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Biochemical analysis of α -synuclein extracted from control and Parkinson's disease colonic biopsies

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h i g h l i g h t s

- Alpha-synuclein deposits are found in the gut in PD.
 - Alpha-synuclein expression levels and phosphorylation status were analyzed by biochemical means in 17 PD patients.
 - No differences in the expression levels, phosphorylation and aggregation status of alpha-synuclein was observed between controls and PD.
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- Our study suggests that the biochemical methods tested are not adequate for the prediction of PD using gastrointestinal biopsies.

a r t i c l e i n f o

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Lewy bodies and neurites, the pathological hallmarks found in the brain of Parkinson's disease (PD) patients, are primarily composed of aggregated and hyperphosphorylated alpha-synuclein. The observation that alpha-synuclein inclusions are also found in the gut of the vast majority of parkinsonian patients has led to an increasing number of studies aimed at developing diagnostic procedures based on the detection of pathological alpha-synuclein in gastrointestinal biopsies. The previous studies, which have all used immunohistochemistry for the detection of alpha-synuclein, have provided conflicting results. In the current survey, we used a different approach by analyzing the immunoreactivity pattern of alpha-synuclein separated by one- and two-dimensional electrophoresis, in colonic biopsies from PD subjects and healthy individuals. We did not observe any differences between controls and PD in the expression levels, phosphorylation or aggregation status of alpha-synuclein. Overall, our study suggests that the two biochemical methods tested here are not adequate for the prediction of PD using gastrointestinal biopsies. Further studies, using other biochemical approaches, are warranted to test whether there exists specific forms of pathological alpha-synuclein that distinguish PD from control subjects.

1. Introduction

In recent years, the neuronal 140 amino acid protein alpha-synuclein has attracted much attention because of its involvement

in neurodegenerative diseases and more specifically in Parkinson's disease (PD). Five independent mutations in human alpha-synuclein cause familial PD, and wild type alpha-synuclein is one of the main components of Lewy bodies, the characteristic intraneuronal inclusions found in the brain of parkinsonian patients [1]. When deposited in Lewy bodies, alpha-synuclein is aggregated and hyperphosphorylated at serine 129 [2,3].

Alpha-synuclein inclusions that resemble those observed in the brain are also found in the gut of the vast majority of PD patients [4,5]. These findings prompted a substantial amount of research to determine if the immunohistochemical detection of aggregated

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Table 1

Demographic data for patients with Parkinson's disease (PD).

Patient number	Gender	Age (y)	Disease duration (y)
PD1	F	56	7
PD2	F	64	10
PD3	M	67	13
PD4	F	55	5
PD5	M	71	12
PD6	M	67	9
PD7	M	70	14
PD8	F	70	11
PD9	F	53	6
PD10	F	52	8
PD11	M	58	10
PD12	F	72	3
PD13	F	48	7
PD14	F	69	12
PD15	M	63	8
PD16	F	61	7
PD17	M	62	6

Table 2

Demographic data for control subjects. CRC: colorectal cancer.

Control number	Gender	Age (y)	Reason for colonoscopy
C1	F	61	CRC screening
C2	F	67	Rectorrhagia
C3	F	63	CRC screening
C4	M	45	CRC screening
C5	F	19	Abdominal pain
C6	M	76	CRC screening
C7	F	63	CRC screening
C8	M	62	CRC screening
C9	F	69	CRC screening
C10	F	63	CRC screening
C11	M	72	CRC screening
C12	F	69	CRC screening
C13	M	74	CRC screening

and/or phosphorylated alpha-synuclein in routine gastrointestinal biopsies could be used for *in vivo* pathological diagnosis of PD [6–11]. These studies gave conflicting results regarding the sensitivity and specificity of colonic biopsies for the detection of pathological alpha-synuclein inclusions. This was confirmed by a more recent multi-center study, which showed limited diagnostic power of quantifying alpha-synuclein deposition in gastrointestinal biopsies by immunohistochemistry [12]. No studies have yet used biochemical methods such as immunoblotting to detect pathological alpha-synuclein in gastrointestinal biopsies, despite the generally higher sensitivity of these techniques when compared to immunohistochemistry. We therefore undertook the current study to determine if analysis of alpha-synuclein expression and its phosphorylation by one- and two-dimensional immunoblotting would allow the differentiation of colonic samples from PD subjects relative to healthy controls.

2. Patients and methods

2.1. Human colonic biopsies and brain samples

Seventeen patients with PD who were diagnosed according to the United Kingdom PD Society Brain Bank criteria (Table 1, mean age 62.3 ± 7.7) and 13 healthy subjects (Table 2, mean age 61 ± 15) participated in the study. Control subjects underwent a colonoscopy for colorectal cancer screening or for investigation of gastrointestinal symptoms (Table 2). All control subjects underwent a detailed neurological examination to rule out PD symptoms and cognitive deficiency. Biopsies were taken in the sigmoid/descending colon during the course of a rectosigmoidoscopy for PD. The study protocol was approved by the local Committee

on Ethics and Human Research (*Comité de Protection des Personnes Ouest IV and VI*), conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was registered on ClinicalTrials.gov (identifier NCT01353183 and NCT01748409). Written informed consent was obtained from each patient and from each normal volunteer. Biopsies were snap frozen in liquid nitrogen at the time of collection and stored at –80 °C until required. Samples of frozen temporal cortex from two postmortem human brains, one with dementia with Lewy bodies (DLB) and one devoid of neurodegeneration, were obtained from the Neuropathology Department of Angers (Dr. Franck Letournel).

2.2. One-dimensional immunoblotting

The biopsies from 10 PD patients (Table 1, PD1 to PD10) and 9 control subjects (Table 2, C1–C9) were lysed in NETF buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF) containing 1% (v/v) Igepal CA-630 (Sigma-Aldrich, Saint-Quentin Fallavier, France), phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and protease inhibitor cocktail (Roche), homogenized using the “Precellys 24” tissue homogenizer (Bertin technologies, Saint Quentin-en-Yvelines, France),

sonicated and centrifugated at 16,000g for 20 min at 4 °C. Supernatants were analyzed by Western Blot as described previously [13] using rabbit polyclonal anti-alpha-synuclein (C20)-R (Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000) and mouse monoclonal anti-alpha-synuclein Syn-1 (BD Bioscience, Le pont de Claix, France, 1:1000) antibodies to detect total alpha-synuclein, a mouse monoclonal Psyn#64 (Wako, Sodioba, France, 1:500) antibody for the detection of phosphorylated alpha-synuclein and mouse monoclonal antibody anti-Protein Gene Product 9.5 (PGP 9.5; Ultraclone, Isle of Wight, UK, 1:1000) as a general neuronal marker. Enhanced membrane retention of alpha-synuclein was achieved by treating the sample lysate with the cross linker dithio-bis[succinimidylpropionate] (DSP) (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 1 mM [13,14]. The relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed using NIH Image J software. The abundance of total alpha-synuclein was normalized to the amount of PGP 9.5 in each sample, and is expressed as percentage average control. Amounts of phosphorylated alpha-synuclein were normalized to total alpha-synuclein in each sample. All data shown are mean standard error of the mean (SEM). For statistical comparison between groups, a Mann–Whitney test was performed. Differences were deemed statistically significant if $p < 0.05$.

2.3. Two-dimensional immunoblotting

The remaining biopsies (7 PD, PD11–PD17 and 4 controls, C10–C13, Tables 1 and 2, respectively) were lysed in UTC buffer (7 M urea, 2 M thiourea, 4% CHAPS, all from Sigma) containing 0.8% ZOOM® Carrier 3–10 or 4–7 ampholyte buffer (NuPAGE, Life technologies, Cergy-Pontoise, France), 60 mM dithiothreitol (DTT) (Sigma) and protease inhibitor cocktail (Roche), then homogenized using the “Precellys 24” tissue homogenizer (Bertin technologies, Saint Quentin-en-Yvelines, France). Samples (48 µl) were then diluted in a rehydration sample buffer with a trace of bromophenol blue and Isoelectric focusing was performed using the ZOOM® IPGRunner™ System on 3–10 or 4–7 immobilized pH Gradient gels (Life technologies). Gels were equilibrated twice for 15 min with Lithium dodecyl sulfate Sample Buffer (NuPage). The first equilibration step contained 50 mM DTT and the second 125 mM iodoacetamide (Sigma). Lysates were separated on 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and proteins electrotransferred to polyvinylidene difluoride (PVDF) membranes (NuPAGE, Life technologies). Post-transfer membranes

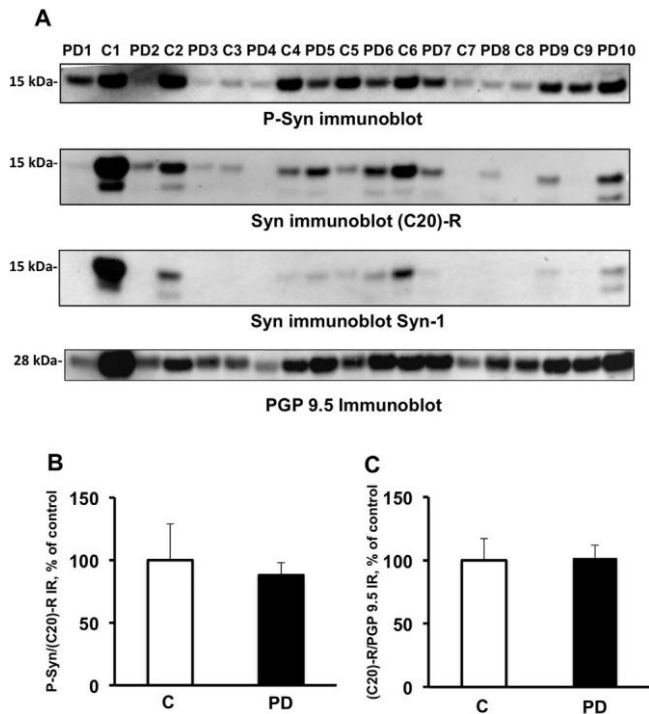


Fig. 1. There are no differences in the expression levels or phosphorylation state of alpha-synuclein in colonic biopsies between control subjects and patients with Parkinson's disease. (A) Colonic biopsies from 10 PD patients (Table 1, PD1–PD10) and 9 control subjects (Table 2, C1–C9) were lysed in NETF/Igepal CA-630 buffer. Biopsy lysates (20 μ g of protein per sample) were subjected to immunoblot analysis using antibodies against total alpha-synuclein (Syn immunoblot, C20-R and Syn-1), phosphorylated alpha-synuclein (P-Syn immunoblot) and PGP 9.5 (PGP 9.5 immunoblot). The optical densities of (C) total alpha-synuclein (C20-R) and (B) phosphorylated alpha-synuclein immunoreactive bands were measured, normalized to total alpha-synuclein immunoreactive bands, respectively, and expressed as percentage control. Data correspond to mean \pm SEM of 9 control subjects (C) and 10 samples for Parkinson's disease (PD) patients. No significant changes were observed between the two groups, ($p = 0.9$ and 0.75 , respectively).

were fixed with 0.4% (v/v) paraformaldehyde (PFA, Sigma) for 30 min and alpha-synuclein immunoblotting was performed as previously described [13].

3. Results

In a first set of experiments, we analyzed by Western blotting the expression levels and phosphorylation status of alpha-synuclein in colonic biopsies from control and PD subjects. A band migrating to 15 kDa on Western blot was observed using (C20)-R and Syn-1 antibodies that recognize different epitopes on alpha-synuclein, thus confirming its identity (Fig. 1A). In some samples, a faster migrating 12–13 kDa band was also observed (Fig. 1A). Such a band has already been described in platelets [15] and cerebrospinal fluid [16] and might correspond to a caspase-truncated form of the protein [17]. Despite equal protein loading, the 15-kDa alpha-synuclein band density was extremely variable (Fig. 1A), with no significant difference between PD patients and controls being found when alpha-synuclein amounts were normalized to PGP 9.5 in each sample (Fig. 1C). Phosphorylated alpha-synuclein was identified in all PD and control samples (Fig. 1A), but in both groups the amount detected was also highly variable and following normalization to total alpha-synuclein no significant differences were found between PD and control cases (Fig. 1B). No difference in the

immunoreactivity of the 12–13 kDa band was observed between PD and controls (data not shown).

The small amount of pathological aggregated and hyperphosphorylated alpha-synuclein present in biopsy samples may have remained largely unsolubilized because of the relatively low-stringency buffer that was used in our one-dimensional immunoblotting experiments. This prompted us to perform a second set of experiments in which samples were lysed with the high stringency UTC buffer then subsequently analyzed by two-dimensional immunoblotting. As previously reported [3], we first showed that this technique was capable of detecting a series of alpha-synuclein species that are unique to DLB brain when compared to control brain, including high molecular weight alpha-synuclein species as well as acidic full-length species (Fig. 2A). However, when this technique was applied to colonic biopsies, the patterns from control subjects and PD patients were qualitatively indistinguishable from each other, with only total unmodified alpha-synuclein being detected in both conditions (Fig. 2B and supplementary Fig. 1).

4. Discussion

Based on the topographic distribution of Lewy bodies and neurites established after autopsy from PD patients, Braak and coworkers hypothesized that the gastrointestinal tract and the olfactory bulb might be trigger sites of alpha-synuclein pathology [18,19]. The pathology would then subsequently spread to the brainstem via the vagus nerve and connected brain regions [20]. This hypothesis has been reinforced by the discovery that alpha-synuclein is capable of spreading transcellularly in a “prion-like” manner, thereby providing a cellular basis for Braak's staging scheme that describes PD progression [21]. These ‘gut to brain’ and ‘nose to brain’ scenarios have ignited heated debates within the movement disorders community and have prompted a large number of studies in both humans and animals. Two recent studies showed that injection of fibrillar alpha-synuclein into the intestinal wall [22] or into the olfactory bulb [23] in rodents leads to propagation of alpha-synuclein pathology in the brain. In human, the accessibility of both the olfactory and enteric nervous systems to biopsies led to interest in utilizing these tissues as more accessible biomarker material for PD and a substantial amount of research has now been conducted to develop original histopathological markers of PD in these tissues [24,25]. Being the only component of the olfactory system accessible to biopsy, the olfactory epithelium has been screened for Lewy pathology. Several independent autopsy studies showed that alpha-synuclein deposits were observed in the olfactory mucosa in subjects with PD [26–29]. However, a pilot *in vivo* study reported no evidence of disease-specific pathology in the olfactory epithelium in seven hypo/anosmic PD patients [30]. Conflicting results have been gained following analysis of gastrointestinal biopsies, all of which have used immunohistochemical methods to detect alpha-synuclein, and there is not yet any fully validated biochemical method for detecting aggregated and/or phosphorylated alpha-synuclein in peripheral tissues [12,31].

Despite the growing interest in peripheral tissue for the detection of PD pathology [24], very few studies have used biochemical approaches including immunoblotting to examine pathological alpha-synuclein in peripheral autonomic networks. One likely explanation is that alpha-synuclein detection by Western blot has proven difficult because the protein is easily washed off from both PVDF or nitrocellulose membranes, making it difficult to detect especially in tissues expressing low levels of the protein [32]. This prompted several groups to develop methods for improving alpha-synuclein retention on blot membranes by increasing its hydrophobicity. This included fixation of membranes with PFA

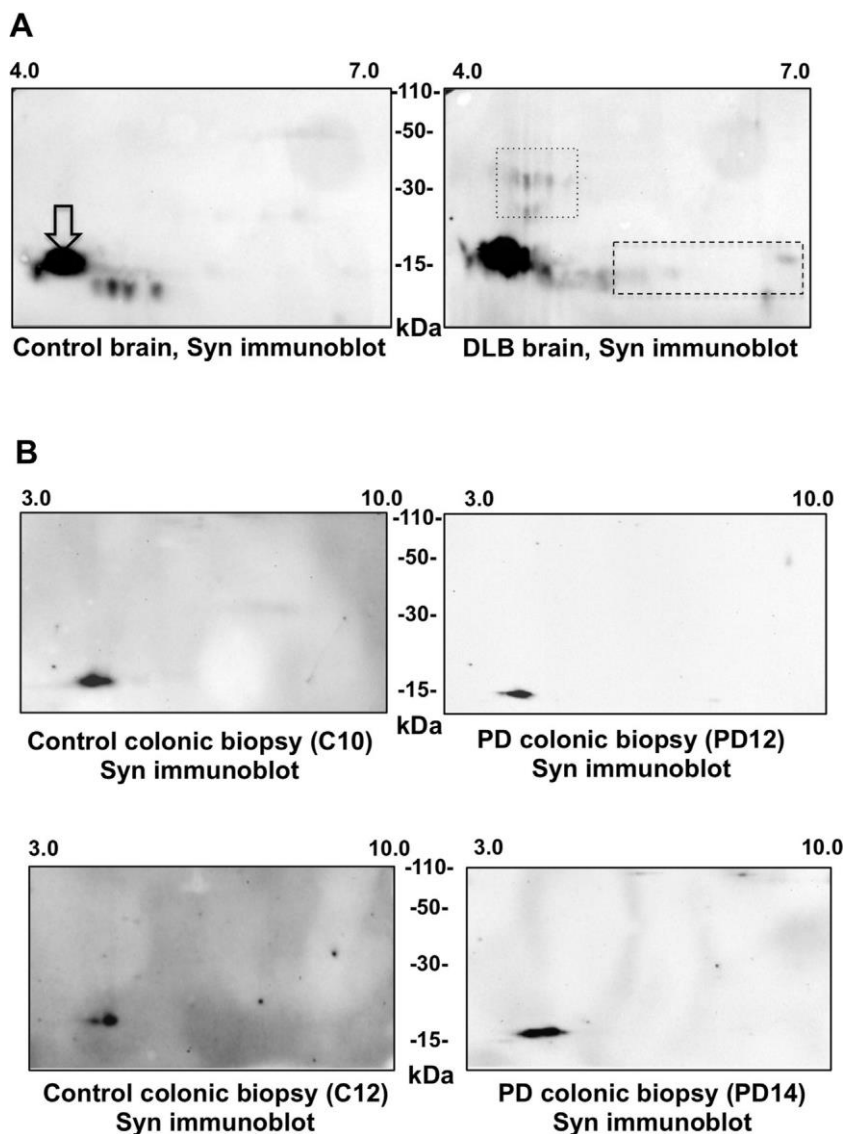


Fig. 2. There are no differences in the two-dimensional immunoblotting pattern of alpha-synuclein extracted from colonic biopsies from control subjects and patients with Parkinson's disease. (A) Immunoblots of brain proteins from a control subject (Control brain) and a DLB patient lysed in UTC buffer and resolved by two-dimensional electrophoresis using pH 4–7 isoelectric focusing gradients were probed with an antibody against Syn-1 (Syn immunoblot). White arrows indicate full-length alpha-synuclein. The boxes in the DLB brain immunoblot indicate differences between control and DLB brain: acidic modifications of full-length monomer (dashed) and high molecular weight species (dotted). (B) Colonic biopsies from 2 PD patients (Table 1, PD 12 and PD14 colonic biopsy) and 2 control subjects (Table 2, C10 and C12 colonic biopsy) were lysed in UTC buffer and resolved by two-dimensional electrophoresis using pH 3–10 isoelectric focusing gradients and probed with the C20-R antibody against total alpha-synuclein. The figure shown is representative of the 2-D immunoblot experiments performed in 7 PD and 4 control subjects.

or treatment of samples with the cross-linker DSP [14,32]. We have recently shown that these two methods are indeed efficient at improving the sensitivity of western blotting to detect alpha-synuclein immunoreactivity in gut samples or primary ENS cultures [13].

We therefore took advantage of these two methods to analyze the expression levels and the phosphorylation status of alpha-synuclein in colonic biopsies from control and PD subjects. The cross-linker DSP was used in experiments involving one-dimensional immunoblotting while PFA-membrane fixation was used in conjunction with two-dimensional immunoblotting. Using one-dimensional immunoblotting, we showed that alpha-synuclein was detected in samples from all PD and control samples, however in both groups the amounts detected were highly variable and no differences between groups were found following normalization of alpha-synuclein amounts to that of the neuronal marker

PGP 9.5. Given that alpha-synuclein is found in nerve terminals and cell bodies of submucosal neurons captured by the biopsy forceps [33–35], it might be postulated that this variability between biopsies relates to variation in the tissues sampled including omission of submucosal neuronal processes [12]. Immunoreactivity for phosphorylated alpha-synuclein was also observed in PD and control, a finding that is likely to be explained by the fact that a small amount (around 4 %) of alpha-synuclein is physiologically phosphorylated at serine 129 in the brain [36] and in the gut [34]. After quantification of phosphorylated alpha-synuclein amounts as a proportion of total alpha-synuclein, we did not observe any difference between PD and healthy subjects.

Two-dimensional immunoblotting is a sensitive technique for identifying protein posttranslational modifications and aggregates, and we therefore used this technique to assess a second set of colonic samples. As previously reported, this approach enabled

the detection of acidic and high molecular weight alpha-synuclein species in DLB brain, that likely represent hyperphosphorylated and aggregated forms of the protein, respectively [3]. However, these species were not observed in colonic samples from PD subjects, which contained only full-length unmodified alpha-synuclein.

Overall, our study suggests that the two biochemical methods investigated here are not sufficient to detect pathological alpha-synuclein species in gastrointestinal biopsies, and therefore have limited utility for predicting PD. This may be due to the relative rarity of neuronal structures and/or alpha-synuclein inclusions in the gastrointestinal samples analyzed, or as a result of the relatively low sensitivity of the methods tested. Such limitations could be overcome in future studies by using ultrasensitive techniques such as protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) assays, which have been recently shown to efficiently amplify aggregated alpha-synuclein [37,38].

Conflict of interest

The authors declare no actual or potential conflict of interest.

Author's contribution

EC performed the colonoscopy and biopsies in PD patients. MRD performed the biopsies for control individuals. AGC, CP and TL performed and analyzed the biochemical experiments. PD and MN designed the study. PD supervised the study, wrote the first draft and the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2017.01.050>.

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