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DO SUBJECTS WITH MINIMAL MOTOR FEATURES HAVE PRODROMAL PD?

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Abstract

Background—Understanding the pathological changes underlying mild motor features of the elderly and defining a patient population with prodromal Parkinson's disease (PD) are of great clinical importance. It remains unclear, however, how to accurately and specifically diagnose prodromal PD. We examined whether older adults with minimal parkinsonian motor features have nigrostriatal degeneration and α -synuclein pathology consistent with prodromal PD.

Methods—Brain sections were obtained from older adults with a clinical diagnosis of PD (N=21) and without a clinical diagnosis of PD (N=27) who underwent motor examination proximate to death. Cases without PD were further dichotomized into no motor deficit (n=9) or minimal motor features (n=18) groups using a modified Unified Parkinson's Disease Rating Scale. We performed quantitative unbiased stereological analyses of dopaminergic neurons/terminals and α -synuclein accumulation in the nigrostriatal system.

Results—In all subjects with minimal motor features, there were significant reductions in dopaminergic neurons and terminals in the substantia nigra and putamen that was intermediate between subjects with no motor deficit and PD. Phosphorylated α -synuclein inclusions were observed in the substantia nigra that were of similar density to what was seen in PD. Furthermore, there was greater Lewy neuritic pathology in the putamen relative to PD patients. Lastly, neurons with α -synuclein inclusions displayed reductions in tyrosine hydroxylase expression that was comparable in subjects with both minimal motor features and PD.

Interpretation—Minimal motor features in older adults may represent prodromal PD and identify at risk individuals for testing putative neuroprotective interventions that could slow or prevent PD progression.

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Authors Contributions

AB and JHK contributed to conception and design of the study; YC, and JHK contributed to the acquisition and analysis of data; and AB, YC, CWO, and JHK contributed to the drafting the text and/or preparing the figures.

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Keywords

Parkinson's disease; minimal motor features; dopaminergic neurons; α -synuclein; substantia nigra; putamen

Introduction

The classic histopathologic hallmarks of Parkinson's disease (PD) are degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and α -synuclein (α -syn) positive Lewy pathology in cell bodies and neurites across the neuraxis.¹ It is now well-recognized that PD pathology is widespread, affecting serotonin, epinephrine, and cholinergic neurons, as well as nerve cells in the olfactory system, cerebral hemispheres, upper and lower brain stem, spinal cord and peripheral autonomic nervous system as well as dopamine neurons in the SNc.² There is also evidence that Lewy pathology accumulates for many years prior to the clinical diagnosis of PD, and that degeneration of dopamine neurons in the SNc likely represents a mid-stage of the disease process.³ Further, recent studies indicate that there is a virtual complete loss of dopamine terminals in the dorsal striatum by 4-5 years following diagnosis,⁴ thereby frustrating attempts to define a neuroprotective therapy in this population of patients. It has thus become a major focus of clinical research to try to define an early or prodromal PD state in which there are sufficient numbers of remaining dopamine neurons and terminals that could be protected or restored by a putative neuroprotective therapy. While several prodromal features such as constipation, REM behavior sleep disorder, and anosmia have been identified,^{5,6} these features are relatively non-specific and it has proven difficult to specifically identify a high percentage of individual patients who will go on to develop PD.

Approximately 15-20 % of clinically normal individuals have incidental Lewy bodies at post mortem and presumably have prodromal PD.⁷ However, it is currently not possible to identify these individuals during life, and there have been very few clinical-pathological studies that have sought to identify clinical factors that could identify these patients during life. Recent studies have suggested that patients with suspected prodromal PD have minimal-mild motor features⁸⁻¹² that are not sufficient to make a formal diagnosis of PD. If it could be confirmed that subjects having minimal motor features (MMF) have associated PD pathology, it could facilitate identifying patients with prodromal PD. To determine whether subjects with MMF have associated PD pathology, we compared the integrity of the nigrostriatal system and the presence of Lewy pathology in 3 groups of elderly subjects; those with no motor deficits (NMD), those with MMF, and those with PD.

Materials and methods

Assessment and Categorization of Subjects

We examined nigrostriatal brain sections from three groups of older adults. The first two groups consisted of older adults without a diagnosis of PD who were participants in the Religious Order Study (ROS), an ongoing clinical-pathologic cohort study¹³ in which participants undergo annual assessments of motor function using a modified version of the

motor Unified Parkinson's Disease Rating Scale (UPDRS).^{10,11} Based upon this score, subjects without PD were divided into two groups; those with (N=18) and those without (N=9) the presence of MMF. In previous publications, the term parkinsonism was used to refer to this group.^{10,11} The third group (N=21) consisted of subjects who were diagnosed as having PD by Movement Disorders Specialists in the Rush Movement Disorders Clinic. The ages of subjects in the three groups were 80+/-7, 90.9+/-5.7, and 77+/-10.2 respectively. During life, all subjects signed an informed consent for clinical assessment and an anatomic gift act for donation of brain at the time of death. Consent for post-mortem was obtained from next of kin or legal representative. The Human Investigation Committee at Rush University Medical Center approved this study.

All cases without PD were assessed within one year prior to their death by trained nurse investigators with a modified version of the UPDRS using a 0-5 rating scale.^{10,14} We have previously demonstrated that nurse clinicians can reliably administer the modified UPDRS with good inter-rater agreement with movement disorders specialists and temporal stability.^{11,15,16} Four established parkinsonian signs including gait, rigidity, bradykinesia and tremor were considered, and a global parkinsonian score was derived from the UPDRS scores^{17,19}. MMF was defined as having 2 or more of these parkinsonian signs with a score of 1, but without clinical features sufficient to meet the definition of PD based on UK brain bank criteria. No motor dysfunction (NMD) was defined as having one or none of these parkinsonian signs.¹¹ All PD cases were diagnosed by a board-certified movement disorder specialist using a battery of tests including the full UPDRS and met UK brain bank criteria. The 26 items included the modified version of the UPDRS were extracted from the full UPDRS.

Tissue processing and postmortem evaluation

At autopsy, the brains were removed from the calvarium and processed as described previously.¹⁸ Briefly, each brain was cut into 1 cm coronal slabs and then hemisected. The slabs were fixed in 4% paraformaldehyde for 5 days at 4°C. The one side brain slabs were used for pathological diagnoses. The other side brain slabs were cryoprotected in 0.1M PBS pH 7.4 containing 2% dimethyl sulphoxide, 10% glycerol for 48 h followed by 2% dimethyl sulphoxide and 20% glycerol in PBS for at least 2 days before sectioning. The fixed slabs containing nigra and striatum were cut into 18 adjacent series of 40 µm-thick sections on a freezing sliding microtome. All sections were collected and stored in a cryoprotectant solution before processing.

A complete neuropathologic evaluation was performed.¹⁹ Dissection of diagnostic blocks included a hemisection of brain, including substantia nigra and striatum. Lewy bodies were examined with H&E staining and further identified with antibodies to α -syn using alkaline phosphatase as the chromogen. A tissue diagnosis of PD was based on the presence of nigral Lewy bodies and moderate or severe nigral neuronal loss.^{3,9}

Immunohistochemistry

An immunoperoxidase labeling method was used to visualize dopaminergic neuron with tyrosine hydroxylase (TH, 22941/ImmunoStar) antibody and phosphorylated α -syn with

phosphor-S129 α -syn (p-S129- α -syn, ab51253/Abcam) antibody. Endogenous peroxidase was quenched by 20 min incubation in 0.1 M sodium periodate, and background staining was blocked by 1 h incubation in a solution containing either 2% bovine serum albumin and 5% normal goat or horse serum. Tissue sections were immunostained for TH (1:5000) and p-S129- α -syn (1:1000) at room temperature. After 6 washes, sections were sequentially incubated for 1 h in biotinylated horse anti-mouse IgG (1:200; Vector, Burlingame, CA) for TH and goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) for p-S129- α -syn followed by the *Elite* avidin–biotin complex (1:500; Vector) for 75 min. The immunohistochemical reaction was completed with 0.05% 3, 3'-diaminobenzidine (DAB) and 0.005% H_2O_2 . Sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and coverslipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

Optical fractionator estimates of TH and p-S129- α -synuclein immunoreactive nigral neuron densities

As only a partial substantia nigra could be obtained from Rush Alzheimer's Disease Center (approximately the caudal 1/2 of the nigra), the density of TH immunoreactive (TH-ir) and p-S129- α -syn immunoreactive (p-S129- α -syn-ir) neurons was evaluated individually for each subject in all groups. An optical fractionator unbiased sampling design was used to estimate the number of TH-ir neurons and p-S129- α -syn-ir inclusions and a Cavalieri's principle to assess the volume within substantia nigra pars compacta.^{20,21,22} In each subject, where possible, we evaluated the substantia nigra pars compacta from the level of midbrain at the exit of the 3rd nerve to the decussation of the superior cerebellar peduncle. Approximately 5 equispaced sections were sampled from each brain. The section sampling fraction (ssf) was 1/0.083. The distance between sections was approximately 0.48 mm. In cross-section, the substantia nigra is located in the ventral midbrain. The substantia nigra pars compacta was outlined according to neuromelanin (NM) using a 1.25 $\times$ objective. A systematic sample of the area occupied by the substantia nigra pars compacta was made from a random starting point (StereoInvestigator v10.40 software; Micro-BrightField, Colchester, VT). Counts were made at regular predetermined intervals ($x = 313 \mu\text{m}$, $y = 313 \mu\text{m}$), and a counting frame ($70 \times 70 \mu\text{m} = 4900 \mu\text{m}^2$) was superimposed on images obtained from tissue sections. The area sampling fraction (asf) was 1/0.05. These sections were then analyzed using a 60 $\times$ Planapo oil immersion objective with a 1.4 numerical aperture. The section thickness was empirically determined. Briefly, as the top of the section was first brought into focus, the stage was zeroed at the z-axis by software. The stage then stepped through the z-axis until the bottom of the section was in focus. Section thickness averaged $16.21 \pm 2.3 \mu\text{m}$ in the midbrain. The disector height (counting frame thickness) was $10 \mu\text{m}$. This method allowed for $1 \mu\text{m}$ top guard zones and at least $2 \mu\text{m}$ bottom guard zones. The thickness sampling fraction (tsf) was 1/0.62. Care was taken to ensure that the top and bottom forbidden planes were never included in the cell counting. Most dopaminergic neurons contain NM in midbrain. NM provides an easily discernible endogenous marker for dopaminergic neurons, allowing for an easy assessment of co-localization with TH-ir and p-S129- α -syn-ir products in dopaminergic neurons. TH-ir/NM-laden, p-S129- α -syn-ir/NM-laden, or NM-laden only (without immunostaining) nigral neurons were separately counted with different markers. Using stereological principles, TH-ir/NM, p-S129- α -syn-ir/NM, or NM-laden neurons in each case were sampled using a uniform, systematic, and random

design. The number of TH-ir/NM, p-S129- α -syn-ir/NM, or NM-laden only neurons within the substantia nigra pars compacta was calculated separately using the following formula: $N = \Sigma Q^{-1} / ssf \cdot 1/asf \cdot 1/tsf$. ΣQ was the estimated number of raw counts.

The Cavalieri estimator (StereoInvestigator software; Micro-BrightField VT) was used to estimate volume of the substantia nigra pars compacta on immunostaining sections. We sampled one serial sections of substantia nigra that extended from the exit of the 3rd nerve to the decussation of the superior cerebellar peduncle using the optical fractionator principle above. The distance between sections interspace was approximately 0.48 mm. According to NM the substantia nigra is outlined in cross-section using a 1.25 $\times$ objective. The section thickness was empirically determined. Section thickness averaged $16.23 \pm 2.2 \mu\text{m}$. The area estimation of substantia nigra pars compacta was performed by means of a $50 \times 50 \mu\text{m}$ point grid with 10 $\times$ objective. The total volume of substantia nigra pars compacta was calculated by Cavalieri estimator software.^{18,22} The densities of TH-ir, p-S129- α -syn-ir, or NM-laden neurons were separately calibrated by estimated nigral neuronal number from optical fractionator/substantia nigra volume from Cavalieri estimator (neuronal number/ mm^3). The coefficients of error (CE) were calculated according to the procedure of Gunderson and colleagues as estimates of precision.^{23,24} The values of CE were 0.10 ± 0.02 (range 0.08 to 0.12) in parkinsonism subjects and non-parkinsonism and 0.12 ± 0.05 (range 0.10 to 0.15) in PD. All Assessments were made by a blinded observer. The density of p-S129- α -syn-ir neurites in putamen was performed using the same method.

Double-label immunofluorescence

We have previously demonstrated that in PD, nigral neurons with α -syn inclusions lose tyrosine hydroxylase expression relative to neighbors without inclusions or age-matched controls.¹⁸ Thus, to see whether subjects with MMF display a similar phenomenon, a double-label immunofluorescence procedure was employed to determine whether α -syn inclusions affected dopaminergic neuronal expression in these cases. Midbrain sections from all three groups were incubated in the first primary antibody p-S129- α -syn (1:1000) overnight and the goat anti-rabbit antibody coupled to DyLight 649 (1:200, Jackson ImmunoResearch) for 1 h. After blockade for 1 h, the sections were then incubated in the second primary antibody (TH, 1:5000) overnight, and the goat anti-mouse antibody coupled to DyLight 488 (1:200) for 1 h. The sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and covered using DPX (Sigma-Aldrich).

Optical density measurements

Optical density measurements of TH immunoreactivity in the striatum—

Quantification of the relative optical density of striatal TH immunoreactivity was performed using a densitometry software program (Image 1.63; NIH), as described previously.²¹ The TH-immunostained putamen was outlined manually, and optical density measurements were automatically performed, in gray-scale (0 represented a maximum bright image and 255 represented a maximum dark image). For each subject, approximately ten equally spaced sections though the entire putamen were sampled and evaluated. To account for differences in background staining intensity, background optical density measurements in each section

were taken from corpus callosum that lacked TH-immunoreactive profile. The mean of these measurements constituted the background optical density that was subtracted from the optical density of TH-immunoreactive intensity measurements to provide a final optical density value. Previous studies have shown that optical density measurements reflect changes in protein expression parallel to those obtained using a biochemical protein assay such as Western blot.²⁵ All measurements were made by a blinded observer.

Fluorescence intensity measurements—Fluorescence intensity measurements were performed according to our previously published procedures.^{18,21,26} All immunofluorescence double-labeled images were scanned with an Olympus Confocal Fluoroview microscope equipped with argon, helium-neon lasers, and transparent optics. With a 20× magnification objective and a 488 and 633 nm excitation source, images were acquired at each sampling site in the substantia nigra pars compacta and were saved to a Fluoroview file. Following acquisition of an image, the stage moves to the next sampling site to ensure a completely non-redundant evaluation. Once all images were acquired, optical density measurements were performed on individual nigral neurons. To maintain consistency of the scanned image for each slide, the laser intensity, confocal aperture, photomultiplier voltage, offset, electronic gain, scan speed, image size, filter and zoom were set for the background level whereby autofluorescence was not visible with a control section. These settings were maintained throughout the entire experiment.^{18,21} The intensity mapping sliders ranged from 0 to 4095; 0 represented a maximum black image and 4095 represented a maximum bright image. The TH-ir perikarya with or without p-S129- α -syn-ir inclusions were identified and outlined separately. Quantitative optical density of immunofluorescence was performed on individual TH-ir soma with or without p-S129- α -syn-ir inclusions in different channels. Five equispaced sections of the substantia nigra were sampled and evaluated. The number of cells per case was analyzed as follows: >100 nigral cells in per non-parkinsonism, 50–70 nigral cells that contained p-S129- α -syn-ir inclusions and >100 nigral cells that did not contain inclusions per subject. To account for differences in background staining intensity, five background intensity measurements lacking immunofluorescent profiles were taken from each section. The mean of these five measurements constituted the background intensity that was then subtracted from the measured optical density of each individual neuron to provide a final optical density value. To confirm co-localization of p-s129- α -syn immunofluorescence, optical scanning through the neuron's z-axis was performed at 1- μ m thickness and neurons suspected of being double labeled were confirmed with confocal cross-sectional analyses. All measurements were made by a blinded observer.

Data analyses

Neuronal counts and optical density measurements were compared across groups with one-way Kruskal–Wallis test followed by Dunn's post hoc tests for multiple comparisons (Prism 4, GraphPad Software, Inc.). The level of significance was set at 0.05 (two-tailed).

Digital illustrations

Conventional light microscopic images were acquired using an Olympus microscope (BX61) attached to a Nikon digital camera DXM1200 and stored as tif files. Confocal

images were exported from the Olympus laser-scanning microscope with Fluoview software and stored as tif files. All figures were prepared using Photoshop 7.0 graphics software. Only minor adjustments of brightness were made.

Results

Parkinsonian signs in subjects with and without a clinical diagnosis of PD

We analyzed brain tissues from 9 older adults with NMD; 18 older adults with MMF; and 21 adults with a clinical diagnosis of PD. The diagnosis of PD was confirmed pathologically in each case and there was no evidence of an atypical parkinsonism (e.g. PSP or MSA) in any of these subjects. Demographics are provided in Table 1. As detailed in Table 1, NMD subjects displayed low global scores as well as individual scores on gait, bradykinesia, rigidity and tremor (Supplementary tables 1 and 2). In contrast, subjects with PD exhibited significantly higher scores on each of these measures (Supplementary table 3). For the MMF cases, scores were intermediate; It is noteworthy that only 1 of 18 subjects with MMF did not have bradykinesia..

Qualitative observations of nigrostriatal TH-immunoreactivity

Substantia Nigra—Cases from the NMD group showed numerous TH-immunoreactive somata with an intricate local plexus of TH-immunoreactive processes within the substantia nigra (Fig. 1A, 1B). A few NM-laden nigral neurons were TH-immunonegative (Fig 2B). This pattern was observed in each of the cases without motor deficits.

In cases diagnosed as having PD, both TH-immunoreactive somata and processes within the substantia nigra (Fig. 1E, 1F) were severely reduced compared to cases without motor deficits. Remaining TH-immunopositive neurons looked atrophic in comparison to the older adults without PD, and many NM-laden nigral neurons were TH-ir negative (Fig. 1F). Most of the remaining neurons appears normal in size with a relatively small number of TH-immunopositive cells appearing atrophic. These changes were noted in each of the PD cases.

Cases with MMF showed extensive and intense TH-immunoreactivities in the substantia nigra (Fig. 1C) that was intermediate between patients with NMD (Fig. 1A) and those with PD (Fig. 1E). Under high-power, some NM-laden nigral neurons displayed dark TH-stained somata and processes (Fig. 1D), while many NM-laden nigral neurons exhibited brown granules without TH-immunoreactivity (Fig 1D) and appeared atrophic. This pattern was observed in each of the cases with MMF.

Putamen—In NMD cases, a dense TH-immunostained fibers pattern was distributed throughout putamen in a mosaic pattern of low- and high-immunoreactive (patch matrix) zones (Fig. 2A) with dense meshed TH-immunoreactive in the putamenal grey matter (Fig. 2B) as has been described in normal individuals. In each of the PD cases, TH-immunoreactive fine fibers were barely detected in the majority of the putamen (Fig. 2E) with the few remaining fibres displaying swollen varicosities and segments (Fig. 2F) as we have previously described.⁴ Within the body of putamen, only a rare individual TH-immunoreactive fiber was observed. In addition, as we have published before in other cases,²¹ the only remaining plexus of TH-fibers seen in PD was in fibers between the medial

putamen and the lateral globus pallidus en route to the caudate nucleus (Fig. 2E). In each subject with MMF, putamenal TH-immunostaining was remarkably decreased (Fig. 2C, 2D) when compared with subjects with NMD (Fig. 2A, 2B). However, the density of TH-immunoreactivity in these individuals was clearly detectable throughout the entire putamen and was greater than that observed in the PD cases. Indeed while reduced in number (Fig. 2D), the general TH-immunostaining mosaic patch-matrix pattern was still preserved. (Fig. 2C). At higher power, some remaining putamenal fibers displayed an abnormal morphology characterized by swollen varicosities (Fig. 2D).

Quantification of nigral neuron number of putamenal TH optical density

Stereological analyses were employed to estimate the number of nigral neurons. The density of NM-laden neurons was reduced by 54.6 % in subjects with MMF (1558.39 ± 803.9) and 73.9% in PD cases (893.58 ± 693.5) in comparison to cases with NMD (3431.77 ± 617.1) ($p < 0.001$ for difference between groups; Fig 3A). Post hoc analyses demonstrated that the densities of NM-laden neurons in subjects with MMF was intermediate between those with NMD and PD; lower than NMD ($P < 0.05$) and higher than PD cases ($p < 0.05$).

The density of TH expression in dopaminergic nigral neurons in the SNc was (3116.47 ± 770.7) in cases with NMD. It was decreased by 64.1% in those with MMF (1137.70 ± 537.9) and by 85.8% in PD (450.19 ± 323.1) (difference between groups = $p < 0.001$; Fig. 3B). The densities of TH-ir neurons in subjects with MMF was significant lower than subjects with NMD ($p < 0.05$) and higher than PD ($p < 0.05$).

Optical density measurement of putamenal TH staining was graded at 101.71 ± 14.9 in individuals with NMD, and was reduced by 47.2% in those with MMF (53.75 ± 23.1) and by 76.8% in PD (23.62 ± 10.3) (Fig. 3C). A Kruskal–Wallis test revealed a statistically significant difference in the optical density of putamenal TH-ir intensities among groups ($P < 0.0001$;). Post hoc analyses revealed that there was a greater reduction in subjects with PD than in those with MMF ($p < 0.001$).

Phosphorylated α -synuclein in nigrostriatal system

In the NMD group, aggregated p-S129- α -syn immunoreactivity was undetectable in the SNc. In contrast, all cases with MMF and PD displayed p-S129- α -syn-ir inclusions in the SNc (Fig. 4C, 4E). Nigral neurons with p-S129- α -syn labeling displayed dark somata with extensive processes (Fig. 4C). Within the putamen, virtually no p-S129- α -syn-ir was observed in any of the NMD cases (Fig. 4B). In contrast, extensive and intensely stained p-S129- α -syn-ir fibres were observed in putamen (Fig. 4D) of subjects with MMF. In these cases, the p-S129- α -syn-ir labeled putamenal profiles were characterized by having swollen varicosities, segments, and punctuated boutons (Fig. 4D'). Nigral p-S129- α -syn-ir appeared round in shape morphologically appearing like Lewy bodies (Fig. 5E). Interestingly, fewer putamenal p-S129- α -syn-ir processes were seen in the putamen of PD cases than in those with MMF probably reflecting relative preservation of striatal terminals in these cases (Fig. 4C, 4D, 4E, 4F).

Stereological analyses verified that the density of p-S129- α -syn-ir in SNc neurons was similar between subjects with MMF and PD with no statistically significant differences

between the groups (NS; Fig. 5A). However, the density of putamenal p-S129- α -syn-ir Lewy neurites were much higher in MMF subjects than in the PD cases ($p < 0.001$ Fig. 5B).

Co-localization and quantitative analysis of TH expression in nigral neurons with α -syn inclusions

We have previously demonstrated that in PD, individual nigral neurons containing α -syn aggregates exhibit reduced TH expression.¹⁸ We used optical density measurements of TH to assess whether there is a similar loss of TH expression associated with α -syn accumulation and aggregation in subjects with MMF. Double-labelling studies revealed that the staining intensity of perikaryal TH immunoreactivity in neurons without α -syn-ir inclusions was similar across all three groups (Fig. 6A, 6D, 6G). In contrast, as we have seen previously reported,¹⁸ there was a significant reduction in TH expression in nigral neurons with α -syn inclusions in PD cases ($p < 0.001$). Interestingly, TH expression was similarly and equally reduced in nigral neurons that contained α -syn-ir aggregates in subjects with MMF and PD (Fig. 6E, 6F). Quantitatively, a Kruskal–Wallis test revealed a significant difference in the optical density of TH immunoreactivity in nigral perikarya (Fig. 6J; $P < 0.0001$) among groups. Relative to cases with NMD, the fluorescent intensity of TH was reduced by ~11.9% in neurons without α -syn-ir inclusions in both PD cases and those with MMF ($p > 0.05$). In contrast, in both of these groups nigral neurons that contained α -syn inclusions had a similar and significant reduction in TH-immunofluorescence intensity (67% for MMF and 71% for PD; both $p < 0.001$) versus nigral neurons without α -syn inclusions (Fig. 6J).

Discussion

We used a unique cohort of individuals to quantify the degree to which individuals with MMF displayed nigrostriatal degeneration and α -synuclein pathology that would indicate that this condition represents prodromal (albeit not premotor) PD. Additionally it should be noted that this paper focused upon the nigrostriatal system when it is clear that PD is a disease affecting numerous systems across the neuraxis and indeed the peripheral nervous system as well³. We did see α -syn aggregation in non-nigrostriatal regions (e.g. the raphe nuclei) of the brain from MMF subjects and we are currently evaluating whether this pathology follows a PD-like pattern.

For purposes of this study, MMF was defined as subjects with more than 1 parkinsonian motor feature (bradykinesia, tremor, rigidity, or gait dysfunction) with a score of 1 on a modified UPDRS, and no clinical evidence of dementia or an alternate explanation for motor deficits. Subjects from the Religious Orders Study without a clinical diagnosis of PD determined to have NMD or MMF were compared to patients that were diagnosed clinically with PD based on UK Brain Bank Criteria. Using a modified UPDRS scale, patients with MMF had a global parkinsonian (motor) score intermediate between the NMD and PD groups. Clinical assessments in the PD group was performed by movement disorder experts while the NMD and MMF cases were assessed by a trained nurse practitioner. However, when directly compared, correlations between nurses and movement disorder expert exceeded 0.90 for the total modified UPDRS, ranged from 0.76 to 0.95 for the four parkinsonian domain scores, and exceeded 0.90 for the global parkinsonian sign score.¹⁵

Thus the variability between groups are unlikely due to differences in assessors. These clinical data raise the possibility that MMF, as defined in this study, might be a prodromal form of PD. It should be noted however, that this study cannot determine the specificity of minimal motor features for the development of PD. In normal aging as well as other disease states such as Alzheimer's disease minimal motor features can be observed⁹. Indeed the seminal paper by Adler and coworkers⁴⁰ found a low concordance between clinical diagnosis of early PD with neuropathological confirmation.

We performed a number of pathological studies to test the hypothesis that the MMF cohort as presently defined is prodromal PD. As known for decades, degeneration of the nigrostriatal system is a hallmark pathology in PD. We hypothesized that if our MMF cohort is prodromal PD, then the nigrostriatal system should degenerate to a degree intermediate between what is seen in PD and healthy controls. This is what we found. The density of both melanin expressing and TH-containing nigral neurons seen in both the non-parkinsonian and PD cohorts was similar to what we¹⁸ and others²⁷ have reported previously using stereological assessments. The number of nigral neurons using either melanin and TH as markers in MMF patients was intermediate between these two groups supporting the concept that the MMF cohort is prodromal PD.

Similarly, we assessed dopaminergic innervation of the putamen in all three groups. The NMD group displayed robust TH-ir throughout the entire putamen with fibers and terminals displaying a characteristic patch-matrix pattern. PD cases, on the other hand, displayed a TH-innervation pattern similar to what we have previously described.⁴ That is, within the body of the putamen there were negligible TH-ir fibers with only individual fibers being able to be visualized except for a plexus that runs in the external medullary lamina of the globus pallidus en route to the caudate nucleus. TH-ir fiber expression in the the MMF group was intermediate between the two other groups. The entire putamen contained appreciable TH-ir fibers, but at a density much lower than the NMD group and higher and with more breadth than in the PD group. This finding too, suggests that MMF is prodromal PD. This finding potentially has significant clinical impact. Numerous disease modifying experimental therapeutic clinical trials such as those employing trophic factor protein and gene delivery^{28,29} have failed to-date in part because the residual dopaminergic fiber innervation to the striatum was negligible at the time patients were enrolled into the study. The prevailing school of thought has been that interventions need to occur at a timepoint in the disease in which sufficient nigrostriatal innervation is available for these therapies to work.²⁷ The fact that the cohort with the mild motor deficits has such a residual innervation, mimics the status of the nigrostriatal systems seen in pivotal and successful preclinical studies,³⁰ and suggests that these therapies might be more successfully applied if tested in an MMF cohort rather than PD. These findings emphasize the importance of identifying subjects with prodromal PD for testing putative neuroprotective interventions at a time when there are still nerve cells and terminals available to be rescued or protected.

Clearly, PD is a synucleinopathy³¹ and α -syn aggregation would be required for our MMF cohort to be considered prodromal PD. Indeed, we found that in the substantia nigra from the MMF cohort, α -syn aggregation and serine 129 phospho- α -syn expression is present in a manner indistinguishable from PD. This is a critical separation point from the MMF just

being advanced aging because we have previously reported in aged humans and nonhuman primates that aged nigral neurons only display a pathological expression of soluble non-aggregated α -syn.³² Another remarkable finding was the robust expression of serine-129 phospho α -syn within the MMF putamen. The degree of expression was far greater than what was seen in PD, likely because there was greater residual dopaminergic innervation within the putamen in these early cases than in those who had gone on to develop PD.

Another important finding is that remaining dopaminergic fiber innervation seen in the MMF group displays robust expression of serine 129 phospho α -syn and this fact can have important therapeutic implications. This suggests that therapies that fail to work in preclinical models that are alpha synuclein based may be ineffective in this patient population. Specifically we are referring to trophic factors such as glial cell derived neurotrophic factor (GDNF) and neurturin; two potent trophic factors that are effective in virtually all PD models except those based upon alpha synuclein over-expression.²⁸ Indeed randomized placebo controlled clinical trials testing trophic factors such as GDNF and neurturin in PD have likely failed to date^{33–36} because these factors do not provide neuroprotection or disease modification in α -syn based models likely due to alpha synuclein's down-regulation of Nurr1 and Ret receptors.^{37,38,39} Thus the concept of going earlier for these therapies will likely require identifying individuals even before they display MMF due to the extensive synucleinopathy already present in the dopaminergic fibers in this cohort. Still, this cohort may be important for testing novel therapies that work via other mechanisms.

Finally, for the MMF cohort to be considered to have prodromal PD, other known biological principles known to be true in the PD nigrostriatal system should also be true in the MMF cohort. We previously established that nigral neurons with aggregated α -syn display a diminished dopaminergic phenotype^{18,21,32}. We hypothesized that this would occur in the MMF cohort in an intermediate fashion much like the alterations in nigral neuron number and dopaminergic fiber innervation. Indeed, the level of phenotypic downregulation seen in the MMF group was virtually identical to what was seen in PD; again supporting the notion that MMF as presently defined is prodromal PD.

There are important limitations to our study. We only studied a relatively small number of subjects – nonetheless it is noteworthy that all subjects in the MMF subgroup demonstrated virtually the same pathology. It remains possible that some subjects with MMF pathology will never develop clinical manifestations of PD or will go on to form a different type of synucleinopathy such as LBD or MSA, and prospective longitudinal studies monitoring subjects with MMF are warranted. It will also be interesting to determine if other factors implicated in prodromal PD such as RBD and anosmia, increase the likelihood and time course of MMF subjects going on to develop classical PD. In addition, individuals with MMF had lower MMSE scores than PD cases. Firstly, the PD cases on average were younger and this in part may explain less cognitive impairment. In addition previous reports in this community cohort reported increased dementia in cases with Lewy pathology suggesting that the incidence of Lewy body dementia may be four fold greater than prior reports. Thus, synucleinopathies may make a larger contribution to loss of motor and cognitive function in older adults. This deserves further investigation.

Prior clinical-autopsy studies in this cohort have reported the association of Lewy bodies and nigral neuronal loss with parkinsonian MMF in older adults without a clinical diagnosis of PD, but did not directly compare the clinical and autopsy findings in cases with and without PD or examine the integrity of the nigrostriatal system in older adults with evidence of PD pathology.^{9,10,11} By comparing older adults with and without PD, the current study extends these prior studies showing the full clinical heterogeneity associated with PD pathology. In addition, the results of the current study suggests that not only do some older adults with MMF accumulate PD pathology but that these adults also show changes in the integrity of the nigrostriatal system that are similar but less severe than PD. Thus, the current results provide important evidence which supports the idea that like other neurodegenerative pathologies such as AD, PD pathology may have similar clinical stages with an asymptomatic PD pathology stage, followed by a prodromal stage in which PD pathology results in MMF, leading to a later stage, when signs are severe enough for the clinical diagnosis of PD. This unique MMF population which may be a motor signature for prodromal PD may be valuable for studying PD pathogenesis and provide critical information regarding the potential for therapeutic, especially regenerative, initiatives.

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Fig. 1. Patterns of tyrosine hydroxylase (TH) immunoreactivity in substantia nigra from NMD (A, B), MMF (C, D), and PD (E,F) subjects. There was a clear reduction of TH immunoreactive neurons in subjects with MMF (C, D) compared with NMD (A, B). PD case (E) displayed severe reduction of TH immunoreactivity relative to MMF (C). Some remaining nigral melanized neurons exhibited TH immunoreactivities (arrows; D, F) while others displayed no detectable TH immunoreactivity (arrowheads; D, F). Scale bar = 100 μm in F (applies to B, D); 500 μm for A, C,E.).

Fig 2.

Patterns of TH staining in the putamen from NMD (A, B), MMF; (C, D), and PD E,F). An loss of TH staining was observed in MMF cases © relative to NMD (A) subjects. However, staining was detectable throughout the putamen in MMF cases, PD patient displayed virtually undetectable TH immunoreactivity in major putamen, except the ventromedial putamen near globus pallidus (arrows; E). At higher magnification, dense TH immureactive fine fibre was observed in NMD subjects (B). In contrast, TH labeled fibres from MMF subjects were displayed swollen varicosities and segments in mild-motor feature subjects

(D). In PD case, remaining TH immunoreactive fibre exhibited swollen segments (F). Scale bar = 20 μ m in F (applies to B and D); 2.0mm for A, C, and E.

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Fig. 3. Histograms showing the density of nigral melanin-laden neurons (A) and nigral tyrosine hydroxylase (TH) immunoreactive neurons (B) and optical density of TH immunoreactivities in putamen (C) from subjects with NMD, MMF, and PD. The density of nigral melanin-laden and TH-immunoreactive neurons and the levels of putamenal TH immunoreactivities were intermediate in MMF subjects as compared with NMD and PD. (* <0.05, ***P<0.001 compared with non-parkinsonism; #p<0.05, ### P<0.001 compared with parkinsonism). Data are mean $\pm$ SD. AGSU = arbitrary grey scale units.

Fig. 4.
Pattern of phosphorylated α -synuclein (p-S129- α -syn) immunoreactivity in substantianigra (A, C, E) and putamen (B, D, D', F) from subjects with NMD (A, B) MMF (C, D, and PD (E, F). PS129- α -syn immunoreactivity was undetectable in the subjects with NMD (A, B). In contrast, p-S129- α -syn immunoreactive nigral neurons (C, E) and putamenal fibres (D, F) were observed in subjects with MMF(C, D) and patients with PD (E, F). Note more p-S129- α -syn immunoreactive processes in subjects with MMF (C, D) than patients with PD (E, F). The p S129- α -syn immunoreactive processes displayed varicosities (arrow; D'), dot (curved

arrow, D'), and segments (arrowhead, D') in putamen. Scale bar = 100 μ m in F (applies to A-D) and 20 μ m for D'.

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Fig. 5.

Histograms showing the densities of phosphorylated α -synuclein (p-S129- α -syn) inclusions in substantia nigra (A) and neurites in putamen (B) from subjects with mild motor features (MMF) and patients with Parkinson's disease (PD). There was no difference on densities of nigral p-S129- α -syn immunoreactive inclusions between subjects with MMF and patients with PD (A). Interestingly, the densities of putamenal p-S129- α -syn immunoreactive inclusions in subjects with MMF were significantly higher than the patients with PD (B).

*** $P < 0.001$ compared with parkinsonism). Data are mean $\pm$ SD.

Fig. 6. Confocal microscopic images of substantia nigra from subjects with NMD (A-C), MMF (D-F), and PD (G-I) illustrating immunostaining for tyrosine hydroxylase (TH; green; A, D, G), α -synuclein inclusions (α -syn; red; B, E, H), and co-localization of TH and α -syn (merged; C, F, I). Note that TH immunofluorescent intensity was extensively reduced in the nigral neurons with α -syn inclusions (arrowheads, E, F, H, I) but not in the nigral neurons without α -syn inclusion (arrows D, F, G, I) in both MMF and PD. Scale bar in I = 30 μ m (applies to all). Measurements of immunofluorescent intensities (J) further revealed that TH expression

was significantly reduced in the neurons with p-S129- α -syn inclusions but not in the neurons with absent α -syn inclusions.*** $P < 0.001$ compared with non-parkinsonism; ## $P < 0.01$ compared with neurons without α -syn inclusion. Data are mean $\pm$ SD, AFU = arbitrary fluorescence units.

Table 1

Clinical and Postmortem Characteristics (Mean±SD)

Measure	No motor impairment		Mild motor features
Parkinson's disease			
Cases number	N=9	N=18	N=21
Clinical Measures			
Age at death (yrs)	80.66±7.0	90.94±5.7 ^{*##}	77.00±10.2
Sex (male/female)	6/3	5/13	10/11
Mini-mental status examine (0-30)	27.77±1.4	11.00±10.4 ^{**###}	26.12±4.2
Number of parkinsonian signs present	0.88±0.3	1.69±0.5 ^{###}	3.50±0.5 ^{***}
Global parkinsonism score	6.51±3.4	15.50±10.5 ^{###}	37.50±10.8 ^{***}
Gait score	3.29±1.9	35.83±20.8 ^{**}	52.14±24.8 ^{***}
Bradykinesia score	3.37±3.3	14.65±12.3 ^{###}	52.98±16.7 ^{***}
Tremor score	4.04±3.0	3.29±5.4 ^{###}	9.69±13.1 ^{***}
Rigidity score	5.00±5.5	13.61±22.4 ^{###}	41.66±20.3 ^{***}
Postmortem interval (hrs)	6.26±2.7	9.20±5.8	7.06±3.8

*
P<0.05,**
P<0.01,***
P<0.001 compared with Non-parkinsonism;#
P<0.05,##
P<0.01,###
P<0.001 compared with Parkinson's disease.