

Bury AG, Pyle A, Elson JL, Greaves L, Morris CM, Hudson G, Pienaar IS.
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Parkinson disease.](#)

Annals of Neurology 2017, Epub ahead of print.

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Date deposited:

11/12/2017

Embargo release date:

17 November 2018

1 **Mitochondrial DNA changes in pedunculopontine cholinergic neurons in**
2 **Parkinson's**

3 ***Running Head: PD affects mtDNA in PPN cholinergic neurons***

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24
25 Character Count (Title): 80, including spaces

26 Character Count (Running Head): 43, including spaces

27 Word Count (Abstract): 100

28 Word Count (Body): 1,651

29 Figure and Table Count: 3 Main Figures (1 Color; 2 Black and white)

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32 **ABSTRACT**

33 In Parkinson's disease (PD), mitochondrial dysfunction associates with nigral dopamin-
34 ergic neuronal loss. Cholinergic neuronal loss co-occurs, particularly within a brainstem
35 structure, the pedunclopontine nucleus (PPN). We isolated single cholinergic neurons
36 from post-mortem PPNs of aged controls and PD patients. Mitochondrial DNA (mtDNA)
37 copy number and mtDNA deletions were increased significantly in PD patients com-
38 pared to controls. Furthermore, compared to controls the PD patients had significantly
39 more PPN cholinergic neurons containing mtDNA deletion levels exceeding 60%, a
40 level associated with deleterious effects on oxidative phosphorylation. The current re-
41 sults differ from studies reporting mtDNA depletion in nigral dopaminergic neurons of
42 PD patients.

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55 **1. Introduction**

56 Parkinson's disease (PD) patients typically present with tremor, bradykinesia and rigid-
57 ity, the onset and progression of which associate with selective loss of nigro-striatal do-
58 paminergic neurons.¹ Interest has grown as to the role of non-dopaminergic neurotrans-
59 mission in PD. Cholinergic degeneration, affecting the basal forebrain, the nucleus ba-
60 salis of Meynert and a rostral brainstem structure called the pedunculo pontine nucleus
61 (PPN), associates with the onset and development of 'axial' signs and cognitive impair-
62 ment seen in PD patients.²⁻⁴ Surviving (but susceptible) cholinergic neurons in these nu-
63 clei contain aggregated α -synuclein fibrils, forming Lewy bodies and Lewy neurites, a
64 neuropathological hallmark of PD.³

65 The links between mitochondria and PD patho-etiology are well studied.⁵ This
66 commenced in the 1980s when the potent respiratory chain inhibitor 1-methyl-4-phenyl-
67 1,2,3,6-tetrahydropyridine (MPTP), serving as a prodrug to the neurotoxin MPP+, was
68 shown to cause parkinsonism in illicit drug users,⁶ through to identification of complex-I-
69 mediated reactive oxygen species forming within PD brains⁷ and continuing with inher-
70 ited and somatic mitochondrial DNA (mtDNA) variants that were shown to affect PD
71 risk.⁸⁻¹⁰ Additionally, changes in mitochondrial DNA copy number (mtCN) was shown to
72 associate with PD pathology, with vulnerable dopaminergic neurons showing depleted
73 levels of wild-type mtCN.^{11,12}

74 Recently, we showed that several mitochondrial respiratory chain proteins are sig-
75 nificantly up-regulated in gamma-aminobutyric acid(GABA)ergic and glycinergic PPN
76 neurons from PD post-mortem brains.³ Conversely, in the same patients, there was a
77 marked reduction of these mitochondrial proteins in the cholinergic neurons of the PPN,

78 suggesting mitochondrial injury. Moreover, we observed a significant reduction in mito-
79 chondrial mass across all three neuronal types, with the most pronounced loss seen in
80 PPN cholinergic neurons.³ Given the links between PPN cholinergic neuronal deteriora-
81 tion, mitochondrial function and the development of PD, we investigated the role of
82 mtDNA maintenance and stability in cholinergic neurons from the PPN of PD patients
83 and controls.

84 **2. Materials and Methods**

85 *Subjects:* Post-mortem human brains (n=6 controls; n=6 PD; male:female ratio: 5:1)
86 were obtained from the Newcastle Brain Tissue Resource in Newcastle upon Tyne,
87 UK, which is covered by Newcastle University's Human Tissue Authority license. Ethical
88 approval was granted for this study in 2016 by the Newcastle and North Tyneside Local
89 Research Ethics Committee. All tissue donors gave written informed consent during life.
90 All cases were assessed locally by a Neuropathologist and met the UK-PD Society's
91 brain bank criteria for diagnosing PD. None of the control cases met such criteria and
92 also did not meet operational criteria for diagnosing Alzheimer's disease, with control
93 brains that showed only minimal, age-related tau and β -amyloid pathology. The mean (\pm
94 standard error of the mean (S.E.M.)) for post-mortem interval (PMI) was 21 ± 4 hours (hrs)
95 for PD cases and 20 ± 3 hrs for controls. The mean (\pm S.E.M.) age of death was 76 ± 3
96 years for PD cases and 84 ± 7 years for controls. Between PD and control cases
97 there was no statistically significant differences in PMI or age of death
98 ($p=5.2\times 10^{-1}$ and $p=2.3\times 10^{-1}$, respectively; independent Student's *t*-test). The
99 mean disease duration for the PD cohort was 8 ± 2 years.

100 *PPN identification:* Serial sections (20 μ m) were cut from the left hemisphere of PPN-
101 containing brain blocks, using a cryostat (Bright Instrument Company Ltd., UK). To dis-
102 tinguish the PPN, pairs of sections (first and last in series) for each case were stained
103 with Haematoxylin and Eosin (H&E) (Fig. 1A) and Luxol Fast Blue (LFB) (Fig. 1B), using
104 standard protocols. Paired stained sections were then used to define the PPN's bound-
105 aries, noting neighboring neural structures, including the lateral lemniscus (LL), medial
106 lemniscus (ML) and superior cerebellar decussation (SCD).¹³

107 *Single cell isolation:* To immunohistochemically stain cholinergic neurons in PPN-con-
108 taining brain tissue sections, the sections were air-dried for 30 min at room temperature
109 (RT) and then fixed for 20 min in 4% paraformaldehyde (PFA) dissolved in phosphate
110 buffered saline (PBS). The sections were washed with PBS before blocking with 5%
111 normal rabbit serum (S-5000; Vector laboratories, UK) for 30 min, then incubated for 2
112 hrs at RT with a primary antibody detecting choline acetyltransferase (ChAT, polyclonal
113 goat, 1:150; AB 144P, Millipore, USA). After further washing in PBS, the secondary anti-
114 body (peroxidase horse anti-goat, 1:200; PI-9500, Vector laboratories, UK) was applied
115 for 1 hr at RT. Following washes in PBS, 3,3',5,5'-tetramethylbenzidine (TMB) stabilized
116 chromagen (Invitrogen, UK), a substrate of horseradish peroxidase that oxides to form a
117 blue chromagen, was applied to the sections for 10 min at RT and then rinsed well with
118 distilled water. All antibody and serum dilutions were made using Tris Buffered Saline
119 (TBS). Individual PPN cholinergic neurons were isolated using the P.A.L.M. MicroBeam
120 Laser-Capture Microdissection system coupled to an inverted Zeiss microscope (Axio-
121 vert 200M, Carl Zeiss, Germany), and individually placed into adhesive cap microfuge

122 tubes (Carl Zeiss, Germany) containing lysis buffer consisting of 50mM Tris-Hydrochloride
123 ride with 1% Tween 20 (pH 8.0) and 20mg/ml proteinase K (Thermo Fisher Scientific,
124 UK). Each lysis was immediately centrifuged at 13,000 revolutions/min and subse-
125 quently incubated at 55°C for 16 hrs, followed by incubation at 95°C for 10 min. In total,
126 144 neurons were isolated (n=72 controls; n=72 PD).

127 *mtDNA analysis:* Quantification of mtDNA was performed as previously described,¹⁴ via
128 a probe-based¹¹ multiplex Taqman quantitative polymerase chain reaction (qPCR) to
129 amplify the mitochondrial genes *MTND1* and *MTND4*. mtCN was calculated by absolute
130 quantification of *MTND1*, using the standard curve method, with serial dilutions of PCR-
131 generated templates. PD and control samples (assayed in triplicate) were randomly as-
132 signed to each run to limit run-specific stratification.

133 *Statistical analysis:* Data were analyzed using SPSS (version 22, SPSS Inc., USA) with
134 data-appropriate tests (detailed in text). Statistical significance was set at $p < 5 \times 10^{-2}$. The
135 choice of which analyses to apply was based on the data type. A correlative analysis
136 was used when two continuous variables were present, for example mtCN and PMI. A
137 one-way ANOVA was used where there was greater than two categories whose means
138 were to be compared, i.e. mtCN versus Braak stage (0-4). Data are expressed as the
139 mean \pm S.E.M. The raw mtCN data is available on request

140 **3. Results**

141 Comparison of mtDNA levels from single PPN cholinergic neurons showed a signifi-
142 cant increase in mtCN in PD cases compared to controls ($p = 2.9 \times 10^{-2}$, two-way

143 ANOVA, PD mean = 10,706±1,441 versus control mean = 7,017±825; Fig. 2A). Im-
144 portantly, no correlation was found between PMI and mtCN in either PD cases or con-
145 trols ($p=5.1\times 10^{-1}$ and $p=8.7\times 10^{-1}$ respectively, linear regression analysis). Furthermore,
146 no significant association was detected between mtCN and PD duration ($p=9.7\times 10^{-1}$, linear regression analysis) nor mtCN and Braak stage ($p=3.1\times 10^{-1}$, one-way
147 ANOVA; Fig. 2B).

149 mtDNA deletion levels were also significantly increased in PD cases com-
150 pared to controls ($p=2.5\times 10^{-2}$, two-way ANOVA, PD mean = 21.60±3.04% versus con-
151 trol mean = 17.15±1.99%; Fig. 2C). Again, no correlation was found between PMI and
152 mtDNA deletions in neither cases nor controls ($p=9\times 10^{-2}$ and $p=3.4\times 10^{-1}$ respectively,
153 linear regression analysis). However, mtDNA deletion levels correlated with higher
154 Braak staging in PD patients ($p=3\times 10^{-3}$, one-way-ANOVA; Fig. 2D). Stratification of in-
155 dividual neurons into high- (i.e. >60% at which level they are likely to exhibit a pathologi-
156 cal phenotype⁸) and lower mtDNA deletion levels (i.e. <60%, and therefore less likely to
157 exhibit a pathological phenotype), revealed that PD cases harbored a disproportionately
158 higher number of PPN cholinergic neurons with high mtDNA deletion load compared
159 to controls ($p<1\times 10^{-4}$, Fisher's exact test; Fig. 3A).

160 Thus, unlike recent studies performed on substantia nigra pars compacta (SNpc)
161 dopaminergic neurons taken from post-mortem PD brains, and which reported mtCN
162 depletion,^{6,9,10,12} the current findings relating to single PPN cholinergic neurons showed
163 significantly elevated mtCN and mtDNA deletion levels in PD patients compared to con-
164 trols. We investigated this relationship further by using linear regression analysis. A sig-
165 nificant negative correlation was observed between mtCN and mtDNA deletion levels in

166 neurons taken from controls ($r=-2.5 \times 10^{-1}$, $p=4.9 \times 10^{-2}$), this finding resulting from very
167 few neurons having higher levels of mtDNA deletions. When all data points were in-
168 cluded, no significant association was detected between deletion levels and mtCN
169 ($r=5.4 \times 10^{-2}$, $p=6.7 \times 10^{-1}$; Fig. 3A).

170 An elevation in mtCN in response to the accumulation of mtDNA deletions might
171 be considered evidence for cells' ability to proliferate mtDNA in order to maintain a criti-
172 cal number of wild-type mtDNA molecules to support a variety of physiological pro-
173 cesses. This regression analysis was repeated, by excluding neurons containing lower
174 deletion levels (30%), as at these lower levels the neuroprotective processes might not
175 have been triggered yet, thereby inhibiting the ability to detect a correlation between the
176 two variables. The exclusion of neurons with lower mtDNA deletion levels did not reveal
177 a significant relationship between deletion levels and mtCN ($r=0.23$, $p=4 \times 10^{-1}$; Fig. 3B).
178 Taken together, our study reveals an elevation of mtCN and mtDNA deletion levels in
179 PD patients compared to controls; however, a correlation was not detected between
180 mtCN and mtDNA deletion levels in the single cholinergic neurons of the PD patients.

181 **4. Discussion**

182 This is the only study to date that characterizes mtCN and mtDNA deletion levels in cho-
183 linergic neurons of the PPN, a neuronal population that is highly vulnerable to cell death
184 in PD patients. The aim was to better understand the role that mtDNA changes play in
185 the loss of PPN cholinergic neurons in PD, which has been shown to be pivotal in the
186 onset and progression of motor and non-motor PD symptoms.

187 Our investigations revealed that in remaining PPN cholinergic neurons of PD pa-

188 tients mtCN is increased compared to controls. This is in contrast to studies which re-
189 ported decreased mtCN levels within remaining SNpc dopaminergic neurons in PD post-
190 mortem brains,^{8,11,12,15} suggesting for neuronal-type and brain region-specific responses
191 to accumulation of mtDNA mutations in PD patients. However, similar to previous studies
192 performed on SNpc dopaminergic neurons,^{8,11,12,15} our data indicate that mtDNA deletions
193 are increased in PD patients compared to controls. Importantly, we show that in PD pa-
194 tients there is a substantial increase in the number of PPN cholinergic neurons contain-
195 ing >60% mtDNA deletion levels, making it highly likely that these neurons should mani-
196 fest a respiratory chain deficiency.⁸

197 Our data shows that cholinergic neurons in the PPN, in contrast to reports of deple-
198 tion in the SNpc of PD cases,^{8,11,12,15} appear to increase their mtCN in response to rising
199 deletion levels. This raises the hypothesis that the PPN has a compensatory mechanism
200 designed to maintain a pool of wild-type mtDNA molecules that is not present in the SNpc,
201 or alternatively that the dopaminergic neurons of the SNpc are more vulnerable to rising
202 mtDNA deletion levels than the cholinergic neurons of the PPN. The former appears to
203 be supported by our data, as we detected a seemingly overall elevation in mtCN in the
204 PD patients who also had higher deletion levels (Figs. 2A & B). This may reflect an ina-
205 bility by PPN cholinergic neurons to maintain the required level of wild-type mtDNA past
206 the threshold deletion level, or might result from the smaller number of neurons with high
207 levels of mtDNA deletions which we observed here. Nevertheless, this exploratory obser-
208 vation should be explored further in future studies.

209 Although not a conclusive indicator of PD, Braak staging indicates disease ad-
210 vancement.¹⁶ In this study, we identified a significant difference between high mtDNA

211 deletion levels and advanced Braak staging, supporting the hypothesis that mtDNA de-
212 letions within PPN cholinergic neurons contribute to PD progression. This is similar to
213 SNpc dopaminergic neurons in PD patients, where Lewy body abundance, associated
214 with advanced Braak staging, coincided with increased mtCN, which was hypothesized
215 as a possible compensatory response against deficient levels of adenosine triphosphate
216 (ATP).¹⁷ In the current study, we found no significant difference in PPN cholinergic neu-
217 ronal mtCN values between the different Braak stages; however, this is an area that
218 merits additional investigation in future studies.

219 In conclusion, we show increased mtCN and mtDNA deletion levels within remain-
220 ing PPN cholinergic neurons in PD patients compared to controls. We further found sig-
221 nificantly more cholinergic neurons harboring mtDNA deletion levels that associate with
222 a mitochondrial dysfunction in PD, compared to controls. These findings support the
223 view that mtDNA deletions are frequent in PPN cholinergic neurons and that these
224 might play a role in the death of these neurons in PD patients. Critically, the data sug-
225 gests that different brain regions and neurochemical cell types vary in their responses to
226 accumulated mtDNA deletions in PD patients, since the data contrasts with prior obser-
227 vations relating to SNpc dopaminergic neurons.

228 **Acknowledgements**

229 ISP receives grant support from the Rosetrees Trust and the British Pharmacological So-
230 ciety. GH is a Parkinson's UK Senior Research Fellow (F-1202).

231 **Abbreviations used:** ATP, adenosine triphosphate; ChAT, choline acetyltransferase; CI,
232 confidence interval; GABA, gamma-aminobutyric acid; H&E, haematoxylin and eosin; hrs,
233 hours; LFB, luxol fast blue; LL, lateral lemniscus; mtCN, mitochondrial DNA copy number;

234 mtDNA, mitochondrial DNA; ML, medial lemniscus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tet-
235 rahydropyridine; PFA, paraformaldehyde; PBS, phosphate buffered saline; PD, Parkin-
236 son's disease; PPN, pedunclopontine nucleus; PMI, post-mortem interval; qPCR, quan-
237 titative polymerase chain reaction; RT, room temperature; S.E.M., standard error of the
238 mean; SNpc, substantia nigra pars compacta; SCD, superior cerebellar decussation;
239 TBS, Tris Buffered Saline; TMB, 3,3',5,5'-tetramethylbenzidine

240 **Author Contributions:** I.-S.P., J.L.E. and G.H. conceived and designed the project.
241 A.G.B., A.P., J.L.E., L.G., C.M.M., G.H., and I.-S.P. collected, analyzed and interpreted
242 the data. A.G.B., A.P., J.L.E., L.G., C.M.M., G.H., and I.-S.P. wrote the paper.

243 **Potential Conflicts of Interest:** Nothing to report.

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303 **Figure and table legends:**

304 **Figure 1:** A low (2.5× air-based) objective lens was used to capture tiled images, **(A)**
305 representing the most rostral and **(B)** immediately adjacent section, along the rostro-
306 caudal axis that cryostat sections were collected. The PPN and surrounding neural
307 structures were visualized with **(A)** H&E and **(B)** LFB staining. Mapped outlines indicate
308 the anatomical location of the PPN in relation to major surrounding structures, including
309 the LL, ML and SCD. The insets show magnified (20× air-based objective) images of
310 the **(Ai)** H&E and **(Bi)** LFB stained sections, illustrating the Ch5 cholinergic neurons of
311 the PPN.¹⁸ Scale bars: **(A, B)** 500 μm and **(Ai, Bi)** 75 μm. **(C)** Isolation of individual cho-
312 linergic neurons from the PPN, using laser-assisted microdissection. **(Ci)** Prior to dis-
313 section, individual cholinergic neurons were viewed with a Brightfield inverted micro-
314 scope (Carl Zeiss) at high (40×) magnification. Neurons were collected based on ChAT
315 immunoreactivity and typical morphology. Neurons were manually circumscribed by us-
316 ing the “draw shape” tool of the software interface. **(Cii)** Laser energy pulses were then
317 applied to separate out an outlined neuron from the surrounding tissue. **(D)** Successful
318 cell capture was confirmed by microscopically viewing the tube’s cap, shown at **(Di)** 5×
319 and **(Dii)** 20× magnification. Scale bars: **(Ci, Cii)** 75 μm, **(Di)** 50 μm and **(Dii)** 75 μm.

320 **Figure 2:** **(A)** A data scatter plot shows a significant increase in mtCN in PPN choliner-
321 gic neurons of PD compared to controls ($p=2.9\times 10^{-2}$). There was no significant differ-
322 ence in these values between individual cases, although more stratification was ob-
323 served between the PD cases ($p= 5.2\times 10^{-1}$). **(B)** A box plot shows no significant trend
324 between increased mtCN and later Braak stage for the PD cohort ($p=3.1\times 10^{-1}$). The er-
325 ror bars represent the 95% confidence interval (CI) of the mtCN values for each case.

326 Dots above the error bars represent values greater than the upper 95% CI. **(C)** A data
327 scatter plot shows a significant increase in %mtDNA deletions in PD patients compared
328 to controls ($p=2.5\times 10^{-2}$), with no significant difference in such values between individual
329 cases ($p=7.8\times 10^{-2}$) in either the PD or control cohorts. Significantly more PPN cholin-
330 ergic neurons harbored mtDNA deletions >60%, compared to controls ($p<1\times 10^{-4}$). **(D)** A
331 box plot of % mtDNA deletion against Braak stage shows a positive, statistically signifi-
332 cant relationship between mtDNA deletion levels and advanced Braak stage ($p=3\times 10^{-3}$).
333 The error bars represent the 95% CI of the mean percentage deletion value for each
334 case. Dots above the error bars represent values greater than the upper 95% CI.

335 **Figure 3:** The figure depicts the relationship between mtDNA deletion levels and mtCN
336 for **(A)** all PPN cholinergic neurons taken from PD patients and controls, as well as **(B)**
337 in PPN cholinergic neurons where mtDNA deletion levels exceeded 30%.

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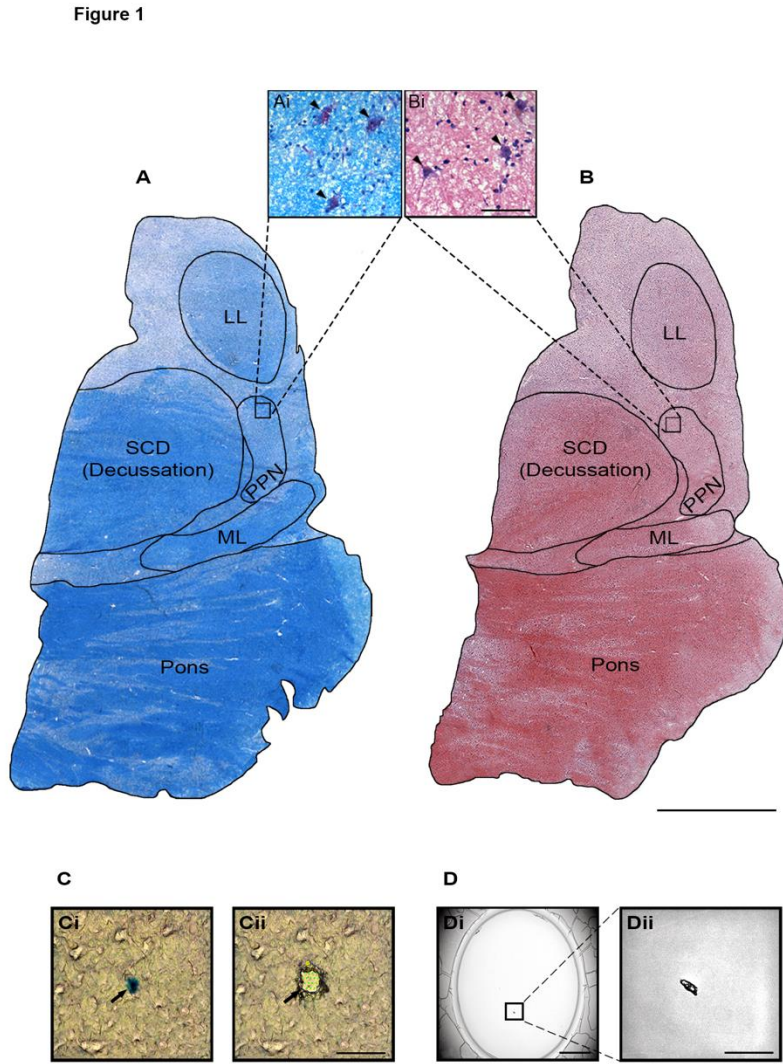
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346 **Figure 1:**

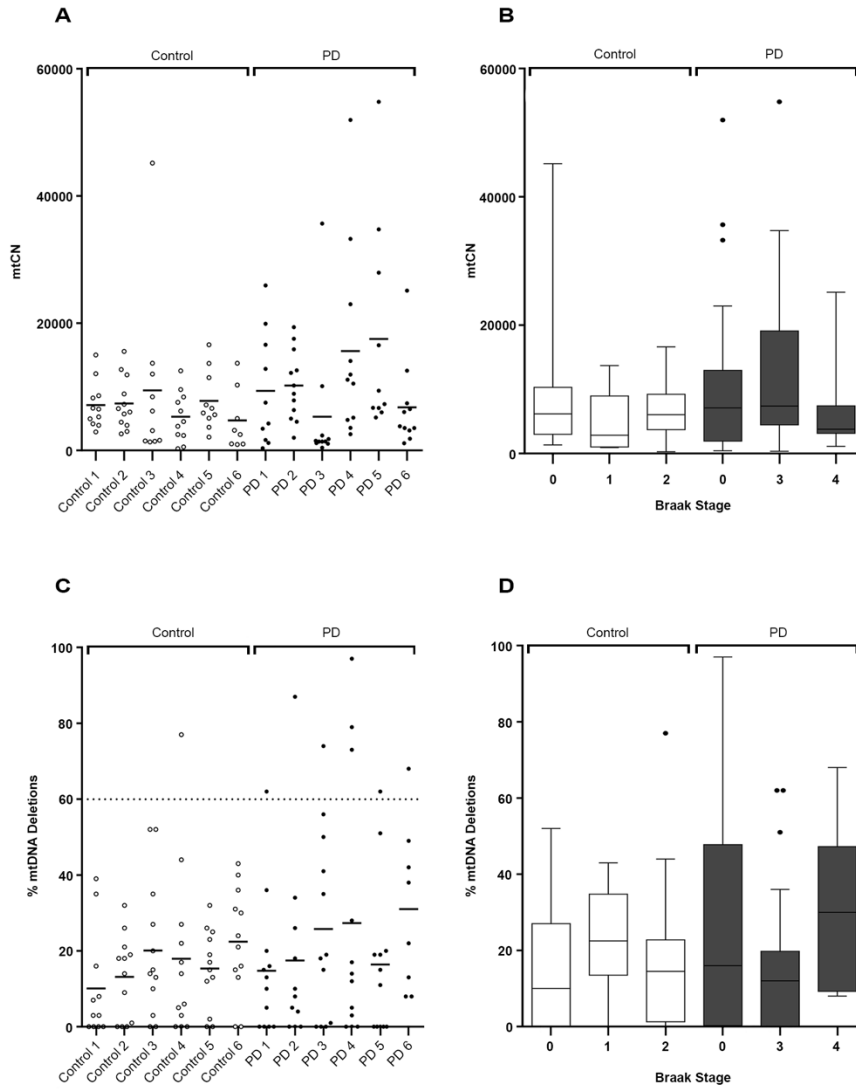


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Figure 2



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