Detection of alpha-synuclein aggregates in gastrointestinal biopsies by protein misfolding cyclic amplification

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1 **Detection of alpha-synuclein aggregates in gastrointestinal biopsies by**

2 **Protein Misfolding Cyclic Amplification**

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Abstract

Lewy bodies and neurites, the pathological signatures found in the central nervous system of Parkinson's disease (PD) patients, are primarily composed of aggregated alpha-synuclein (aSyn). The observation that aSyn aggregates are also found in the enteric nervous system has prompted several studies aimed at developing a diagnostic procedure based on the detection of pathological aSyn in gastrointestinal (GI) biopsies. The existing studies, which have all used immunohistochemistry for the detection of pathological aSyn, have had conflicting results. In the current survey, we analyzed the seeding propensity of aSyn aggregates from GI biopsies.

A total of 29 subjects participated to this study, 18 PD patients and 11 controls. For each patient, 2 to 4 GI biopsies were taken from the same site (antrum, sigmoid colon or rectum) and used to seed the aggregation of recombinant aSyn in an assay inspired from the Protein Misfolding Cyclic Amplification (PMCA) method. In a subset of patients and controls (14 and 3, respectively), one or two additional biopsies were analyzed by immunohistochemistry for the presence of phosphorylated aSyn histopathology (PASH) using antibodies against phosphorylated aSyn and PGP 9.5.

Except for one subject, none of the control samples seeded aSyn aggregation in PMCA reaction. GI biopsies from patients with PD seeded aSyn aggregation in 10 out of 18 cases (7 from the sigmoid colon, 2 from the antrum and one from the rectum). There was good agreement between PMCA and immunohistochemistry results as, except for two cases, all PMCA-positive PD patients were also PASH-positive.

Our findings show that the PMCA method we implemented is capable of detecting aSyn aggregates in routine GI biopsies. They also suggest that rectum biopsies do not contain sufficient amounts of aggregated aSyn to detect seeded assembly by PMCA. While

49   encouraging, our findings indicate that further studies are needed to establish the diagnostic

50   potential of the PMCA method we implemented to detect aSyn aggregates in upper GI

51   biopsies.

52   **Keywords**

53   Alpha-synuclein; Parkinson's disease; Protein Misfolding Cyclic Amplification; gut; enteric

54   nervous system; biopsy

55   **List of abbreviations**

56   aSyn – alpha-synuclein

57   CNS – central nervous system

58   CSF – cerebrospinal fluid

59   ENS – enteric nervous system

60   FFPE – Formalin-fixed, paraffin-embedded

61   GI – gastrointestinal

62   PASH – phosphorylated aSyn histopathology

63   PD -Parkinson's disease

64   PGP9.5 -Protein Gene Product 9.5

65   PMCA – Protein Misfolding Cyclic Amplification

66   ThT - Thioflavine T

67   **Introduction**

68   The *postmortem* detection by immunohistochemistry of aggregated alpha-synuclein

69   (aSyn) in the brain along with neuronal loss in the *substantia nigra* remain the gold standard

70   for the definite diagnosis of Parkinson's disease (PD). Over the last 12 years, several studies

71   have shown that aSyn aggregates can also be detected outside the central nervous system,

72   particularly in the enteric nervous system (ENS) [1–4]. The ENS is a neuronal network

73   embedded in the lining of the gastrointestinal (GI) tract from the lower third of the

74   esophagus to the rectum, whose distinguishing feature is its accessibility and suitability for

75   routine GI biopsies [5]. These findings logically prompted a substantial amount of research

76   to determine if the immunohistochemical detection of pathological aSyn in routine GI

77   biopsies could be used for pathological diagnosis of PD within patients lifespan (reviewed in

78   [6,7]).

79   In our seminal study, using microdissection and whole-mount preparation of colonic

80   biopsies, we detected aSyn pathology in 21 out of 29 PD patients and in none of the controls

81   [8]. Although the microdissection technique we have developed provides outstanding

82   information on the morphology of enteric neurons, it nonetheless has several limitations as

83   it needs to be performed immediately after the endoscopic procedure and requires technical

84   expertise and training [5]. Most subsequent studies on GI biopsies in PD have therefore been

85   performed on formalin-fixed, paraffin-embedded (FFPE) tissue, which has the main

86   advantages of being readily accessible to most hospital-based laboratories and to allow

87   retrospective analyses. Nevertheless, the diagnostic utility of FFPE GI biopsies in PD remains

88   unclear, as these studies yielded conflicting results regarding the sensitivity and specificity of

89   GI biopsies in detecting pathological aSyn [9–17].

90   One possible explanation for the observed discrepancies between existing studies on

91   immunohistochemical detection of aSyn pathology in the ENS is the very low amount of aSyn

92   aggregates usually found in GI samples. Protein Misfolding Cyclic Amplification (PMCA)

93   enables ultrasensitive detection of the pathogenic prion protein PrP in Creutzfeldt-Jakob

94   disease through amplification of its misfolding and aggregation *in vitro* under different

95   experimental conditions [18]. This technique has been recently used to detect aggregated

96   aSyn in the CSF of PD patients [19] and in formaldehyde-fixed samples from multiple system

97 atrophy patients [20]. We implemented this method to assess the presence of pathogenic

98 aSyn in GI biopsies.

99 **Methods**

100 *Subjects, GI biopsies and study design*

101 A total of 29 subjects participated to this study, 18 patients with PD and 11 controls. PD

102 patients who were diagnosed according to the United Kingdom PD Society Brain Bank

103 criteria were recruited from the movement disorder clinic at Nantes University Hospital,

104 France. 16 out of 18 PD patients had a rectosigmodoscopy or a colonoscopy, while the

105 remaining 2 had upper GI endoscopy for placement of an administration tube for continuous

106 levodopa enteral infusion. As controls, we used GI biopsies from 2 groups : (1) 7 subjects

107 who had colonoscopy for colorectal cancer screening, (2) 4 subjects with Crohn's disease

108 who underwent either rectosimoidoscopy or colonoscopy for disease management and

109 surveillance (Table 1). All control subjects had a detailed neurological examination to rule

110 out PD symptoms and cognitive deficiency. Two to 4 biopsies per subject were snap frozen

111 in liquid nitrogen at the time of collection and stored at −80°C. 14 PD cases and 3 control

112 cases had additional biopsies (taken from the same site, either in the sigmoid colon [2

113 biopsies] or in the rectum [1 biopsy]) that were analyzed by immunohistochemistry (see

114 below). The study protocol was approved by the local Committee on Ethics and Human

115 Research (*Comité de Protection des Personnes Ouest IV and VI*), conformed to the Code of

116 Ethics of the World Medical Association (Declaration of Helsinki) and was registered on

117 ClinicalTrials.gov (identifier NCT01353183 and NCT01748409). Written informed consent

118 was obtained from each patient and from each control.

119 *Reagents and chemicals*

120   Unless otherwise stated all reagents were purchased from Sigma (Saint-Quentin-Fallavier,

121   France).

122   *Microdissection and immunohistochemistry*

123   Microdissection was performed as previously described in one biopsy (when taken in the

124   rectum) or two biopsies (when taken in the sigmoid colon) [6]. Each whole-mount

125   preparation of submucosa obtained from a single biopsy was permeabilized for 3 hours in

126   phosphate buffered saline (PBS)/NaN_3 containing 1% (v/v) Triton X-100 and 10% (v/v) horse

127   serum and then incubated with antibodies against phosphorylated aSyn (1:5,000, WAKO,

128   Osaka, Japan) and PGP9.5 (1:10,000; Ultraclone Limited, UK). A patient was noted as positive

129   (phosphorylated aSyn histopathology, PASH+) when at least one structure immunoreactive

130   for both phosphorylated aSyn and PGP9.5 was observed.

131   *Biopsies treatment for amplification*

132   2 to 4 Biopsies were weighted in a Safelock 2mL Biopur tube (Eppendorf, Montesson,

133   France). The biopsies were resuspended 5% (weight: volume) in buffer (150mM KCl, 50mM

134   Tris-HCl pH7.5) and disrupted by sonication using a Branson SFX 150 Cell Disruptor sonicator

135   equipped with a 3.17mm microtip probe (Emerson, Bron, France). Sonication was applied for

136   10 seconds at 70% of the maximum amplitude. The resulting homogenate was aliquoted and

137   flash frozen in liquid nitrogen before storage at -80°C.

138   *PMCA assay*

139   Biopsies homogenates were diluted in 150mM KCl, 50mM Tris-HCl pH7.5 to a final

140   proportion of 2% (weight: volume). Monomeric aSyn concentration was adjusted to 100μM

141   in a total volume of 300μL. The solution was split in 2 replicates in a PCR strips (BIOplastics,

142   Landgraaf, The Netherlands) tubes (150μl in each). PMCA amplification was performed on 2

143   replicates for each patient using the Q700 generator and a 431MPX horn (both from

144   Qsonica, Fisher scientific, Illkirch, France). The power of the horn was set to 30% of the

145   maximum amplitude. The sonication protocol consisted of 15sec of sonication and 315sec of

146   rest at 31°C. Every hour, 5µL from each tube were collected and diluted into 300µL

147   Thioflavine T (ThT, 10µM). The amplification was monitored by measuring ThT fluorescence

148   increase using a Cary Eclipse Fluorescence Spectrophotometer (Agilent, les Ulis, France) with

149   fixed excitation and emission wavelength at 440 and 480nm, respectively. The sensitivity of

150   the method was assessed using recombinant preformed fibrils. The detection threshold was

151   determined to be in the range of 10 pM of aggregated aSyn (Figure 1). PMCA efficacy was

152   derived from the lag phase length and was considered high when the lag phase was shorter

153   or equal to 300 minutes and was considered medium to low when it lied within the time

154   frame 300 to 425 minutes. Statistical analysis were perfomed using ANOVA, on the duration

155   of the lag phase or the ThT fluorecence at 480 minutes (Figure 2 C and D).

156   **Results**

157   A total of 29 subjects participated to this study, 18 PD patients (12 males, mean age ±

158   standard deviation: 63.7±7.7 years) and 11 controls (7 males, 52.4±15.3 years). Age and sex

159   did not differ significantly between patients and controls (Table 1).

160   Two to 4 biopsies were obtained from each PD cases and control cases. For each

161   subject multiple biopsies were pooled and homogenized as follow: 2 pooled biopsies for 6

162   PD cases, 3 pooled biopsies for 3 PD cases and 3 controls, 4 pooled biopsies for 9 PD cases

163   and 8 controls (Table 1). Homogenates were blindly analyzed by means of PMCA (Figure 2).

164   Except for subject #1, none of the GI biopsies homogenates from the control group,

165   regardless of the biopsy site (sigmoid colon or rectum), seeded the aggregation of

166   monomeric aSyn. Subject #28 was initially enrolled in 2008 as a control and as such had no

167   signs or symptoms suggestive of PD. She underwent upper GI endoscopy for the

168   investigation of anemia and 4 antrum biopsies were taken and stored as part of our research

169   protocol. Because of the positive PMCA result, we decided to clinically reevaluate this

170   patient 10 years after GI biopsies collection. Neurological examination showed that she had

171   developed in the meantime typical signs and symtoms of PD, including right bradykinesia

172   and mild bilateral rest tremor.

173   GI biopsies homogenates from patients with PD seeded monomeric aSyn aggregation

174   in 10 out of 18 cases (Table 1). Among these PMCA+ biopsies, 2 sets of biopsies were taken

175   from the antrum, 7 sets of biopsies were taken in the sigmoid colon while the remaining one

176   was from the rectum. Of note rectal biopsies from 3 out of 4 PD patients yielded negative

177   results with PMCA. Identical results were observed for the rectal biopsies from control

178   patients (Table 1).

179   In 14 patients with PD and 3 controls, one or two additional biopsies were

180   immunohistochemically assessed for the presence of PASH (Figure 3). All 3 controls as well

181   as the 4 PD patients who had rectal biopsies were PASH- (Table 1). When sigmoid biopsies

182   were analyzed, 5 out of 10 PD patients were PASH+ and among these 5 cases, 4 were also

183   PMCA+ (Table 1).

184   **Discussion**

185   Here, we have used GI biopsies from PD patients to show that aSyn aggregates from

186   peripheral autonomic nervous system can be amplified through PMCA. Only one control

187   biopsy induced aSyn aggregation suggesting that the seeding method we implemented has a

188   high specificity. Regarding PD, 2/2 upper GI biopsies, 7/12 sigmoid biopsies and only 1/4

189   rectal biopsies were PMCA+. Although our sample size is too small to draw firm conclusions,

190   our findings are in line with previous reports that showed a rostro-caudal distribution of

191   aSyn pathology in the gut, with the lower esophagus and stomach having the highest

192 frequency of PASH, followed by small bowel regions, colon and rectum [2,21,22]. They also

193 suggest that rectal biopsies are not suited for PMCA-based PD diagnosis. There was

194 significant agreement between PMCA and immunohistochemistry as 4 out of 5 PASH+ PD

195 patients were also PMCA+. Samples from the remaining PMCA+ patients were either not

196 assessed by immunohistochemistry (4 cases) or were PMCA+ but PASH- (2 cases). All PMCA+

197 and PASH+ samples originated from the sigmoid colon (4 cases). Limited agreement

198 between PMCA and immunohistochemistry was observed for rectal samples as none was

199 PASH+ while 1 out of 4 was PMCA+. The same figure was observed for control samples.

200 Altogether, our results suggest that rectal biopsies are not suited for PMCA-based PD

201 diagnosis.

202 An interesting observation is the positive detection of aSyn aggregates in GI samples

203 initially collected as controls from one subject who subsequently developed clinical

204 symptoms of PD (subject #28). In a recent report that used aSyn PMCA in CSF, Shahnawaz *et*

205 *al.* identified two subjects originally enrolled as controls who were subsequently diagnosed

206 as having PD 1 and 4 years after sample collection [19]. These findings suggest that PMCA

207 might be used as a preclinical or premotor diagnostic method for PD.

208 In conclusion, our preliminary study supports the idea that PMCA could be used to

209 detect aSyn aggregates in GI biopsies from PD patients. Further studies are needed to

210 establish the diagnostic potential of PMCA to detect aSyn aggregates in GI biopsies

211 especially in the upper GI tract.

212 **Declarations**

213 *Ethics approval and consent to participate*

214 Regarding sigmoid biopsies sampling, the study protocol was approved by the local

215 Committee on Ethics and Human Research (*Comité de Protection des Personnes Ouest IV and*

216 *VI*) and was registered on ClinicalTrials.gov (identifier NCT01353183 and NCT01748409).

217 Written informed consent was obtained from each patient and from each control.

218 *Consent for publication*

219 Not applicable.

220 *Availability of data and materials*

221 The datasets used and/or analysed during the current study available from the

222 corresponding author on reasonable request.

223 *Competing interests*

224 None.

225 *Funding*

235 *Author Contributions*

236 AF, RM, TC, PD and LB performed the experiments and analyzed the data. LLV and MN

237 managed the biobanking and dissected the colonic samples. EC performed the endoscopy.

238 AF, RM, PD and LB designed the research and wrote the manuscript.

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Legends to Figures

Figure 1: PMCA of preformed recombinant aSyn fibrils. Increasing concentration of recombinant seeds were added in the reaction to assess the sensitivity of the PMCA method we implemented. The fluorescence due to the seeds is indicated at time -30 minutes corresponding to the mixing of seeds and monomeric substrate for 1µM, 10µM and, as a dashed line, for 100 µM of aSyn fibrils. Time zero corresponds to the time where PMCA amplification is initiated. The concentrations of recombinants fibrils are expressed as initial monomeric concentration. The curves represent the average of 4 replicates +/- SD.

Figure 2: PMCA assay in controls and PD. PMCA was performed using gastrointestinal biopsies homogenates from controls (A) or PD patients (B). aSyn seeded assembly reactions obtained in the presence of homogenates from rectum colon biopsies are coloured in blue (controls) and orange (PD patients). Those obtained in the presence of sigmoid colon biopsies homogenates are coloured in purple (controls) and red (PD patients). Those obtained in the presence of antrum biopsies homogenates are coloured in brown (PD patients). PMCA efficacy was considered high when the assembly kinetics lied within the grey surface area. PMCA efficacy was considered medium or low when the assembly kinetics lied within the hatched grey surface area. The curves represent an average of 2 replicates, +/- SD. (C) Box plot representation of the distribution of ThT fluorescence at time 480 min, (D) Distribution of the lag phase duration. One-way analysis of variance (ANOVA) test was used for statistical analysis; * : P<0.05.

Figure 3. Representative photomicrographs of phosphorylated alpha-synuclein and PGP 9.5 staining in the submucosa of PD patients and controls. Sigmoid biopsies from one PD patient (PD, #22; upper panels) and one control (CTL, #10; lower panels) were microdissected and analyzed by immunohistochemistry with antibodies against PGP 9.5 and phosphorylated alpha-synuclein (P-aSyn). The representative photomicrographs from PD

344 patient show multiple PASH in whole-mount of submucosa immunoreactive for
345 PGP9.5 (green) that were also positive for P-aSyn (red) in PD. By contrast, no
346 immunoreactivity for P-aSyn was observed in control, with the exception of some faint
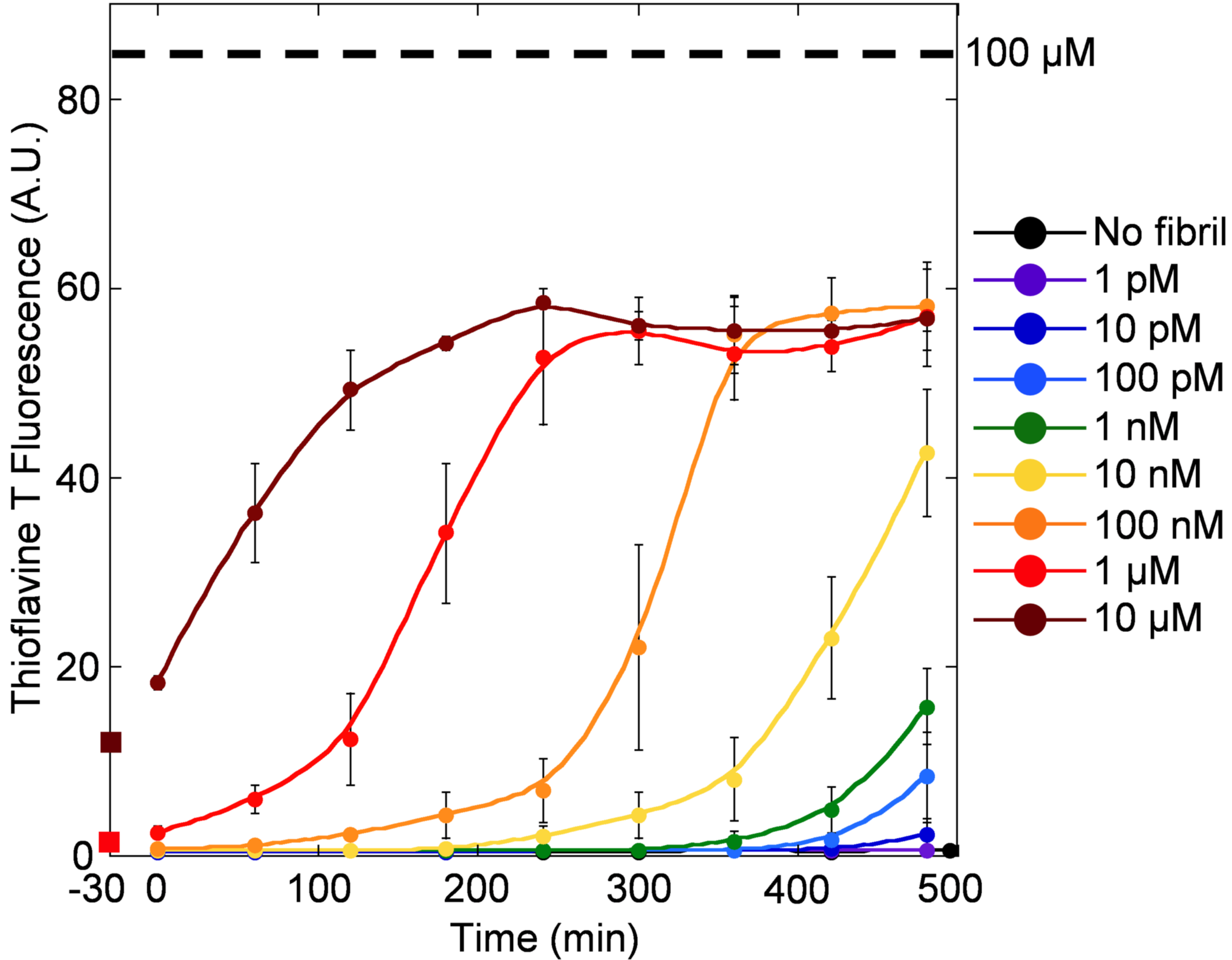347 nonspecific background labeling. Scale bar: 75 µm.

348 Suplementary FIGURE 1. **PMCA kinetics raw data.** PMCA amplification of gastrointestinal
349 biopsies homogenates from controls (rectum and sigmoid colon) or PD patients (rectum and
350 sigmoid colon and antrum) followed by ThT fluorescence measurement. PMCA reactions
351 were performed in duplicate. Individual replicates and mean values are drawn for the 29
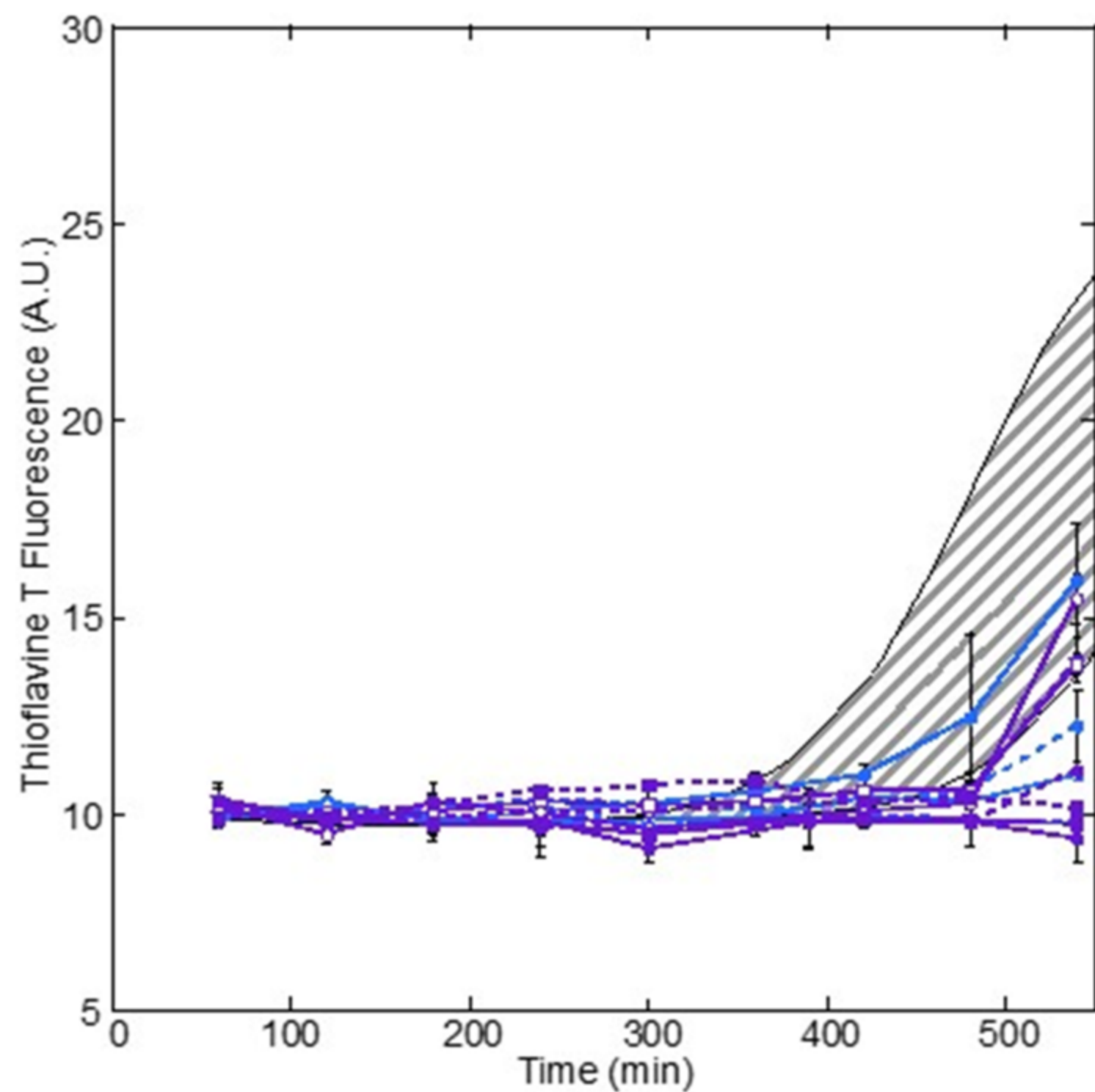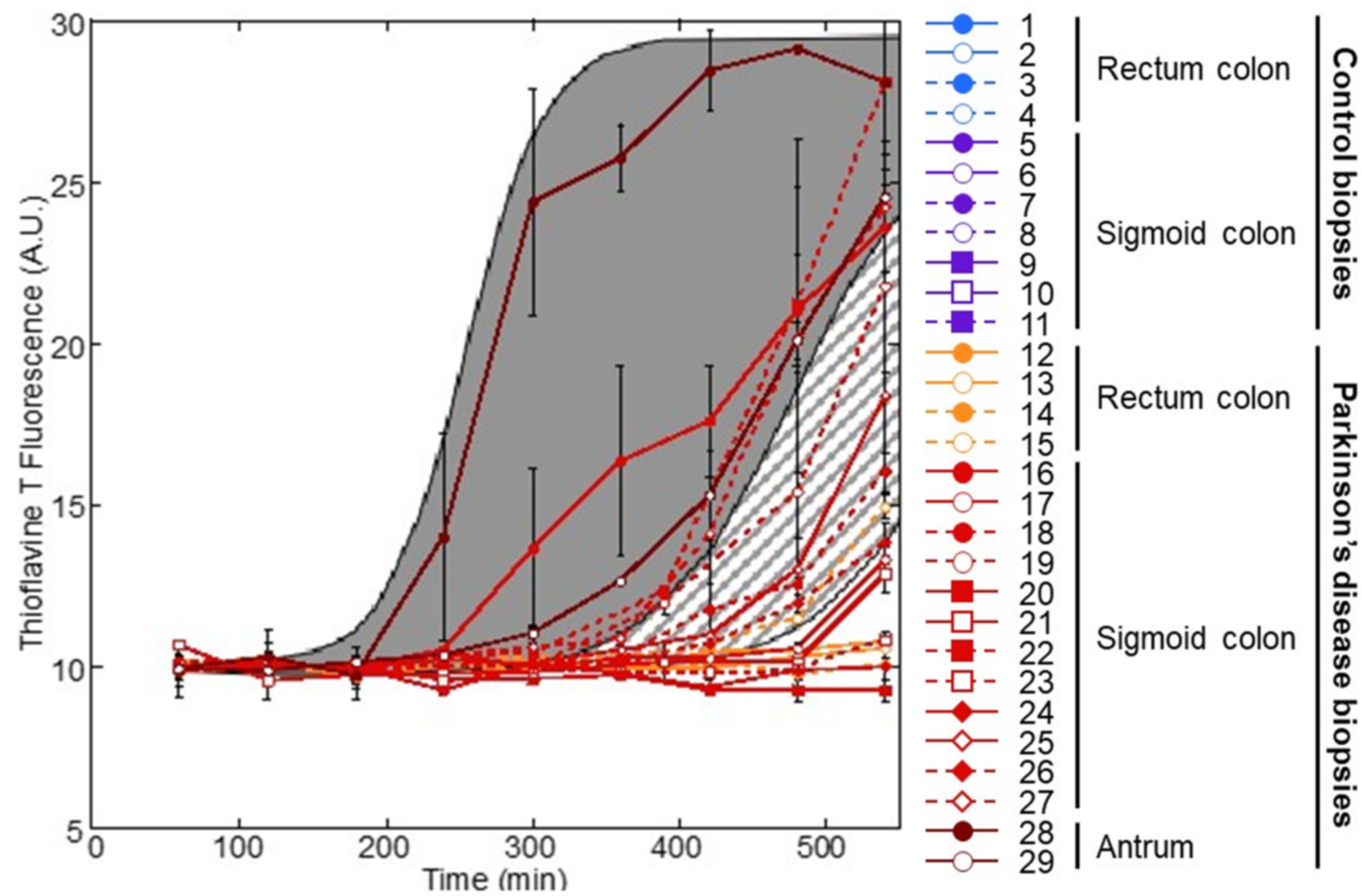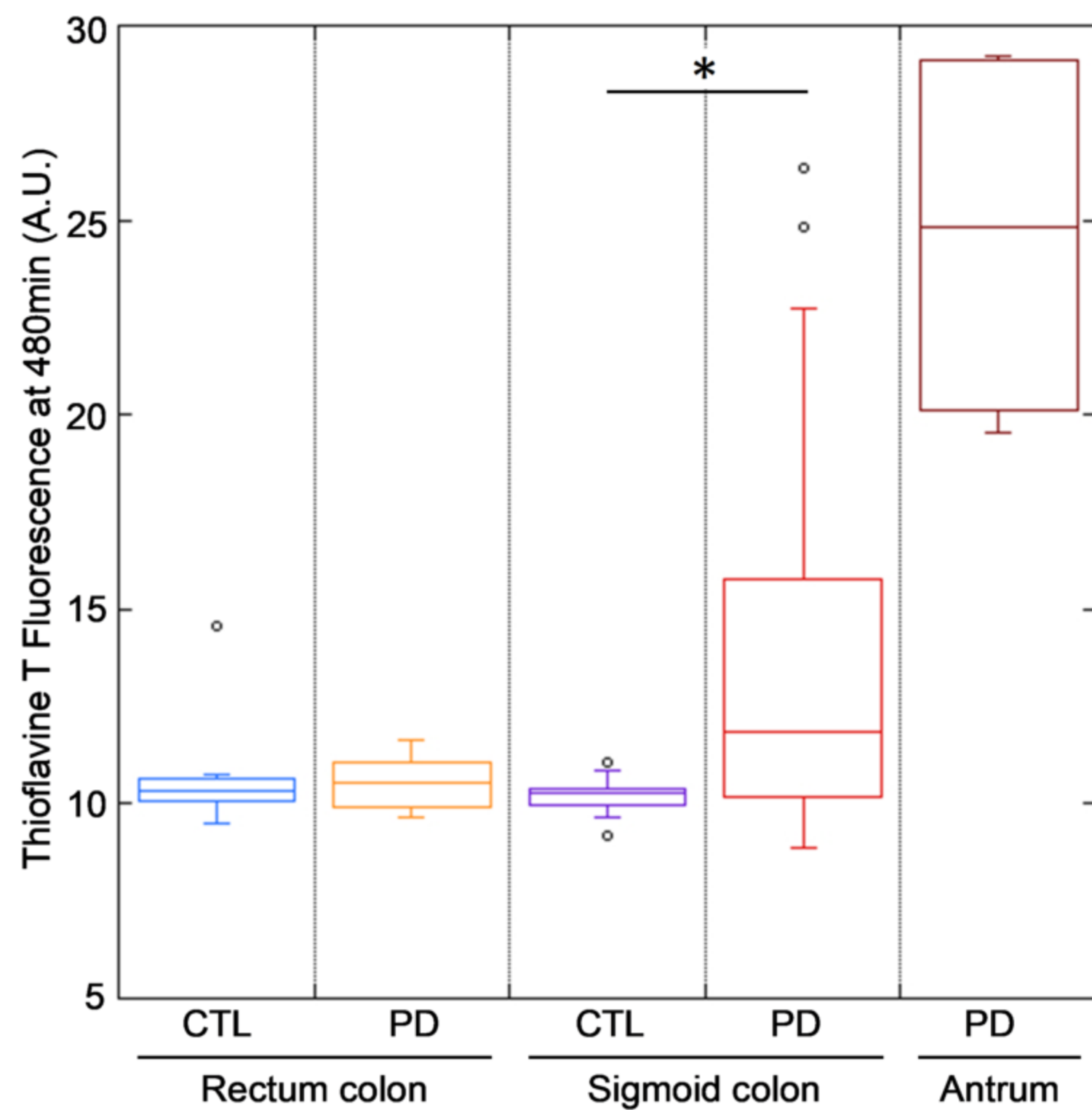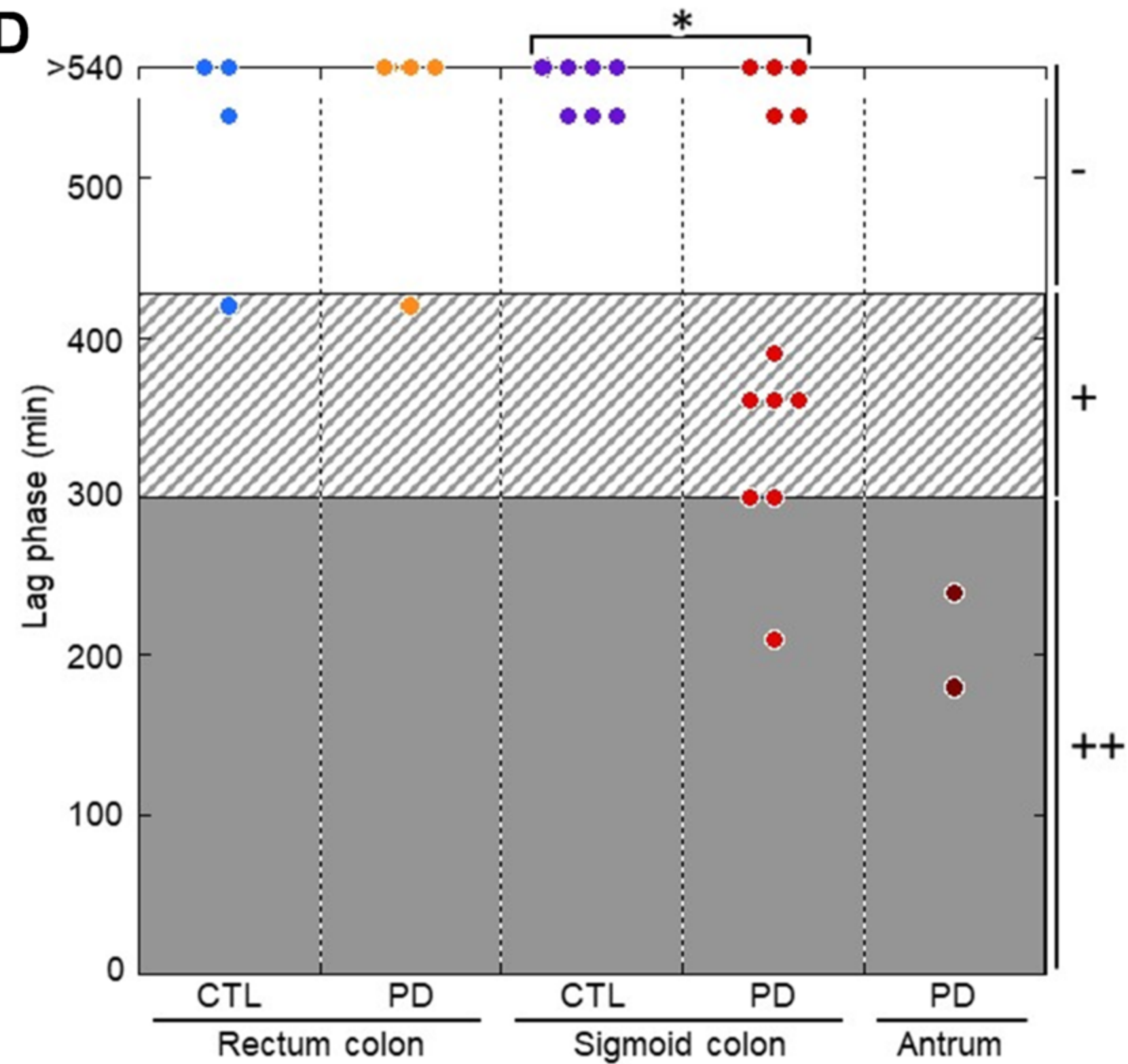352 subjects in separated graphs.

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	Age, sex	Diagnosis	Biopsy			PMCA results			
			Site	Number	PASH	Amplification	Lag time	ThT increase at 480min for each duplicate	
1	68/M	Control	Rectum	4	(0/1)	+	420	**4,4**	<1
2	58/F			4	(0/1)	-	>540	<1	<1
3	38/F			4	NA	-	540	<1	<1
4	43/M			4	NA	-	>540	<1	<1
5	62/M		Sigmoid	3	NA	-	>540	<1	<1
6	66/M			3	NA	-	540	<1	<1
7	61/F			3	NA	-	>540	<1	<1
8	72/M			4	NA	-	540	<1	<1
9	56/F			4	NA	-	>540	<1	<1
10	53/M			4	(0/2)	-	540	<1	<1
11	33/M			4	NA	-	>540	<1	<1
12	67/M	Parkinson's disease	Rectum	4	(0/1)	-	>540	<1	<1
13	66/M			4	(0/1)	-	>540	<1	<1
14	55/M			4	(0/1)	-	>540	<1	<1
15	68/F			4	(0/1)	+	420	**1,4**	**1,2**
16	70/M		Sigmoid	4	(1/2)	++	210	**12,6**	**9,1**
17	70/M			4	(0/2)	-	540	<1	<1
18	55/M			4	NA	+	360	**2,6**	**2,0**
19	47/F			3	NA	+	300	**8,9**	**1,5**
20	53/M			2	(0/2)	-	>540	<1	<1
21	67/M			3	(0/2)	-	540	<1	<1
22	58/M			3	(2/2)	++	300	**16,1**	**5,8**
23	69/M			2	(2/2)	-	>540	<1	<1
24	72/F			2	(0/2)	-	>540	<1	<1
25	59/M			2	(0/2)	+	390	**3,8**	**1,8**
26	58/F			2	(2/2)	+	360	**2,1**	**1,5**
27	70/M			2	(2/2)	++	360	**14,6**	**5,4**
28	72/F		Antrum	4	NA	++	180	**19,0**	**18,8**
29	70/F			4	NA	++	240	**10,5**	**9,3**

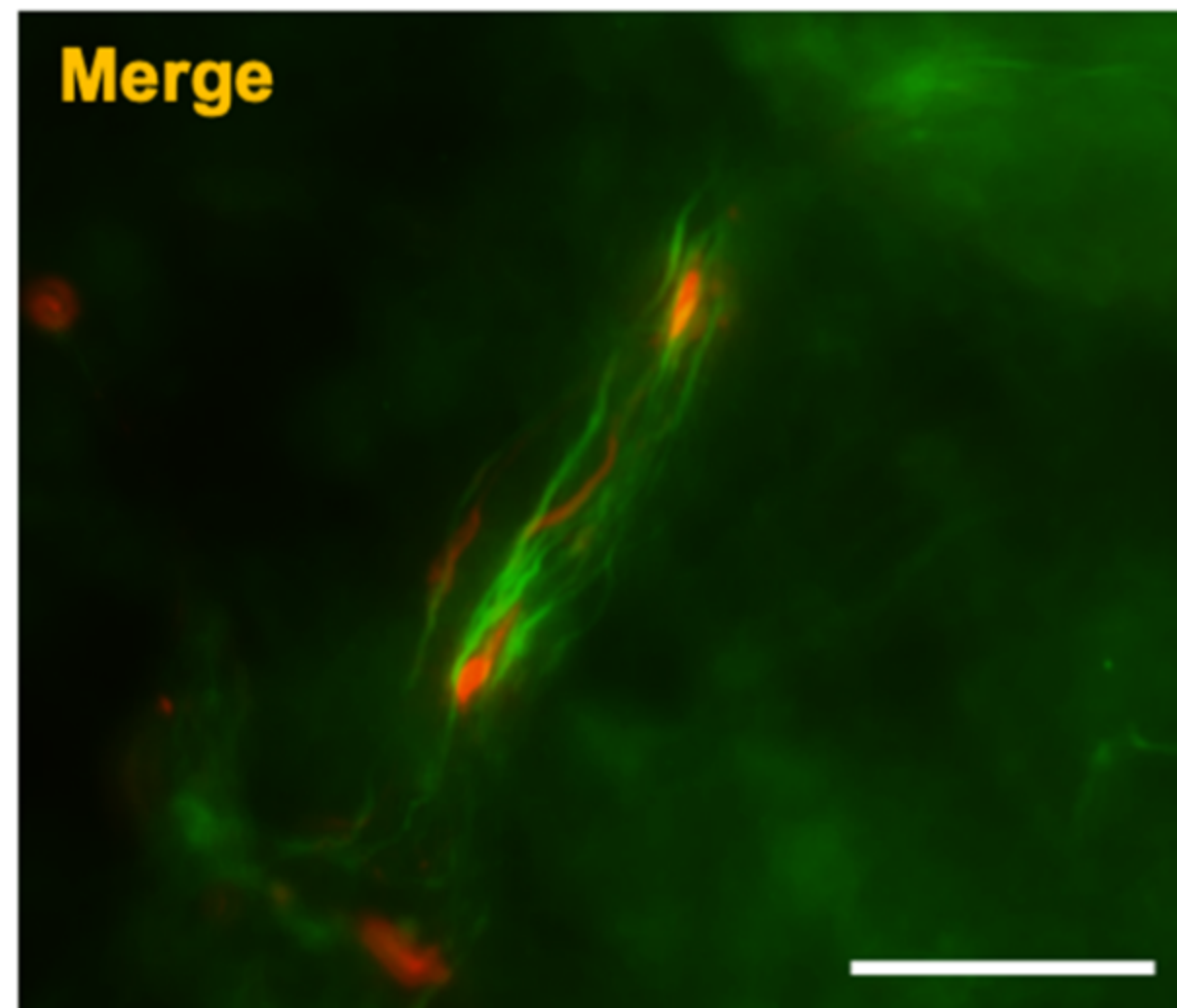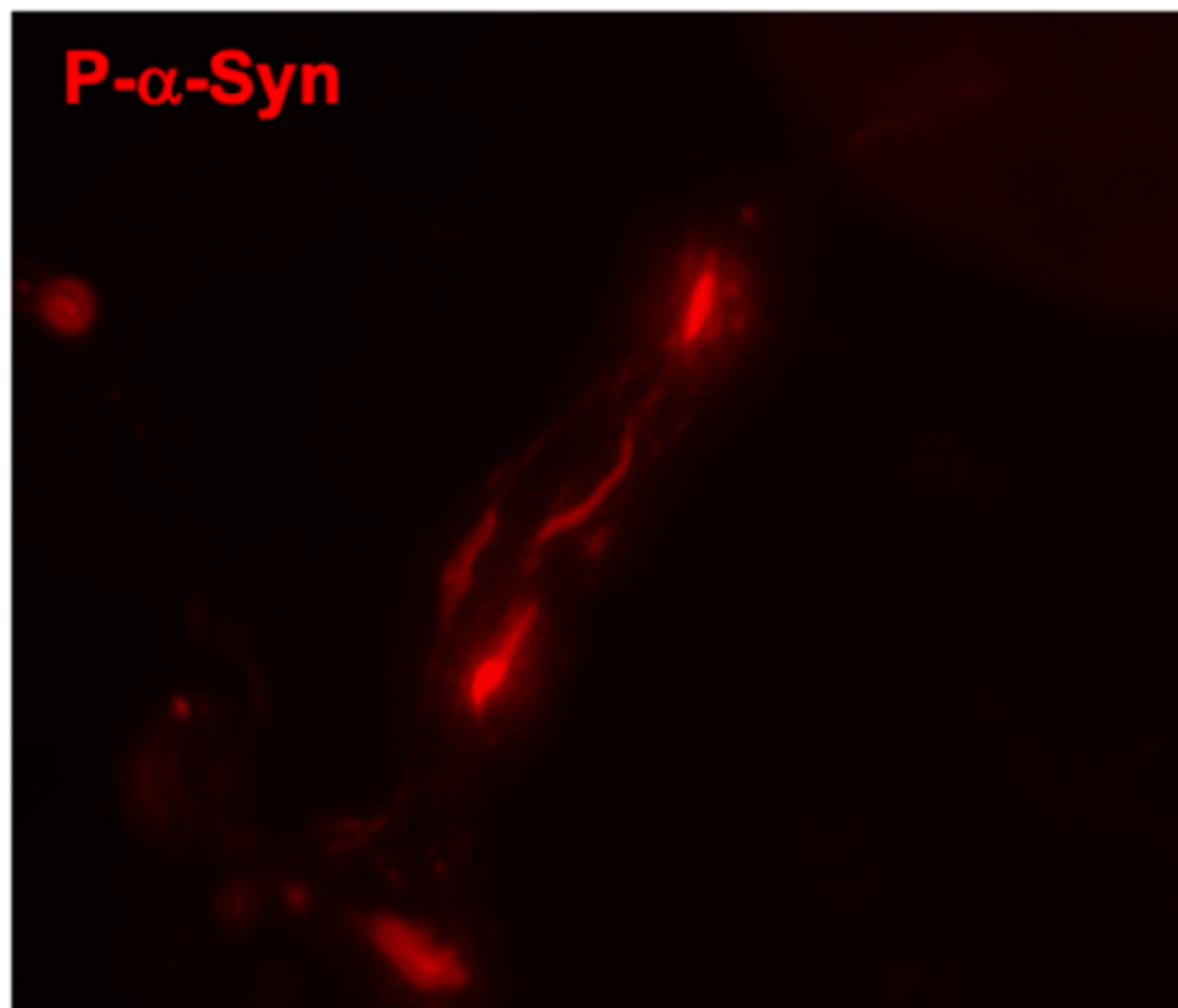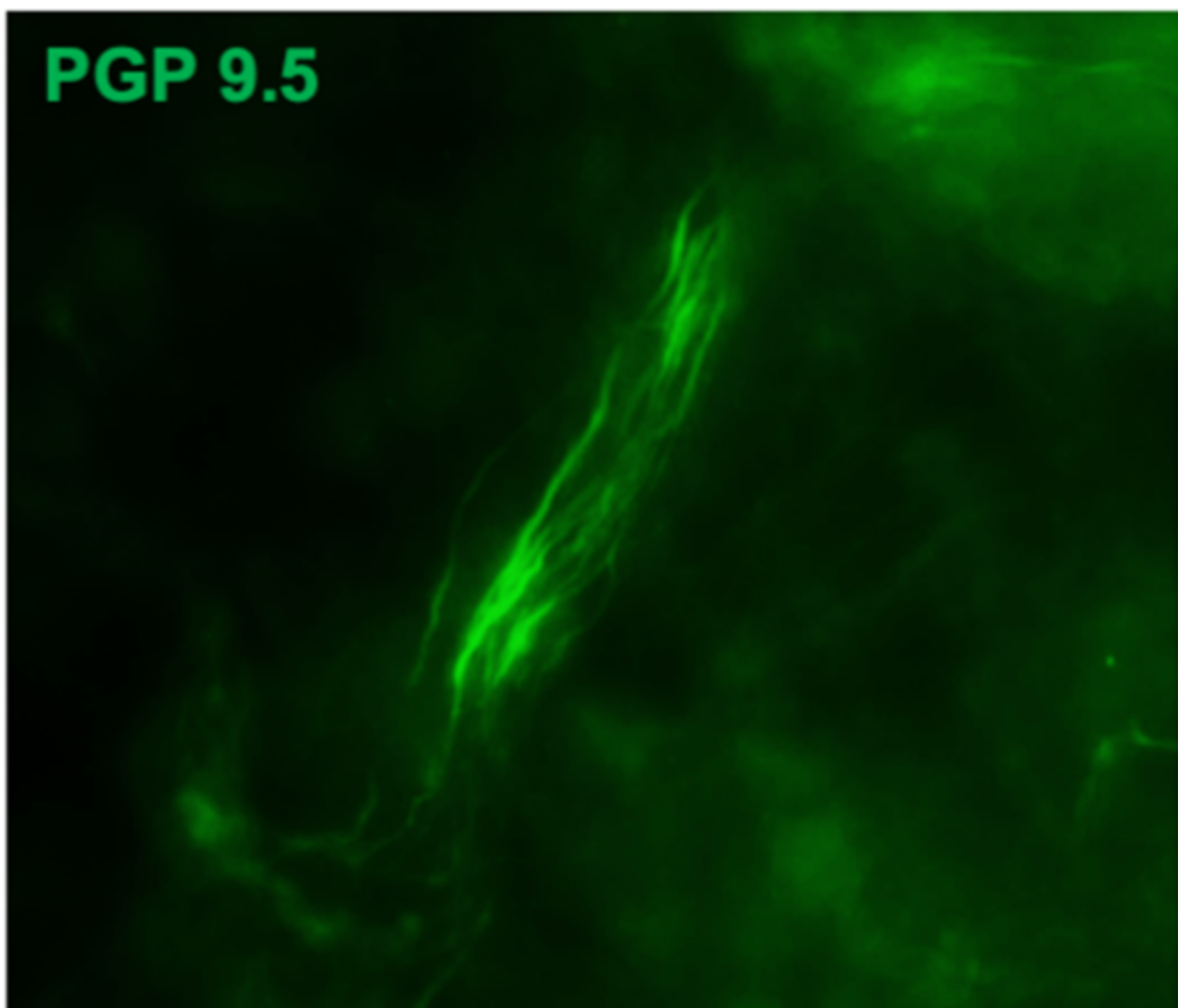354 **Table 1 : Histology and amplification summary.**

355 Age and sex: age of patients at the time of the biopsies and gender; Diagnosis: differentiates

356 controls from parkinsonian patients; Biopsy: site of the biopsy (Site), number of biopsies

357 used in the PMCA assay (Number), result of the histological analysis (PASH; NA = not

358 assessed; (X/Y) = number of positive biopsy[ies]); PMCA results: amplification efficacy ranked

359 base on lag phase duration and ThT increase (Amplification; ++ : high amplification; + : low

360 amplification; - : no amplification detected), lag phase duration in minutes (Lag Time), ThT

361   increase calculated between time point 60 minutes and 480 minutes (ThT increase at

362   480min for each duplicate).