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Supplementary Materials for

The vermiform appendix impacts the risk of developing Parkinson's disease

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The PDF file includes:

References (*92*–*96*)

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/465/eaar5280/DC1)

Data file S1 (Microsoft Excel format). Source data for biochemical assays.

Materials and Methods

Measuring proteinase K resistant α-synuclein

Formalin-fixed, paraffin-embedded appendix samples were obtained from the Van Andel Biorepository and Pathology Core. Sections from each tissue were reviewed by a trained pathologist. Protocols were approved by the Van Andel Research Institute review board (IRB # 15025).

Fixed and paraffin-embedded appendix were sectioned 5 µm thick onto glass slides. Slides were heated for 10 min at 50°C and placed into fresh xylene three times, 5 minutes each time. Rehydration was then performed by placing slides in 100% ethanol three times, 3 min each. Followed by 95% ethanol for 3 min and 70% ethanol for 3 min. Slides were then rinsed with tap water and placed in 1 X PBS.

Slides were then incubated with PBS containing 0.2% Triton X-100 for 10 minutes and washed in PBS three times for 2 minutes each. Each slide was then incubated with proteinase K (Enzo Life Sciences) diluted in TE buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% Triton X-100) to a final concentration of 20 µg proteinase K / mL for 30 min at 37°C. After, the slides were washed in PBS buffer three times, for 2 minutes each. Slides were then incubated for 1 hour with ready-to-use (2.5%) normal horse serum (Vector Labs) at room temperature. The sections were then incubated 1 h at room temperature with MJFR1 (Abcam) diluted 1:400 in wash buffer (PBS and 0.1% Tween-20) containing 2% normal goat serum (Gibco). Following incubation with MJFR1, slides were washed three times in wash buffer and incubated with alkaline phosphatase conjugated horse anti-rabbit IgG (Vector Labs) for 1 h at room temperature. Slides were washed again three times in wash buffer and then incubated with ImmPACT Vector Red AP substrate

(Vector Labs) for 8 min. Slides were counterstained with 50% Hematoxylin QS Nuclear Counterstain (Vector Labs) for 15 seconds and then rinsed under running water.

The slides were then dehydrated in increasing ethanol solutions and xylene, mounted (Vectamount permanent mounting media; Vector Labs) and coverslipped (Globe Scientific). Two experimenters blind to the sample key assessed staining in each slide. Experimenters determined the presence/absence of proteinase K resistant α-synuclein in neuronal structures of each appendix sample using a Leica DM5500 B microscope. Staining was also scored (0=none, 1=low, 2=moderate, 3=high) in the mucosa, submucosa, and muscularis externa of the appendix.

Dual-labeling immunohistochemistry

For dual labeling experiments paraffin removal and rehydration was performed on slides as described above. Sections stained for synaptophysin were incubated with epitope retrieval buffer (10 mM Tris pH 9, 1 mM EDTA, 0.05% Tween 20) at 95°C for 20 min and allowed to cool for 20 min. These sections were then treated with proteinase K (Enzo Life Sciences) 10 µg / mL diluted in PBS for 10 min at room temperature. For peripherin staining sections were treated with proteinase K as described above. Then sections were incubated in PBS containing 10% normal goat serum and 0.3% triton X-100 for 1h at room temperature. Sections were then incubated with either anti-peripherin (diluted 1:100; Abcam) or anti-synaptophysin (1:20; Nordic BioSite) and MJFR1 (diluted 1:400; Abcam) diluted in PBS containing 10% normal goat serum and 0.3% triton X-100 overnight at 4°C. Sections were then washed three times for 10 min each in PBS containing 0.1% triton X-100. Sections were then incubated with a mixture of Alexa 647 conjugate goat anti-chicken IgG (diluted 1:400; Abcam; for peripherin staining) or Alexa 680 conjugate goat anti-mouse IgG (diluted 1:400, Invitrogen; for synaptophysin staining), Alexa 594

goat anti-rabbit IgG (diluted 1:400; Jackson Immunoresearch) and DAPI (diluted 1:10,000; Sigma) in PBS containing 2% normal goat serum and 0.3% Triton X-100 for 2 h at room temperature. Sections were then rinsed with PBS containing 0.1% triton X-100 two times for 10 min each. Slides were then rinsed in PBS for 10 min, mounted with glass coverslip and Everbrite hardset mounting media (Biotium). Sections were imaged using Nikon A1plus-RSi scanning confocal microscope and images were analyzed with NIS-Elements software.

Human appendix and brain tissues

Surgical samples of appendix from healthy individuals were obtained from the Cooperative Human Tissue Network and Spectrum Health Universal Biorepository. Surgical samples were from individuals undergoing incidental appendectomies and are clinically normal (non-inflamed, without histological signs of appendicitis). Post-mortem brain and PD samples were obtained from the Oregon Brain Bank. The post-surgical interval was less than 45 minutes, and the post-mortem interval was ≤24 h. All fresh tissues were snap frozen and stored at -80°C until time of processing. This study examined 8 control and 6 PD appendix tissues along with 6 control and 6 PD SN tissues. The study protocol was ethically approved by the institutional review board at the Van Andel Research Institute (IRB #15025).

Detergent Solubility Assay

All procedures were conducted at 4°C. Frozen appendix and SN tissues weighing 30-70 mg were sonicated (10 x 2 sec pulses at medium amplitude) in ice cold lysis buffer (1 X PBS, 1% Triton-X 100 v/v, 1 X protease inhibitor cocktail, 2 mM EDTA). Immediately following sonication 2 mM PMSF was added to each sample, vortexed vigorously, and incubated for 30 min

at 4°C with inversion. Samples were then centrifuged at 22,000 × g for 30 min and the supernatant collected (S1). The pellet was resuspended in 500 µl lysis buffer, vortexed vigorously, and incubated for 30 min at 4°C with inversion. Samples were then centrifuged at 22,000 × g for 30 min and the supernatant (S2) was added to S1, together these represent the triton x-100 soluble fraction. The pellet was then resuspended in 500 µl freshly made 8M urea, vortexed vigorously, and incubated for 1 h at 4°C with inversion. The sample was then centrifuged at 22,000 × g for 30 min and the supernatant (S3) was retained. The pellet was then washed once with 1 X PBS, resuspended in 300 µl 6M guanidine HCl, vortexed vigorously, and then incubated overnight at 4°C with inversion. The sample is then centrifuged at 22,000 x g for 30 min and the supernatant (S4) retained. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific). Then 30 µg of protein from each sample was cleaned using a previously described method (*92*), resuspended in 30 µl of SDS-page sample buffer, and heated to 98°C for 5 min. Samples were then resolved by SDS-PAGE and blotted. Following blotting gels were stained using colloidal coomassie blue dye and imaged with near infrared fluorescence (sensitivity <1 ng) (*93*) using Chemidoc MP system (Biorad).

SDS-PAGE and immunoblotting

Samples were heated to 70°C for 10 min in 1 X Laemmli sample buffer (Biorad) containing 2.5% 2-mercaptoethanol, mixed vigorously, and resolved on a 4-20% SDS-PAGE gel (Thermo Fisher Scientific) using SDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine, 10% m/v sodium dodecyl sulfate). Resolved proteins were then blotted onto methanol activated 0.2 micron-pore size polyvinylidene fluoride (Thermo Fisher Scientific) using transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol). Proteins were blotted using the XCell II blot

module (Thermo Fisher Scientific) with 10 V applied potential for 16 h at 4°C. Following transfer all blots were rinsed with ultrapure Milli-Q water and incubated with 0.4% paraformaldehyde for 15 min at room temperature. Blots were then rinsed in ultrapure water, dried completely (2 h), and reactivated with methanol. Protein blotting quality was assessed by incubating the PVDF membrane in Ponceau S solution (Sigma-Aldrich) solution (1% Ponceau S, 1% acetic acid) for 5 min and rinsing in ultrapure water. The membrane was then incubated in blocking buffer, which consisted of TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20) and 5% non-fat milk, for 1 h at room temperature. Membranes were then incubated overnight at 4°C with one of several primary antibodies; Clone 42/α-Synuclein SYN-1 (BD Biosciences), MJFR1 (Abcam) or anti-actin clone C4 (Millipore), anti-aggregated a-synuclein antibody clone 5G4 (Millipore), diluted 1:1000 in blocking buffer. Membranes were then wash 3 times for 10 min each. Membranes were then incubated for 1 h at room temperature with either anti-mouse IgG or anti-rabbit IgG horse radish peroxidase conjugated antibodies (Cell Signaling Technology) diluted in blocking buffer. Membranes were then washed 3 times for 10 min each and developed using Supersignal West Pico ECL substrate (Thermofisher Scientific). Blots were imaged using either Fujifilm LAS 4000 (GE HealthCare Lifesciences) or Chemidoc MP System (Biorad).

Immunoprecipitation of α-synuclein

Samples containing extracted proteins from patient tissues were diluted to a concentration of 1 mg protein / mL using lysis buffer containing a combination of protease inhibitors (1 X protease inhibitor cocktail, 2 mM EDTA, 2 mM PMSF). Then 2.4 µg MJFR1 was added to 600 µl of each sample. The sample was incubated overnight (~16 h) with gentle inversion at 4°C. Each sample was then incubated with 25 µl of protein A/G magnetic beads (Thermo Fisher Scientific)

for 1 h at 4°C with inversion. Samples were placed on a magnet (Thermo Fisher Scientific) for 1 min and the supernatant retained. The protein A/G magnetic beads were then washed with 1 mL of lysis buffer by gently inverting the sample. The sample was placed on the magnet and wash buffer completely removed. The sample tube containing protein A/G magnetic beads were then centrifuged at 22,000 × g for 1 min at 4°C. Proteins were then eluted off beads with 1 X Laemmli sample buffer with heating for 70°C for 10 min.

α-Synuclein cleavage assay

The shaking assay with purified human α-synuclein added to appendix or substantia nigra tissue lysates was performed similarly to as previously described (*94*). Tissue lysates were diluted to 0.8 mg protein / mL in lysis buffer containing 1 X protease inhibitor cocktail. Full-length purified human α-synuclein (kind gift from Dr. Jiyan Ma) was then added to the sample to a final concentration of 1 mg α-synuclein / mL. For some experiments several protease inhibitors (PI) or combinations of protease inhibitors were added to the sample including; 2 mM EDTA, 2 mM PMSF, 1 X complete PI cocktail (Roche), 5 µM pepstatin A (Tocris), Asparagine endopeptidase inhibitor (AENK, EMD Millipore), and/or 10 mM marimastat (Tocris). Samples were then incubated at 37°C with shaking at 1250 rpm using a Vortemp 56 (Labnet). At time points indicated 0.5 µl of the reaction was removed, placed into 20 µl 1 X SDS-PAGE sample buffer, and immediately placed on dry ice. The entire aliquoted sample, containing 500 ng α-synuclein, was then used for analysis by SDS-PAGE.

LC–MS/MS and data processing

α-Synuclein was immunoprecipitated from normal appendix tissues, as described above. Prior to LC-MS/MS samples were precipitated using a mixture of methanol/chloroform/H_2O as described previously (*95*). Resulting pellets were re-solubilized in 0.1% formic acid. Proteoforms were then separated by nanocapillary LC system (Dionex RSLCnano) using trap (2 cm × 150 μm i.d.) and analytical (20 cm × 75 μm) columns both in-house packed using PLRP-S stationary phase (5 μm particle size, Agilent, Santa Clara, CA) coupled with a custom electrospray ionization (ESI) source equipped with a PicoTip spray emitter (catalog no. FS360-50-15-N-20-C12; New Objective, Woburn, MA). Proteoforms were analyzed online after nano-ESI on an LTQ Velos Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) operated in targeted and data-dependent modes using established instrument methods (*96*). Resulting raw data files were deconvoluted using Xtract software (Thermo Fisher Scientific) to obtain molecular weight and relative abundance of proteoforms. Additionally raw files were processed through TDPortal (http://nrtdp.northwestern.edu/resource-software) a search environment on the Quest high-performance computing cluster at Northwestern University, to identify intact proteins and to characterize proteoforms. TDPortal generated linked sets of precursor and fragmentation data from .raw files, which were searched against a highly annotated version of the Human UniProt Knowledge Base using a three-tiered search tree (raw data files and process top down results are accessible at ftp://massive.ucsd.edu/MSV000082353).

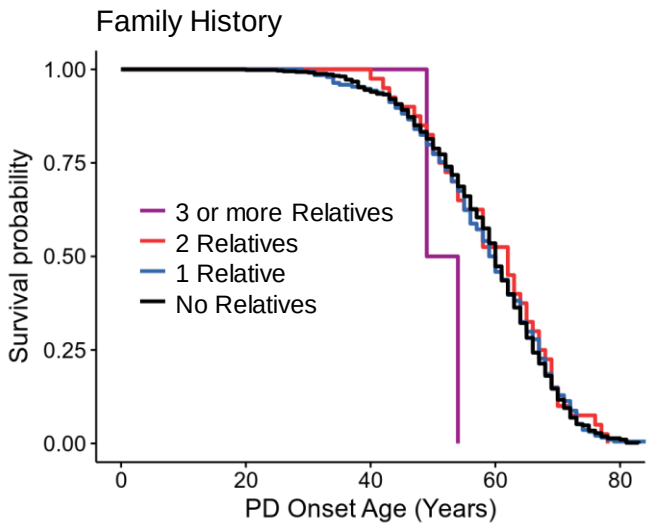

Fig. S1. Age of PD onset in individuals with and without a family history of PD in the PPMI data. Kaplan-Meier plot for PD onset times for individuals with 0, 1, 2, or >2 relatives (parents/siblings) with PD. Individuals that had 3 or more relatives with PD have a much earlier PD onset (median difference 8.5 years; p=0.063, based on a log-rank test). This group (comprised of n=2 control patients and no appendectomy patients) was excluded to avoid an imbalance in the covariates and subsequent biasing of analyses away from the null. All other groups (no relatives, 1-2 relatives with PD) were used in the analysis.

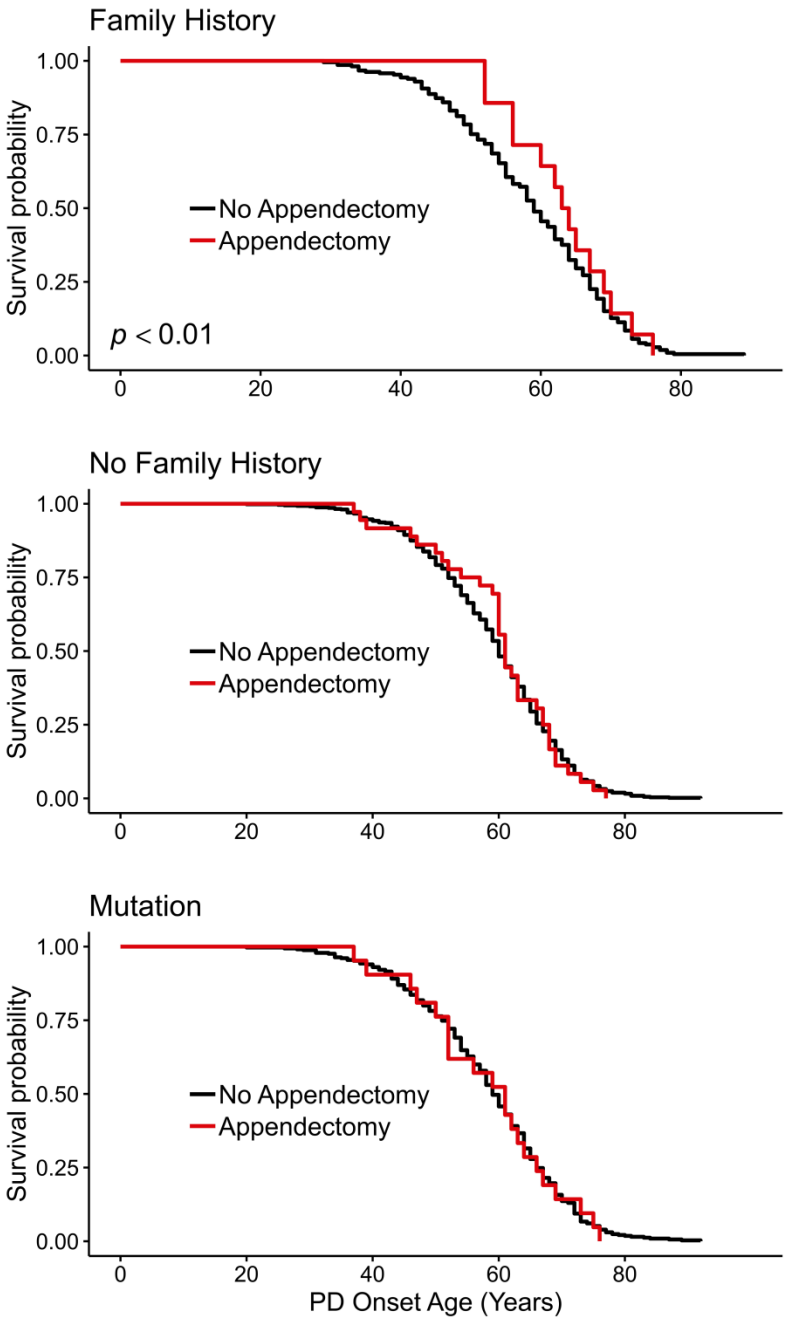

Fig. S2. An appendectomy delays the age of PD onset in individuals with a family history of PD that is not explained by genetic risk factors of PD. Patients that had an appendectomy before PD onset were examined in the PPMI dataset. An appendectomy postponed the age of PD onset in individuals with family history of PD (p<0.01). Analyses involved a log-normal survival

regression with robust standard errors, adjusted for sex, ethnicity, education years, and mutation status. n=14 PD patients that had an appendectomy, median age of PD onset 63.2 (CI [59.5, 67.0]); n=213 PD patients without an appendectomy, median age of PD onset 58.4 (CI [57.0, 59.9]). Family history of PD is defined as having 1-2 parents, siblings or half-siblings with PD. An appendectomy did not benefit the age of PD onset in patients with no family history (no parent, sibling or half-sibling with PD). n=36 PD patients that had an appendectomy, median age of PD onset 60.1 (CI [56.9, 63.3]); n=567 PD patients without an appendectomy, median age of PD onset 59.2 (CI [58.3, 60.1]). Similarly, an appendectomy did not alter the age of PD onset in individuals carrying a mutation in α-synuclein, leucine-rich repeat kinase 2 and/or beta-glucocerebrosidase. n=21 PD patients that had an appendectomy, median age of PD onset 58.4 (CI [53.8, 63.0]); n=336 PD patients without an appendectomy, median age of PD onset 58.4 (CI [57.2, 59.6]).

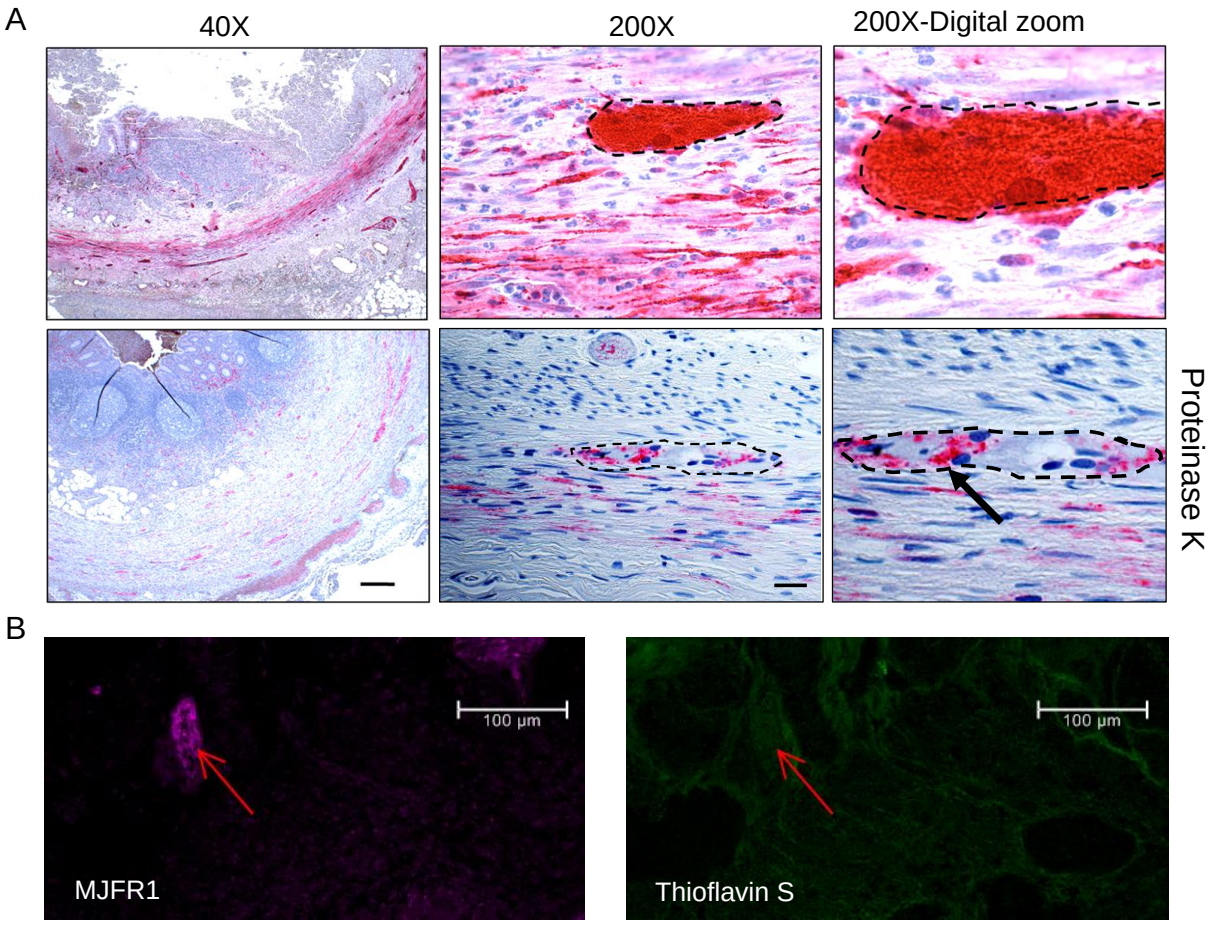

Fig. S3. Validation of proteinase K digestion protocol in the human appendix used to assess aggregated α-synuclein. (**A**) Formalin-fixed appendix tissues (5 µm sections) were incubated with or without proteinase K, and then immunostained for α-synuclein using the MJFR1 antibody. Top panels show appendix tissue without exposure to proteinase K. Bottom panels depict appendix tissue incubated with proteinase K. Black arrow highlights remaining immunoreactivity within myenteric plexus of proteinase K treated appendix tissue. Scale bar left panels = 250 micron; Scale bar middle panel = 100 micron. (**B**) Aggregated α-synuclein in the appendix does not stain for thioflavin S. Appendix tissue showing proteinase K resistant α-synuclein aggregates (left) and co-stained for thioflavin S (right). Red arrow highlights a myenteric plexus. Scale bar = 100 micron. Representative sections from n=4 individuals. Thioflavin S binds β-sheet structures

including α-synuclein fibrils, and so a lack of thioflavin S staining signifies that the healthy human appendix contains prefibrillar α-synuclein aggregates.

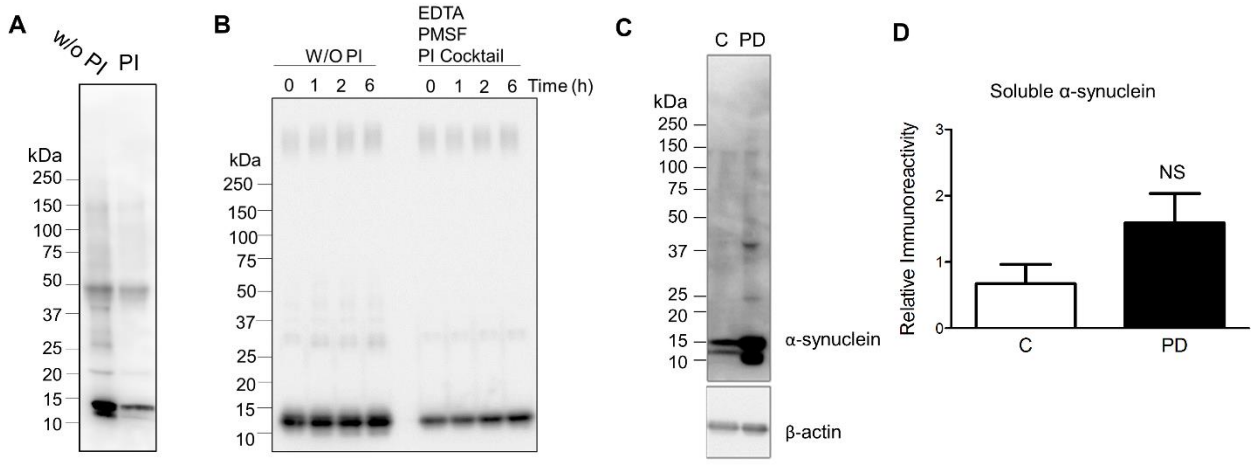

Fig. S4. Evaluation of α-synuclein proteolysis under different tissue processing conditions. (**A**) Blot showing endogenous α-synuclein from appendix tissue processed without protease inhibitors (w/o PI) or with protease inhibitors (PI): 10 mM EDTA, 10 mM PMSF, and 1 X Roche protease inhibitor cocktail. Triton X-100 soluble proteins extracted from appendix samples were resolved by SDS-PAGE and immunoblotted using MJFR1. There is minimal α-synuclein cleavage in the presence of PI. (**B**) Blot showing proteolytic cleavage of full-length α-synuclein over time in the presence of human appendix tissue lysates, with and without PI. Samples contained appendix tissue lysates (diluted to 0.8 mg protein / mL) and purified α-synuclein (1 mg α-synuclein / mL), and were incubated on ice with or without PI. Samples were maintained on ice to recreate protein extraction procedures. Aliquots of 0.5 µL (i.e. 500 ng purified α-synuclein) from each sample were collected at 0 (baseline), 1, 2, and 6 h. The aliquots were resolved by SDS-PAGE and immunoblotted with the MJFR1 antibody. In the presence of protease inhibitors there is no detectable α-synuclein cleavage product. (**C**) Triton X-100 soluble α-synuclein in the human appendix. Proteins were extracted from the appendix of healthy (C) and PD patients. Soluble proteins were resolved via SDS-PAGE and immunoblotted using the anti-α-synuclein antibody MJFR1. Values relative to β-actin loading control. (**D**) Densitometric analysis revealed

no significant (NS) difference in triton X-100 soluble α-synuclein between healthy individuals and PD patients. n=5 healthy controls and 6 PD. $p>0.05$ by one-way ANOVA.

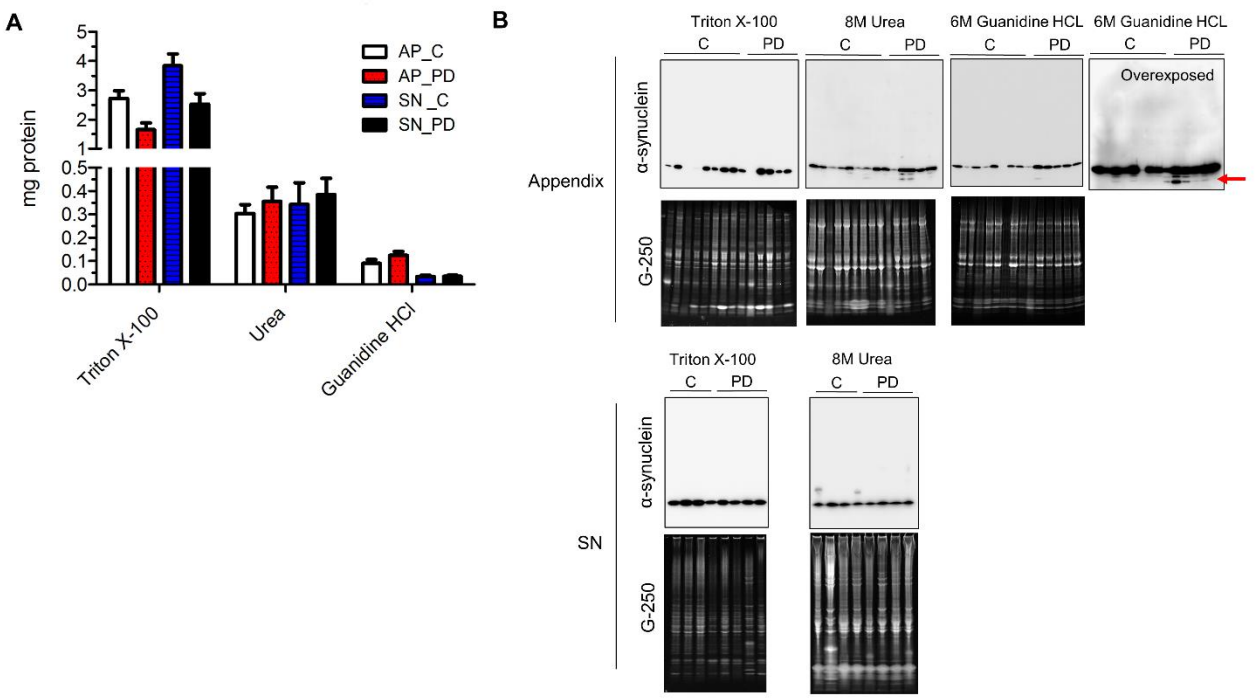

Fig. S5. Extraction of detergent-soluble and -insoluble α-synuclein from appendix and brain tissue. Protein from the appendix (AP) or substantia nigra (SN) of healthy individuals (C) and PD patients was sequentially extracted in the presence of PI. n=8 appendix of healthy individuals, 7 appendix of PD patients, 4 substantia nigra of healthy individuals, 4 substantia nigra of PD patients (**A**) Total protein amounts in the soluble (Triton X-100), insoluble (urea), and highly insoluble (guanidine HCl) fraction, as quantified by BCA assay. The majority of protein is found in the soluble tissue fraction. Subsequent urea and guanidine extractions yielded 8.6-fold and 48.5-fold less protein when compared to the initial Triton X-100 extraction. (**B**) Representative blots showing α-synuclein distribution between the soluble, insoluble, and highly insoluble fractions from the appendix and substantia nigra. Blotted proteins were probed with an antibody specific for α-synuclein (MJFR1). Coomassie blue (G-250) staining of gels following transfer shows protein distribution and abundance. In the substantia nigra, protein extracted by 6 M

guanidine (<3%) was below the detection threshold limit. Cleaved α-synuclein (red arrow) is evident in the insoluble and highly insoluble fraction of the healthy and PD appendix.

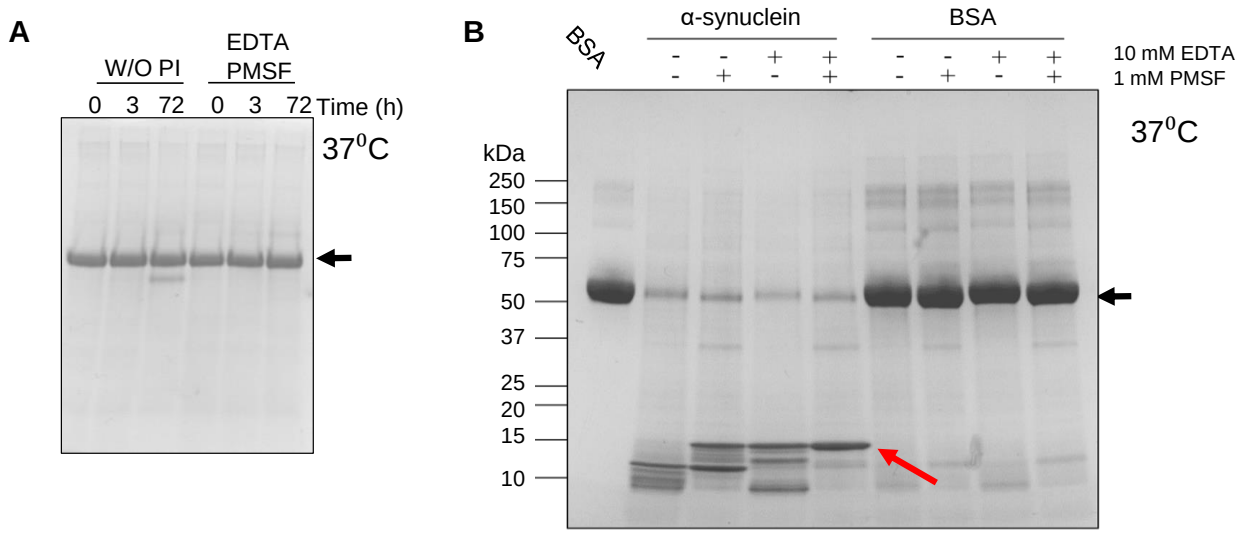

Fig. S6. Active cleavage of α-synuclein in the in vitro shaking assay with appendix tissue lysates. (**A**) Full-length bovine serum albumin (BSA) was incubated with appendix tissue lysate in shaking assay for 0, 3, or 72 h; both in the presence (EDTA and PMSF) or absence of protease inhibitors (w/o PI). No cleavage or oligomerization of BSA was detected, supporting the specificity of cleavage for α-synuclein. (**B**) Shaking assay with either full-length BSA or full-length α-synuclein in the presence of appendix lysate. These samples were all incubated for 6 h with shaking and then resolved by SDS-PAGE. Black arrow highlight position of full-length BSA (66 kDa) and red arrows depict position of full-length α-synuclein (14 kDa). Both images are trans-illumination images of in-gel Coomassie blue staining. α-Synuclein, but not BSA, is rapidly cleaved in the presence of appendix lysate.

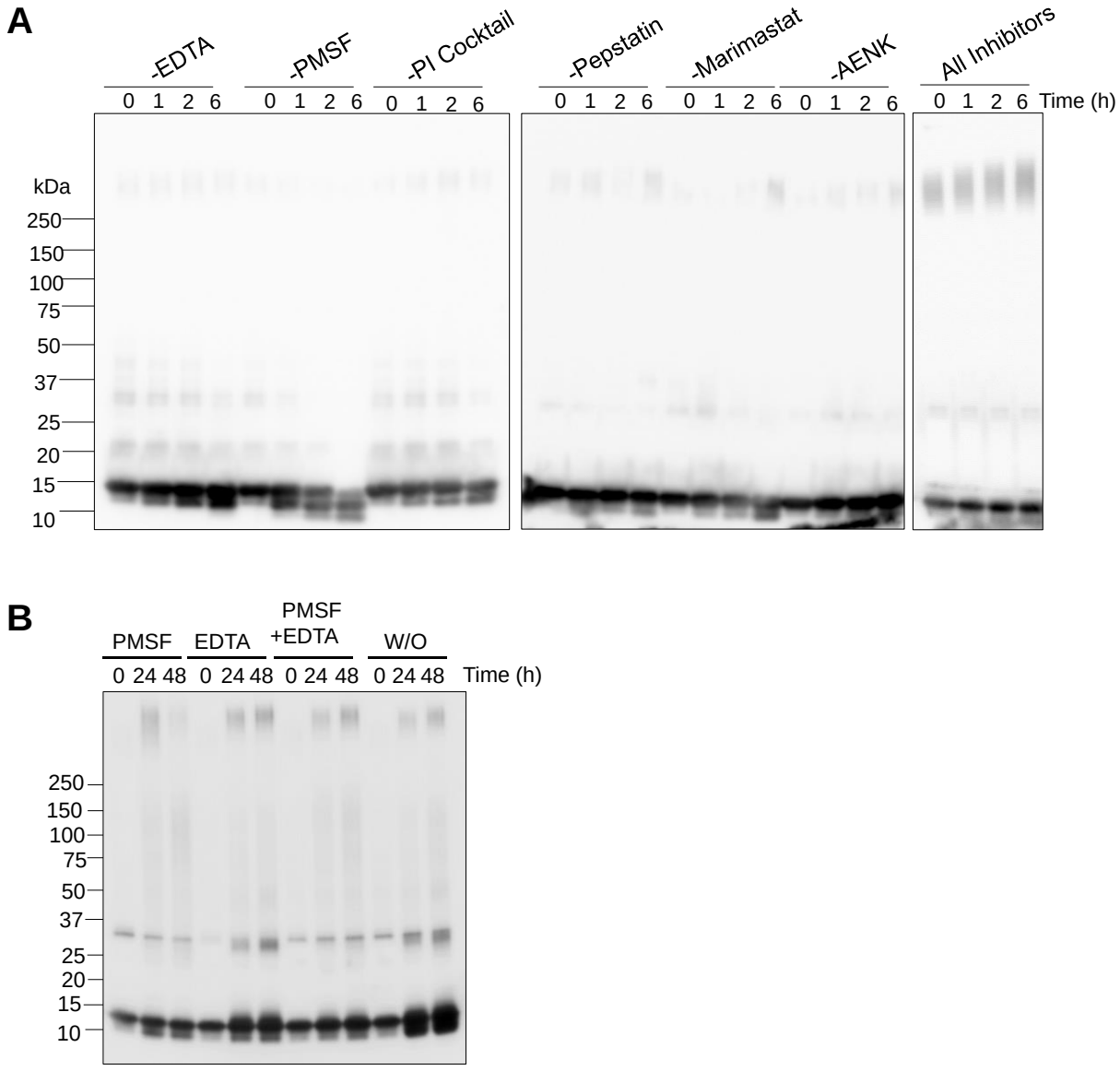

Fig. S7. Effect of protease inhibition on α-synuclein cleavage and aggregation induced by appendix lysates. Full-length purified human α-synuclein (1 mg / mL) was incubated with appendix lysate (0.8 mg protein / mL) for up to 48 h in shaking assay. Aliquots collected at various time points were resolved by SDS-PAGE and blotted for α-synuclein with MJFR1. (**A**) Reactions were conducted in the presence of a combination of protease inhibitors, including EDTA (inhibits metalloproteases), PMSF (inhibits serine protease), 1 X protease inhibitor

cocktail (Roche; inhibits cysteine and serine proteases), pepstatin (inhibits aspartyl proteases), marimastat (inhibits a subset of metalloproteases), and AENK (asparagine endopeptidase inhibitor). Seven separate reactions were conducted, each separate reaction lacked one of the protease inhibitors. The omitted protease inhibitor from each reaction is noted above the blot image. Absence of EDTA or PMSF from the inhibitor mixture resulted in the most α-synuclein cleavage. (**B**) Similar reactions were conducted in presence of a select protease inhibitor(s) (PMSF, EDTA, PMSF + EDTA) or without protease inhibitors (w/o). The combination of PMSF and EDTA was most effective at inhibiting α-synuclein cleavage. Representative blots of 3 experiments.

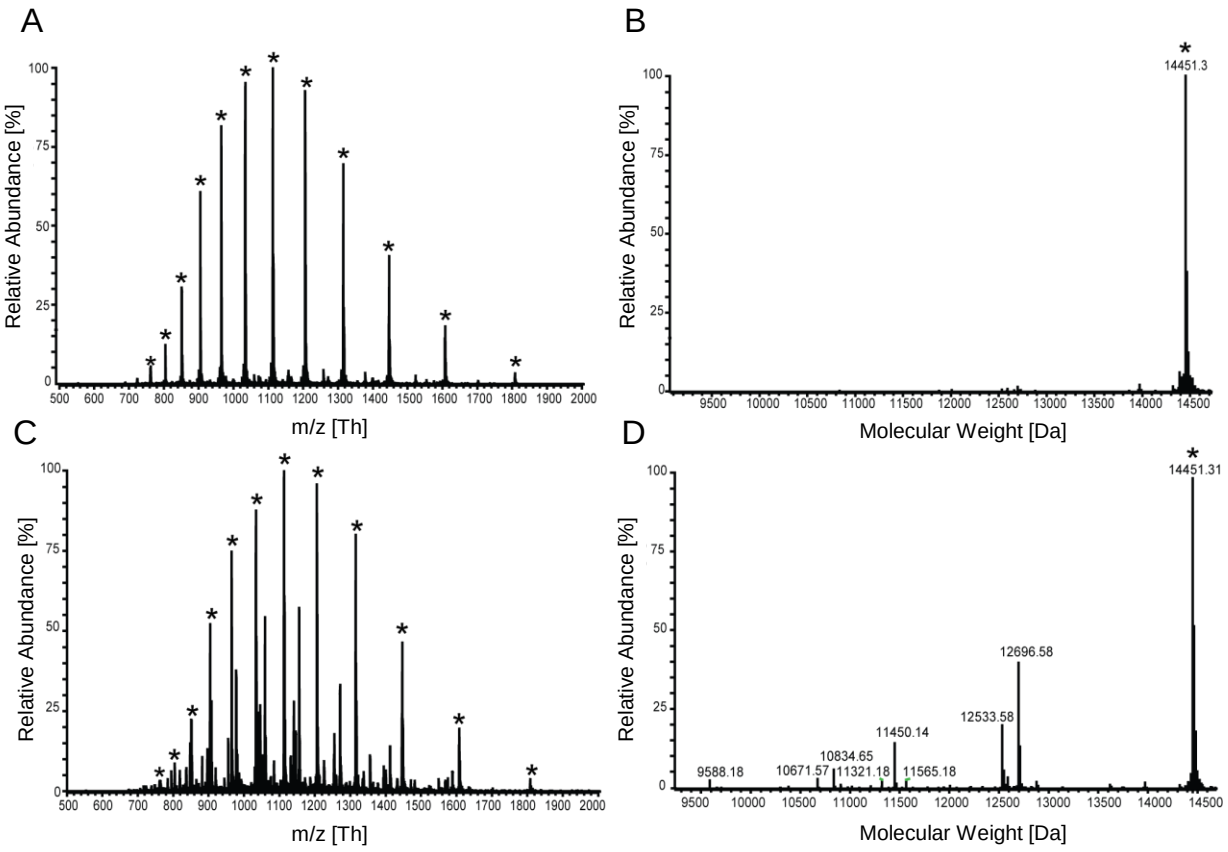

Fig. S8. Identification of α-synuclein using TD-MS. (**A**) TD-MS spectrum for purified recombinant full-length α-synuclein, (**B**) and corresponding deconvoluted spectrum. A single full-length α-synuclein proteoform was detected, supporting high specificity. (**C**) TD-MS spectrum of full-length α-synuclein incubated with appendix tissue lysate for 6 h in shaking assay. (**D**) Deconvoluted MS spectrum of showing α-synuclein proteoforms generated in the shaking assay. The shaking assay yielded α-synuclein truncation products that were analogous the identified endogenous truncation products in the human appendix. Mass of α-synuclein proteoforms given at top of peak and *highlights full-length α-synuclein.

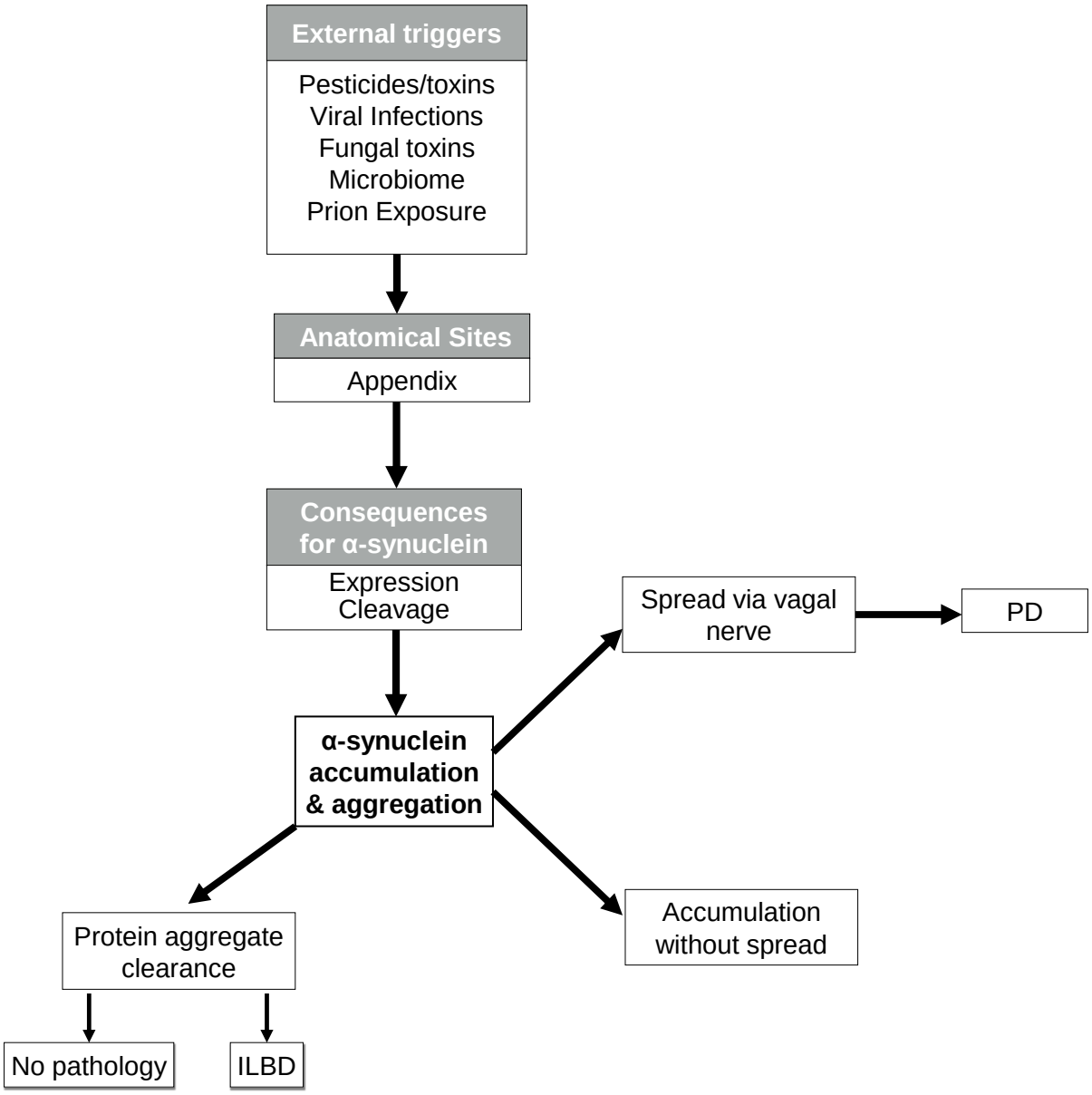

Fig. S9. Schematic of proposed model for the contributions of the vermiform appendix to PD. The appendix may contain aberrant forms of α-synuclein. Exposure to environmental triggers may accelerate the accumulation of α-synuclein aggregates in the vermiform appendix. These aggregates could then spread to the brain via the vagal nerve to cause PD. In individuals that do not develop PD, α-synuclein aggregates from the appendix may be effectively cleared and/or

there is limited spread of the aggregates to the brain, resulting in no brain pathology or incidental Lewy Body disease (ILBD).

Table S1. Characteristics of study samples from the SNPR.

	Appendectomy	Controls
Total, n	551,647	1,146,353
Females, n (%)	297,590 (53.95%)	614,883 (53.64%)
Year of birth, mean (± s.e.m.)	1957.94 (± 0.031)	1958.56 (± 0.022)
Age at Appendectomy, mean (± s.e.m.)	32.45 (± 0.027)	N/A
PD, n (%)	644 (0.12%)	1,608 (0.14%)
Age at PD diagnosis, mean (± s.e.m.)	74.94 (± 0.508)	76.27 (± 0.255)
Rural residency, n (%)	342,582 (62.10%)	704,072 (61.42%)

Table S2. Appendectomy in relation to age of PD onset for patients with PD in the SNPR study.

Age at PD diagnosis	PD diagnosis, n	Age at PD diagnosis (years)[ǂ]	Hazard Ratio [95% CI[‡]]	*p*-value
Appendectomy >0 years before PD				
Appendectomy	479	74.9 ± 0.51	0.990 [0.890, 1.101]	0.851
Controls	1176	74.9 ± 0.31		
Appendectomy ≥10 years before PD				
Appendectomy	306	74.6 ± 0.56	0.948 [0.835, 1.076]	0.409
Controls	1107	74.2 ± 0.30		
Appendectomy ≥20 years before PD				
Appendectomy	101	74.9 ± 0.81	0.793 [0.642, 0.980]	**0.027**
Controls	658	73.2 ± 0.33		
Appendectomy ≥30 years before PD				
Appendectomy	5	73.5 ± 1.88	0.249 [0.079, 0.778]	**0.005**
Controls	37	70.1 ± 0.90		
Binned analysis				
Appendectomy 0-10 years before PD				
Appendectomy	173	75.5 ± 0.99	1.071 [0.909, 1.261]	0.415
Controls	874	76.0 ± 0.41		
Appendectomy 11-20 years before PD				
Appendectomy	205	74.5 ± 0.74	1.002 [0.862, 1.164]	0.984
Controls	1011	74.7 ± 0.32		
Appendectomy 21-30 years before PD				
Appendectomy	96	74.9 ± 0.85	0.794 [0.639, 0.986]	**0.032**
Controls	653	73.4 ± 0.34		

Refers to [ǂ]mean ± s.e.m; [‡]95% confidence interval

Table S3. Incidence of PD in males and females living in rural and urban areas, SNPR study.

Group	Appendectomy[ł]	Controls[ł]	PD incidence change[¥] p-value
Rural[‡]			
All	1.49 [1.31, 1.68]	2.00 [1.87, 2.15]	25.4% decrease **2.93×10^{-5}**
Male	1.75 [1.47, 2.05]	2.27 [2.06, 2.50]	23.0% decrease **6.8×10^{-3}**
Female	1.30 [1.10, 1.54]	1.79 [1.61, 1.96]	27.2% decrease **1.3×10^{-3}**
Urban			
All	1.77 [1.55, 2.02]	1.97 [1.79, 2.16]	N.S. 0.218
Male	1.85 [1.48, 2.27]	1.99 [1.74, 2.28]	N.S. 0.557
Female	1.73 [1.42, 2.09]	1.96 [1.75, 2.22]	N.S 0.263

[ł]Incidence of PD per 100,000 person-years [confidence interval].
[¥]PD incidence change in appendectomy group.
[‡]Males and females differed in their incidence of PD in rural ($p<10^{-4}$), but not urban areas ($p<0.69$). In rural areas, both males and females with an appendectomy showed a decrease in PD incidence. There was no benefit of an appendectomy in males and females dwelling in urban areas.

Table S4. Demographic and clinical information of patients with PD in the PPMI.

Sample cohort	Appendectomy	Immune condition (non-GI)	Other surgery	No immune condition or surgery	Overall Total
PD patients, n	54 (6.4%)	177	245	373	849
Females, n (%)	15 (27.78%)	83 (46.89%)	119 (48.57%)	141 (37.80%)	358 (42.17%)
Years of Education[ł]	15.75 (± 0.600)	15.91 (± 0.227)	15.71 (± 0.219)	14.94 (± 0.195)	15.43 (± 0.123)
Ethnicity, n (%)					
White	41 (75.93%)	164 (92.66%)	213 (86.94%)	321 (86.06%)	739 (87.04%)
Non-White	12 (22.22%)	10 (5.65%)	29 (11.84%)	45 (12.06%)	96 (11.31%)
Not specified	1 (1.85%)	3 (1.69%)	3 (1.22%)	7 (1.88%)	14 (1.65%)
PD Mutation Carrier, n (%)	23 (42.59%)	69 (38.98%)	109 (44.49%)	161 (43.16%)	362 (42.64%)
PD Family History, n (%)	15 (27.78%)	51 (28.81%)	55 (22.45%)	113 (30.29%)	234 (27.56%)
PD Onset Age[ł]	60.67 (± 1.283)	59.02 (± 0.693)	60.71 (± 0.638)	58.13 (± 0.605)	59.13 (± 0.367)
Disorder Diagnosis/Surgery Age[ł]	22.07 (± 2.066)	33.70 (± 1.579)	36.80 (± 1.334)		

Values are given as number of individuals (percentage of PD patients for respective surgery or condition) or for[ł] as mean (±s.e.m.). [¥]Non-White ethnicities include Black, Asian, Hispanic/Latin, Hawaiian/Pacific, and Indian/Inuit.

Table S5. Appendectomy in relation to age of PD onset for patients with PD in PPMI.

Years	Group	n	PD age of onset[ł]	*p* value[ǂ]
Appendectomy				
>0 Years From PD Onset	Appendectomy	50	61.0 [58.4, 63.5]	0.129
	No appendectomy	780	59.0 [58.2, 59.7]	
≥10 Years From PD Onset	Appendectomy	48	61.1 [58.5, 63.8]	0.121
	No appendectomy	780	59.0 [58.2, 59.7]	
≥20 Years From PD Onset	Appendectomy	45	61.2 [58.4, 64.0]	0.132
	No appendectomy	780	59.0 [58.2, 59.7]	
≥30 Years From PD Onset	Appendectomy	39	62.6 [60.1, 65.0]	**0.023**
	No appendectomy	780	59.0 [58.2, 59.7]	

[ł]Refers to restricted mean and 95% confidence interval. [ǂ]Determined using a log-normal regression model, adjusted for sex, ethnicity, education years, family history, and mutation status.

Table S6. PD age of onset is delayed in individuals with an appendectomy but not in individuals with non-GI immune conditions or other surgeries. Data from PPMI.

PPMI Group[¥]	n	PD age of onset[ł]	HR[‡]	*p* value[¥]
Immune condition, non-GI				
Appendectomy	39	62.6 [60.1, 65.0]	0.73 [0.46, 1.17]	**0.038**
Immune condition	39	58.6 [55.7, 61.5]		
No condition/surgery	366	58.0 [56.8, 59.2]	1.07 [0.76, 1.49]	0.479
Immune condition	39	58.6 [55.7, 61.5]		
Other surgery				
Appendectomy	39	62.6 [60.1, 65.0]	0.33 [0.18, 0.62]	**0.003**
Other surgery	22	57.7 [55.4, 59.9]		
No condition/surgery	366	58.0 [56.8, 59.2]	1.54 [0.99, 2.39]	0.955
Other surgery	22	57.7 [55.4, 59.9]		

[¥]Analyses limited to appendectomy, immune condition, or other surgery occurring ≥30 years from PD onset. There is also the no condition/surgery group consisting of individuals without an immune condition or surgery including appendectomy.
[ł]Refers to restricted mean and 95% confidence interval.
[‡]HR refers to hazard ratio and 95% confidence interval. Determined using a weighted Cox regression model adjusting for sex, ethnicity, education years, family history, mutation status, and when applicable, age of condition/surgery.
[¥]Determined using a weighted log-normal survival regression model adjusting for sex, ethnicity, education years, family history, mutation status, and when applicable, age of condition/surgery.

Table S7. Patients who had an appendectomy 30 or more years before PD do not show changes in PD symptom severity, as measured by the Hoehn and Yahr scale and UPDRS.

Measurement	Regression coefficient ± standard error	*p*-value	95% confidence interval
Hoehn and Yahr scale	0.527 ± 0.345	0.792	[-0.63,1.69]
Unified Parkinson's Disease Rating Scale (UPDRS)			
I. Non-motor aspects of experiences of daily living			
Intellectual impairment	0.098 ± 0.321	0.837	[-0.98,1.18]
Hallucinations and psychosis	0.307 ± 0.434	0.81	[-1.16,1.77]
Depressed mood	-0.056 ± 0.347	0.912	[-1.23,1.11]
Anxious mood	0.32 ± 0.324	0.81	[-0.77,1.41]
Apathy	0.067 ± 0.339	0.899	[-1.08,1.21]
Sleep problems (night)	0.143 ± 0.293	0.827	[-0.84,1.13]
Daytime sleepiness	0.282 ± 0.311	0.81	[-0.77,1.33]
Pain and other sensations	0.106 ± 0.309	0.837	[-0.94,1.15]
Urinary problems	0.843 ± 0.304	0.384	[-0.18,1.87]
Constipation problems	-0.176 ± 0.323	0.815	[-1.26,0.91]
Lightheadedness on standing	0.389 ± 0.333	0.801	[-0.73,1.51]
Fatigue	0.2 ± 0.306	0.81	[-0.83,1.23]
II. Motor aspects of experiences of daily living			
Speech	0.095 ± 0.305	0.837	[-0.93,1.12]
Saliva and drooling	0.297 ± 0.308	0.81	[-0.74,1.33]
Chewing and swallowing	-0.219 ± 0.374	0.811	[-1.48,1.04]
Eating tasks	-0.197 ± 0.328	0.811	[-1.3,0.91]
Dressing	0.449 ± 0.302	0.792	[-0.57,1.47]
Hygiene	0.59 ± 0.315	0.648	[-0.47,1.65]
Handwriting	0.564 ± 0.306	0.648	[-0.47,1.59]
Doing hobbies and other activities	0.207 ± 0.308	0.81	[-0.83,1.24]
Turning in bed	0.238 ± 0.309	0.81	[-0.8,1.28]
Tremor	0.675 ± 0.31	0.648	[-0.37,1.72]
Getting out of bed, car, or deep chair	0.524 ± 0.299	0.648	[-0.48,1.53]
Walking and balance	0.718 ± 0.301	0.585	[-0.29,1.73]
Freezing	0.187 ± 0.368	0.827	[-1.05,1.43]
III. Motor examination			
Speech	-0.098 ± 0.316	0.837	[-1.16,0.97]
Facial expression	0.241 ± 0.294	0.81	[-0.75,1.23]
Rigidity - Neck	0.509 ± 0.289	0.648	[-0.47,1.48]
Rigidity - RUE	0.114 ± 0.297	0.837	[-0.89,1.11]
Rigidity - LUE	0.211 ± 0.297	0.81	[-0.79,1.21]
Rigidity - RLE	0.218 ± 0.312	0.81	[-0.83,1.27]
Rigidity - LLE	0.241 ± 0.297	0.81	[-0.76,1.24]
Finger Tapping Right Hand	0.45 ± 0.308	0.792	[-0.59,1.49]
Finger Tapping Left Hand	0.061 ± 0.297	0.899	[-0.94,1.06]
Hand movements - Right Hand	0.354 ± 0.295	0.801	[-0.64,1.35]
Hand movements - Left Hand	0.182 ± 0.293	0.811	[-0.8,1.17]
Pronation-Supination - Right Hand	0.136 ± 0.305	0.837	[-0.89,1.16]

Pronation-Supination - Left Hand	-0.107 ± 0.301	0.837	[-1.12,0.91]
Toe tapping - Right foot	0.23 ± 0.29	0.81	[-0.75,1.21]
Toe tapping - Left foot	0.041 ± 0.294	0.917	[-0.95,1.03]
Leg agility - Right leg	0.211 ± 0.302	0.81	[-0.81,1.23]
Leg agility - Left leg	0.215 ± 0.304	0.81	[-0.81,1.24]
Arising from chair	0.419 ± 0.328	0.801	[-0.69,1.52]
Gait	0.363 ± 0.314	0.801	[-0.7,1.42]
Freezing of gait	-0.203 ± 0.627	0.837	[-2.31,1.91]
Postural stability	0.467 ± 0.371	0.801	[-0.78,1.72]
Posture	0.158 ± 0.296	0.815	[-0.84,1.16]
Global spontaneity of movement	0.259 ± 0.296	0.81	[-0.74,1.26]
Postural tremor - Right Hand	0.307 ± 0.345	0.81	[-0.86,1.47]
Postural tremor - Left hand	-0.268 ± 0.379	0.81	[-1.55,1.01]
Kinetic tremor - Right hand	0.21 ± 0.36	0.811	[-1,1.42]
Kinetic tremor - Left hand	-0.264 ± 0.396	0.81	[-1.6,1.07]
Rest tremor amplitude - RUE	0.326 ± 0.336	0.81	[-0.81,1.46]
Rest tremor amplitude - LUE	0.574 ± 0.327	0.648	[-0.53,1.68]
Rest tremor amplitude - RLE	-1.518 ± 1.025	0.648	[-4.97,1.93]
Rest tremor amplitude - LLE	-0.171 ± 0.55	0.837	[-2.02,1.68]
Rest tremor amplitude - Lip/jaw	-1.005 ± 1.03	0.801	[-4.47,2.46]
Constancy of rest tremor	0.284 ± 0.285	0.81	[-0.68,1.24]
Were dyskinesias present	-0.268 ± 0.31	1	[-1.31,0.78]
IV: Motor complications			
Time spent with dyskinesias	-0.648 ± 0.554	0.801	[-2.51,1.22]
Functional impact of dyskinesias	-0.803 ± 0.762	0.801	[-3.37,1.76]
Time spent in the OFF state	0.457 ± 0.351	0.801	[-0.73,1.64]
Functional impact of fluctuations	-0.004 ± 0.389	1	[-1.31,1.31]
Complexity of motor fluctuations	0.367 ± 0.372	0.81	[-0.89,1.62]
Painful OFF-state dystonia	-0.172 ± 0.55	0.837	[-2.03,1.68]

Values from a proportional odds logistic regression adjusted for sex, ethnicity, education years, family history, and mutation. *P*-values are false discovery rate adjusted.

Table S8. α-Synuclein aggregates are detected in the appendix of both young and older adult individuals and are present in normal and inflamed appendix.

	Group	n[¥]	Age Range, years[§]	Positive Staining[ł]			Staining Score[‡]		
				Mucosa	Submucosa	Muscularis Externa	Mucosa	Submucosa	Muscularis Externa
Normal appendix	Young	12	0-19	75.0%	83.3%	100.0%	1.25 ± 0.30 (1)	1.75 ± 0.28 (2)	2.92 ± 0.08* (3)
	Older Adult	9	52-70	66.7%	77.8%	88.9%	1.56 ± 0.44 (2)	1.11 ± 0.31 (1)	1.89 ± 0.31 (2)
Acute appendicitis	Young	9	6-19	55.6%	77.8%	100.0%	1.11 ± 0.39 (1)	1.11 ± 0.26 (1)	2.00 ± 0.29 (2)
	Older Adult	6	61-84	100.0%	66.7%	100.0%	1.50 ± 0.22 (1.5)	0.83 ± 0.31 (1)	1.67 ± 0.33 (1.5)
Chronic appendicitis	Young	6	8-20	66.7%	50.0%	66.7%	1.17 ± 0.48 (1)	0.83 ± 0.40 (0.5)	1.33 ± 0.56 (1)
	Older Adult	6	48-81	83.3%	83.3%	100.0%	1.67 ± 0.49 (1.5)	1.83 ± 0.48 (2)	1.67 ± 0.33 (1.5)

[¥]Number of individuals; [§]post-natal years

[ł]Positive staining for proteinase K resistant α-synuclein, using antibody MJFR1. % refers to the number of individuals showing positive staining within each group.

[‡]Average staining score of individuals ± s.e.m (Median). Scoring scale is 0=none, 1=low, 2=moderate, and 3=high for proteinase K resistant α-synuclein.

*$p<0.05$ compared to acute and chronic appendicitis in young individuals, and normal appendix in older adults; as determined by cumulative logit mixed-effects regression.

Staining assessed by two experimenters blind to the sample key.

Table S9. Demographic and clinical information of sample sets used in PPMI study.

Group	n	% Females	n, ethnicity			% Appendectomy	PD age of onset[ǂ]		
			W[‡]	NW[‡]	NS[‡]	Age of Appendectomy[ǂ]	All	No appendectomy	Appendectomy
De novo	478	35.77%	439	34	5	6.49% 23.55 ± 3.02	59.76 ± 0.46	59.57 ± 0.48	62.48 ± 1.36
Genetic cohort	190	54.21%	148	37	5	6.32% 18.17 ± 3.43	57.06 ± 0.84	57.04 ± 0.87	57.33 ± 3.20
Genetic registry	181	46.41%	152	25	4	6.08% 22.18 ± 4.16	59.64 ± 0.84	59.67 ± 0.87	59.18 ± 3.52

[ǂ]Mean ± s.e.m. [‡]W = White; NW = Non-White; NS = ethnicity not-specified.

Table S10. Appendectomy delays age of PD onset in patients with de novo PD of PPMI.

Years	Group	n	PD age of onset[¥]	*p* value[‡]
Appendectomy				
>0 Years From PD Onset	Appendectomy	29	62.8 [60.1, 65.5]	0.070
	No appendectomy	442	59.5 [58.6, 60.4]	
≥10 Years From PD Onset	Appendectomy	27	63.2 [60.5, 66.0]	0.059
	No appendectomy	442	59.5 [58.6, 60.4]	
≥20 Years From PD Onset	Appendectomy	25	63.4 [60.5, 66.4]	0.058
	No appendectomy	442	59.5 [58.6, 60.4]	
≥30 Years From PD Onset	Appendectomy	22	64.1 [61.8, 66.5]	**0.028**
	No appendectomy	442	59.5 [58.6, 60.4]	

[ł]Data from de novo PD patients from the PPMI study.
[¥]Refers to mean and 95% confidence interval.
[‡]Determined using a weighted log-normal survival regression model, adjusted for sex, education years, ethnicity, and family history.