in this study. Dyskinetic patients were those with the highest LEDD values. Patients with motor complications were at advanced stages of the disease, as revealed by the Hoehn-Yahr staging and disease duration in years. Therefore, quantifiable high level of salivary ATP13A2 in patients with motor complications could be accounted for by pharmacological effects of levodopa and dopaminergic agents and advanced disease.

Sustained dopaminergic stimulation is known to induce oxidative damage and inflammation in vascular endothelial cells [28], and the histological study showed very intense expression of ATP13A2 in vascular endothelial cells. High dopaminergic stimulation is also associated with excess of reactive oxidant species and lysosome dysfunction [29], mechanisms that are linked to ATP13A2 activity [2,6,7]. Another possibility is that we are measuring ATP13A2 coming from intestinal

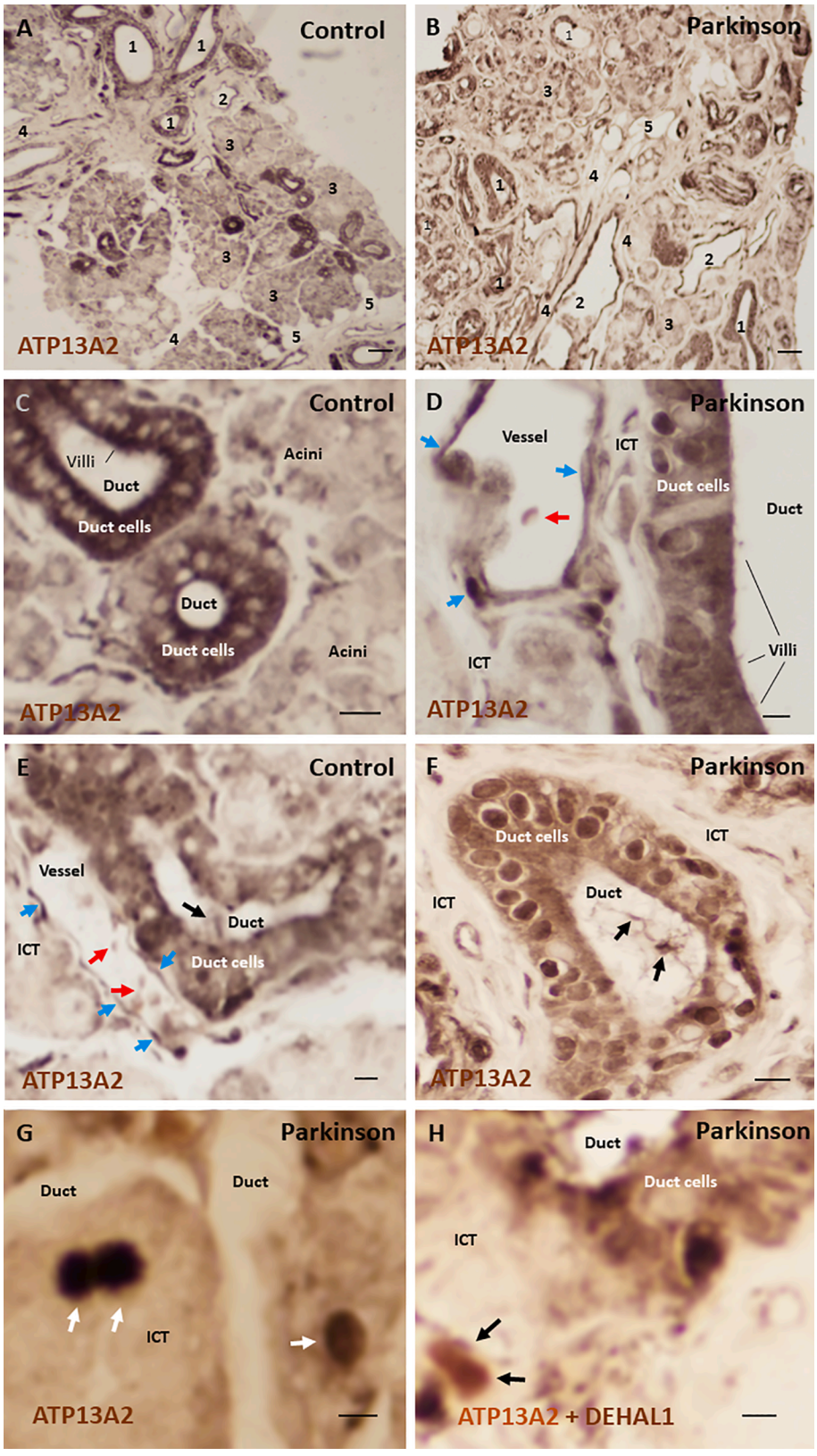

Fig. 2. Representative photomicrographs of submandibulary gland sections in patients with idiopathic Parkinson's disease and controls, after immunostaining against ATP13A2 and DEHAL1. (A, B) Low magnification submandibulary gland sections in a patient and a control participant showing main glandular regions and cell types, as well as intensity of ATP13A2 expression (1, duct cells; 2, vessels with vascular endothelial cells; 3, acinar cells; 4, interlobular connective tissue; 5, adipocytes). (C, D) High-magnification sections of the submandibulary gland showing main cell types that express ATP13A2 in the patients and controls (vascular endothelial cells, blue arrows; red blood cells, red arrows). In C, the difference in staining intensity between excretory duct cells and secretory acinar cells can be observed. In C and D, duct cells show an intracellular gradation of ATP13A2 immunoreactive staining, and the basal region shows less intensity of ATP13A2 expression than the apical region and villi in both the patients and controls. Mild intensity of staining is observed in the interlobular connective tissue. (E-F) ATP13A2-positive deposits inside duct lumen are noticed in the patients and controls (black arrows). These luminal inclusions show amorphous morphology with some fibrils spreading over the lumen. (G-H) Rounded inclusions of 10–20 μm in diameter are observed in the interlobular connective tissue of the submandibular gland in patients (black and white arrows). Abbrev.: ATP13A2, ATPase Cation Transporting 13A2; DEHAL1, dehalogenase type 1; ICT, interlobular connective tissue. Bars: 10 μm.

epithelial cells because ATP13A2 is highly expressed in intestinal cells [30]. However, the role of intestinal ATP13A2 in the saliva might be low, since patientś salivary glands presented abundant rounded aggregates expressing ATP13A2, likely reflecting the glandular origin of salivary ATP13A2. To sum up, the presence of high salivary ATP13A2 level would reflect oxidative and inflammatory effects of dopaminergic therapy on vascular endothelial cells of submandibulary glands. The data allow proposing that salivary ATP13A2 might be a reliable marker for the occurrence of therapy-induced motor complications in PD patients. The relevance of salivary ATP123A2 as a marker of oxidative/

Table 2
Demographic, clinical, and neuropathological data from all cases studied with idiopathic PD and controls.

Demographic and clinical parameters					Intensity of ATP13A2 staining				Density of ATP13A2-positive inclusions (location)	
Case	Gender	Age (y)	H-Y	Disease duration (y)	Duct cells (basal–apical cytoplasm)	Vascular endothelial cells	Acinar cells & Red blood cells	Interlobular connective tissue	Rounded inclusions (Inter-lobular tissue)	Lumenal aggregates (Ducts)
PD1	F	76	2	6	3–4	4	2	1	1	1
PD2	M	73	2.5	8	3–4	4	2	1	1	1
PD3	M	56	2	6	3–4	4	2	2	1	1
PD4	M	55	2	7	3–4	4	2	2	1	2
PD5	M	70	1.5	5	3–4	4	2	1	1	0
PD6	F	71	2.5	7	3–4	4	2	1	0	1
CT1	M	66			3–4	4	2	1	0	1
CT2	F	55			3–4	4	2	1	0	0
CT3	F	57			3–4	4	2	1	0	1
CT4	F	66			3–4	4	2	2	0	0
CT5	M	77			3–4	4	2	1	0	1
CT6	M	60			3–4	4	2	1	0	1

The intensity of immunoreactive ATP13A2 staining within different cell regions of the submandibular gland was assessed in contiguous tissue sections according to a five-point rating scale: no expression (0), mild (1), moderate (2), intense (3), and very intense (4). The presence of aggregates was also analyzed, and the density of immunoreactive inclusions within different regions of the submandibular glands was assessed according to a five-point rating scale: not detectable (0), mild (1), moderate (2), frequent (3), and very frequent (4). Abbrev.: PD, Parkinson's disease; CT, control; F, female; M, male; H-Y, Hoehn-Yahr stage; y, years.

inflammatory effects of dopaminergic medication and motor complications in PD patients is worthy of further investigation.

As regards the histological study, ATP13A2 was differentially expressed by most cell types in the submandibulary gland. This finding fits well with the ubiquitous bodily distribution of the protein [12,13]. Tissue distribution and intensity of ATP13A2 staining seem to be similar in both the patients and controls. The most intense ATP13A2 staining was observed in glandular duct cells and the vascular endothelium. The strong expression of ATP13A2 in duct cells and vascular endothelium would indicate that this protein might subserve important functions in these regions. The expression of ATP13A2 in secretory acinar cells was found to be fainter than that in excretory duct cells. ATP13A2 is involved in several crucial steps of excretory and secretory activity such as cargo trafficking, handling of metals and lipids, endo-/lysosome function, and exosomal release [6–11].

Two patterns of deposits expressing ATP13A2 were observed in the submandibulary gland: amorphous aggregates in duct lumen, and rounded inclusions in the connective tissue. ATP13A2-positive deposits with amorphous shape and fibrils were observed inside lumen of gland ducts in both the patients and control participants. Lumenal aggregates that express α-synuclein have been described by other authors in salivary glands in patients with PD [18], but ATP13A2-positive lumenal inclusions have not been described so far. The observation that these deposits are observed in all subjects suggest that they are not of pathological significance, and it is possible that they represent an aging or involutional change in salivary ducts.

A novel result of this study is that the interlobular connective tissue of the submandibulary gland contained aggregates of spheroid/ovoid shape with 10–20 μm in diameter. This morphology resembles previous descriptions of phosphorylated-αSyn-positive inclusions of rounded morphology within the interlobular tissue in salivary glands [17,18,20]. Previous double-staining studies with antibodies against neural markers such as neurofilaments or protein-G product indicate the neuronal identity of these rounded inclusions within the interlobular tissue [18]. We are unable to confirm the neuronal identity of these inclusions, and their location within the interlobular connective tissue (where salivary neural fibers are located) would suggest that these deposits derive from neuronal secretion. ATP13A2 has been reported to be expressed by Lewy bodies in the brain, but this is the first description of ATP13A2-expressing rounded inclusions outside the nervous system [6,8,11]. This is an important issue because formation of rounded proteinaceous aggregates is linked to the etiology of Parkinson's disease and other neurodegenerative disorders [1,2].

In conclusion, the sensitivity of the ELISA assay was a major limitation for quantifying ATP13A2 in the saliva. However, salivary ATP13A2 was detected in all patients with motor complications, where a direct relationship among ATP13A2 concentration, levodopa equivalent daily dose, and MDS-UPDRS was found. Therefore, salivary ATP13A2 might be a reliable index of therapy-induced motor complications. Finally, we have detected, for the first time, rounded ATP13A-positive aggregates in the submandibulary gland, an organ that is located outside the nervous system.

This study has some limitations that should be acknowledged. We are unable to infer causation because it is a cross-sectional design, not a longitudinal study. The cohorts of participants were relatively small, and the detection threshold of the ELISA kit was a major limitation for quantifying ATP13A2 concentration in the saliva. It is also possible that salivary ATP13A2 reflects dopamine liver metabolism in patients taking tolcapone/entacapone or in subjects with unknown liver disease (liver function was not checked) [30]. The study also included some patients under 50 years of age, an approach that increases the chance of including monogenic forms of PD. The results must be confirmed by means of larger samples in future studies, and the use of more sensitive methods. Strengths of our study include well-characterized patients with PD, rigorous collection of data, and careful selection of participants.

5. Ethics approval

All protocols were approved by the internal ethics and scientific boards of Hospital Universitario Valme (CEI Valme, #10/05/2018 & #1694-M1-19), Hospital Universitario Macarena (CEI #19/05/2010 & #2149-29/10/2013), Comité de Etica de Investigación de la Junta de Andalucia (PEIBA, CEI Sevilla-Sur #201510520554-1 & #2017121418738), and University of Seville (CEE-27/05/2010 & SeccionInvestigacion/Gd-21/06/2010). The subjects' consent was obtained according to the Declaration of Helsinki (BMJ 1991; 302: 1194).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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