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Mitochondrial damage-associated inflammation highlights biomarkers in *PRKN/PINK1* parkinsonism

Max Borsche,[1,2] Inke R. König,[3,*] Sylvie Delcambre,[4,*] Simona Petrucci,[5,6] Alexander Balck,[1,2] Norbert Brüggemann,[1,2] Alexander Zimprich,[7] Kobi Wasner,[4] Sandro L. Pereira,[4] Micol Avenali,[8] Christian Deuschle,[9,10] Katja Badanjak,[4] Jenny Ghelfi,[4] Thomas Gasser,[9,10] Meike Kasten,[1,11] Philip Rosenstiel,[12] Katja Lohmann,[1] Kathrin Brockmann,[9,10] Enza Maria Valente,[8,13] Richard J. Youle,[14] Anne Grünewald[1,4,*] and Christine Klein[1,*]

*These authors contributed equally to this work.

There is increasing evidence for a role of inflammation in Parkinson's disease. Recent research in murine models suggests that parkin and PINK1 deficiency leads to impaired mitophagy, which causes the release of mitochondrial DNA (mtDNA), thereby triggering inflammation. Specifically, the CGAS (cyclic GMP-AMP synthase)-STING (stimulator of interferon genes) pathway mitigates activation of the innate immune system, quantifiable as increased interleukin-6 (IL6) levels. However, the role of IL6 and circulating cell-free mtDNA in unaffected and affected individuals harbouring mutations in *PRKN/PINK1* and idiopathic Parkinson's disease patients remain elusive. We investigated IL6, C-reactive protein, and circulating cell-free mtDNA in serum of 245 participants in two cohorts from tertiary movement disorder centres. We performed a hypothesis-driven rank-based statistical approach adjusting for multiple testing. We detected (i) elevated IL6 levels in patients with biallelic *PRKN/PINK1* mutations compared to healthy control subjects in a German cohort, supporting the concept of a role for inflammation in *PRKN/PINK1*-linked Parkinson's disease. In addition, the comparison of patients with biallelic and heterozygous mutations in *PRKN/PINK1* suggests a gene dosage effect. The differences in IL6 levels were validated in a second independent Italian cohort; (ii) a correlation between IL6 levels and disease duration in carriers of *PRKN/PINK1* mutations, while no such association was observed for idiopathic Parkinson's disease patients. These results highlight the potential of IL6 as progression marker in Parkinson's disease due to *PRKN/PINK1* mutations; (iii) increased circulating cell-free mtDNA serum levels in both patients with biallelic or with heterozygous *PRKN/PINK1* mutations compared to idiopathic Parkinson's disease, which is in line with previous findings in murine models. By contrast, circulating cell-free mtDNA concentrations in unaffected heterozygous carriers of *PRKN/PINK1* mutations were comparable to control levels; and (iv) that circulating cell-free mtDNA levels have good predictive potential to discriminate between idiopathic Parkinson's disease and Parkinson's disease linked to heterozygous *PRKN/PINK1* mutations, providing functional evidence for a role of heterozygous mutations in *PRKN* or *PINK1* as Parkinson's disease risk factor. Taken together, our study further implicates inflammation due to impaired mitophagy and subsequent mtDNA release in the pathogenesis of *PRKN/PINK1*-linked Parkinson's disease. In individuals carrying mutations in *PRKN/PINK1*, IL6 and circulating cell-free mtDNA levels may serve as markers of Parkinson's disease state and progression, respectively. Finally, our study suggests that targeting the immune system with anti-inflammatory medication holds the potential to influence the disease course of Parkinson's disease, at least in this subset of patients.

1 Institute of Neurogenetics, University of Lübeck, Lübeck, Germany
2 Department of Neurology, University of Lübeck, Lübeck, Germany

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3 Institute of Medical Biometry and Statistics, University of Lübeck, Lübeck, Germany
4 Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Esch-sur-Alzette, Luxembourg
5 Department of Clinical and Molecular Medicine, Sapienza University of Rome, Rome, Italy
6 Division of Medical Genetics, IRCCS-Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy
7 Department of Neurology, Medical University of Vienna, Vienna, Austria
8 IRCCS Mondino Foundation, Pavia, Italy
9 Department of Neurodegenerative Diseases, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany
10 German Center for Neurodegenerative Diseases (DZNE), University of Tübingen, Tübingen, Germany
11 Department of Psychiatry, University of Lübeck, Lübeck, Germany
12 Institute for Clinical Molecular Biology, Christian-Albrechts-University Kiel, Kiel, Germany
13 Department of Molecular Medicine, University of Pavia, Pavia, Italy
14 Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA

Correspondence to: Christine Klein, MD
Institute of Neurogenetics, University of Lübeck, Maria-Goeppert-Str. 1, 23562 Lübeck,
Germany
E-mail: christine.klein@neuro.uni-luebeck.de

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Abbreviations: ccf-mtDNA = circulating cell-free mitochondrial DNA; STING = stimulator of interferon genes

Introduction

Multiple lines of evidence support an association between Parkinson's disease and inflammatory processes (Hirsch and Hunot, 2009). In particular, pro-inflammatory cytokines are elevated in serum and CSF samples from patients with Parkinson's disease (Dzamko *et al.*, 2015). These studies are in keeping with the epidemiological observation that intake of anti-inflammatory drugs lowers the risk of developing Parkinson's disease (Noyce *et al.*, 2012). However, it remains unclear if inflammation originates from peripheral tissue or the CNS. If the latter is true, additional studies are required to clarify if the observed inflammation triggers the loss of dopaminergic neurons as a primary event or whether it is a secondary consequence of neurodegeneration. Either way, there is evidence that cytokines, such as interleukin-6 (IL6), overcome the blood–brain barrier and are transported from brain tissue to the blood and vice versa (Banks, 2015). Despite more than two decades of intense research, the molecular pathways connecting interleukin release and anti-inflammatory treatment with Parkinson's disease remain largely elusive.

Biallelic mutations in parkin (encoded by *PRKN*) and *PINK1* cause Parkinson's disease with typical clinical features, albeit with an earlier age of onset (Kasten *et al.*, 2018). The role of heterozygous mutations as a risk factor for Parkinson's disease is still under debate as epidemiological analyses provided controversial results (Reed *et al.*, 2019). Moreover, data on the occurrence of Lewy bodies as a hallmark of Parkinson's disease is limited and must be interpreted with caution in the context of monogenic Parkinson's disease (Schneider and Alcalay, 2017). However, there is evidence that heterozygous mutations increase the risk of developing Parkinson's disease with an age of onset falling between that of biallelic mutation carriers and idiopathic Parkinson's disease patients (Klein *et al.*, 2007; Grünewald *et al.*, 2013; Huttenlocher *et al.*, 2015). Parkin and PINK1 are part of the molecular pathway that controls mitophagy (Narendra *et al.*, 2012). Intriguingly, parkin or PINK1 deficiency leads to CGAS (cyclic GMPD-AMP synthase)/STING (stimulator of interferon genes)-dependent activation of a type I interferon response, associated with increased IL6 levels in biallelic *PRKN*/*PINK1* knockout mice (Sliter *et al.*, 2018).

IL6 is a pro-inflammatory cytokine, which acts in the initiation of innate immune responses, induces the release of C-reactive protein (CRP) (Tanaka *et al.*, 2014), and is elevated in serum samples from patients with Parkinson's disease (Qin *et al.*, 2016). While the particular role of IL6 and CRP in inflammation is controversial (Del Giudice and Gangestad, 2018), there is convincing evidence that IL6 increases with age (Puzianowska-Kuźnicka *et al.*, 2016). Circulating cell-free mitochondrial DNA (ccf-mtDNA) plays a role in the aetiology of various (systemic) diseases including immune responses (West and Shadel, 2017). Connecting impaired mitochondrial clearance and inflammation, the release of ccf-mtDNA into the cytosol triggers innate immunity activation (West and Shadel, 2017) through CGAS/STING (Lood *et al.*, 2016), TLR9 (Toll-like receptor 9) or inflammasomes such as NLRP3 (nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing-3) (Zhong *et al.*, 2018). In idiopathic Parkinson's disease, reduced mtDNA levels have been observed in the CSF in early-stage idiopathic Parkinson's disease (Pyle *et al.*, 2015). However, no reports have indicated an association between serum ccf-mtDNA levels and neurodegeneration in general and with Parkinson's disease in particular.

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With the aim to decipher the role of the cGAS-STING pathway in neuroinflammatory processes in monogenic Parkinson's disease, we have previously analysed IL6 levels in a small pilot sample of *PRKN* and *PINK1* mutation carriers, idiopathic Parkinson's disease patients, and healthy control subjects, demonstrating increased IL6 levels in patients with biallelic *PRKN* mutations (Sliter *et al.*, 2018). In light of the molecular mechanism identified in mice, in the current study, we hypothesized that STING signalling is triggered by ccf-mtDNA as a consequence of impaired mitophagy and results in IL6 elevation in human *PRKN* and *PINK1* mutation carriers. Using a hypothesis-driven statistical approach, we investigated, for the first time, ccf-mtDNA along with IL6 and CRP levels in sera from a large sample of patients with monogenic or idiopathic Parkinson's disease and control subjects.

Materials and methods

Patient recruitment and collection of samples

In the context of the present cross-sectional study, participants were recruited at tertiary movement disorder referral centres in Lübeck, Tübingen (Germany), and Pavia/Rome (Italy). Samples from the University of Lübeck were collected within the SysMedPD study, the EPIPARK cohort (Kasten *et al.*, 2012), and the Transregional Collaborative Research Center 134. All participants gave written informed consent, and the local ethics committees approved the study. Patients were neurologically examined by a movement disorder specialist. Demographic and clinical data, such as sex, age at examination, and regarding patients with Parkinson's disease, age at onset and disease duration were assessed. For a subset of patients, more detailed phenotypic information was obtainable including data referring to the disease state [Hoehn and Yahr stage, Movement Disorder Society Unified Parkinson's Disease Rating Scale part III (MDS-UPDRS III)], and olfactory impairment. Results from patients examined using the previous version of the UPDRS III were transformed into MDS-UPDRS III values as described (Goetz *et al.*, 2012). Olfactory impairment was assessed using the Brief Smell Identification Test (BSIT) or via TDI scores from the Sniffin' Sticks test, respectively, and age-adjusted.

Mutation carriers were identified by Sanger or gene panel sequencing, multiplex ligation-dependent probe amplification (MLPA) analysis, or real-time PCR analysis. We identified *PRKN* variants in 57 study participants and *PINK1* variants in 25 participants. Unaffected individuals harbouring nucleotide changes, which were classified as variants of unknown significance according to ACMG recommendations (Richards *et al.*, 2015), were excluded from the study.

Venous blood was obtained for preparation of serum and DNA extraction. Blood draw and clinical examination took place between 2004 and 2019. In total, samples from 245 participants were analysed. Samples from Lübeck and Tübingen were combined to form the 'German cohort' including 15 biallelic *PRKN/PINK1*, 19 affected heterozygous *PRKN/PINK1*, 15 unaffected heterozygous *PRKN/PINK1* mutation carriers, 59 idiopathic Parkinson's disease patients and 90 healthy, mutation-free control subjects. We aimed to replicate the results of the German cohort in an independent cohort from another centre. Therefore, we additionally investigated the so-called 'Italian cohort' consisting of 19 *PRKN/PINK1* biallelic, five affected heterozygous, nine unaffected heterozygous mutation carriers, as well as five idiopathic Parkinson's disease patients and nine healthy control subjects.

IL6 values from 45 individuals already reported previously (Sliter *et al.*, 2018) were included in the present study, but their serum samples were re-measured in a different laboratory (Supplementary Table 1). Since on rare occasions selected measurements (IL6, CRP or ccf-mtDNA) failed for individual study participants, sample numbers differ between analyses and are therefore indicated for each experiment.

Sample processing

At all study centres, venous blood was collected in serum tubes. Samples were centrifuged at 4°C for 10 min at 2000–4000*g* dependent on the centre within 2 h after blood collection. After centrifugation, samples were immediately frozen at –80°C.

IL6 and CRP measurements

IL6 was measured using an ELISA (Cobas Elecsys IL6 assay). High-sensitive CRP was investigated using particle-enhanced immunonephelometry (Cobas CRPHS assay). Both analyses were performed at the certified diagnostic laboratory LADR Centrallab Dr Kramer and Colleagues, Geesthacht, Germany. Samples from Lübeck and Tübingen (German cohort) were measured together in one run, those of the Italian cohort in a second run. Possibly due to batch effects, the Italian samples exhibited considerably lower IL6 levels (Italian cohort: 2.9 ± 2.9 pg/ml, $n = 47$; German cohort: 5.90 ± 1.90 pg/ml, $n = 181$). Because of these batch-dependent differences in IL6 levels, correlation analyses included only samples from the German cohort. As rank-based statistics were used throughout, values below the detection limit (IL6: 1.5 pg/ml; CRP: 0.3 mg/l) were set to arbitrarily small values of 1.4 pg/ml and 0.2 mg/l, respectively. IL6 and CRP levels were measured in a blinded fashion.

Quantification of serum circulating ccf-mtDNA levels

Levels of ccf-mtDNA in the serum samples from the German cohort were analysed in an equally blinded fashion at the Luxembourg Centre for Systems Biomedicine, University of Luxembourg.

DNA from serum of the German cohort was extracted using the QIAamp 96 DNA blood extraction kit (Qiagen) according to the manufacturer's instructions. Digital PCR (dPCR) was used to quantify ccf-mtDNA. The dPCR assay was performed using TaqMan™ technology. Primers and probes coupled with non-fluorescent quenchers were used to quantify fragments within the mitochondrial gene *MT-ND1* and the nuclear single-copy gene *B2M* (Phillips *et al.*, 2014; Rygiel *et al.*, 2015). Digital PCR was performed using the QuantStudio™ 3D Digital PCR System (Applied Biosystems), and samples were prepared following the manufacturer's instructions. Samples were loaded on a QuantStudio™ 3D digital PCR Chip v2 using

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the QuantStudio™ 3D Digital PCR Chip loader. The PCR was then performed on the ProFlex™ 2X Flat PCR System using a previously published programme (Rygiel *et al.*, 2015). The chips were read using the QuantStudio™ 3D Digital PCR Instrument and the data were analysed using the QuantStudio™ 3D AnalysisSuite, version 3.1.6-PRC-build2. Samples with a *B2M* copy number >200 copies/µl were considered as contaminated with nuclear DNA and were not considered for further analysis.

Statistical analysis

The phenotypic characteristics of the study cohort, correlation analyses, and differences in CRP levels are presented at a descriptive level.

In one individual, the IL6 value was implausibly elevated [>10 standard deviations (SD) above the mean] and thus set to missing. The following group differences were tested in the German cohort: (i) IL6 between *PRKN/PINK1* biallelic, *PRKN/PINK1* heterozygous affected, *PRKN/PINK1* heterozygous unaffected, and healthy mutation-negative controls; (ii) IL6 between *PRKN/PINK1* biallelic, idiopathic Parkinson's disease, and healthy controls; and (iii) ccf-mtDNA between *PRKN/PINK1* biallelic, *PRKN/PINK1* heterozygous affected, *PRKN/PINK1* heterozygous unaffected, idiopathic Parkinson's disease, and healthy controls. For the first two comparisons, an ordered effect was assumed and Jonckheere-Terpstra tests performed; for the latter, no specific order was assumed, so that a Kruskal-Wallis test was performed. When significant, results were followed-up with pairwise Wilcoxon rank sum tests. To account for multiple testing (Harrington *et al.*, 2019) of three hypotheses, the overall significance level of 0.05 was adjusted to 0.0167. To evaluate whether the observed effects might be confounded by different ages at examination of the probands, we performed a sensitivity analysis taking age at examination into account. Specifically, we first performed linear regressions to predict log(IL6) and log(ccf-mtDNA), respectively, from age at examination and saved the residuals. Second, we tested for differences in the resulting residuals between groups as before. Moreover, we analysed the utility of ccf-mtDNA levels to discriminate between idiopathic Parkinson's disease and Parkinson's disease associated with heterozygous *PRKN/PINK1* mutations using a receiving operating characteristic (ROC) curve. Finally, we investigated a possible dependency of IL6 on disease duration in idiopathic and parkin/PINK1-associated Parkinson's disease patients after adjusting for age at examination. Thus, we performed a linear regression to predict disease duration from age and saved the residuals. The latter were then used in a correlation analysis with IL6. Data from the Italian cohort were used for replication purposes only and thus required no additional adjustment. Analyses were performed using R version 3.5 (R Core Team, 2018). Figures were created with GraphPad Prism 8.

Data availability

The data used in this study are available from the corresponding author, upon reasonable request.

Results

The following three hypotheses were addressed in this paper. First, we aimed to re-evaluate serum IL6 levels in *PRKN* mutation carriers in a larger study cohort and to extend our analysis to *PINK1* mutation carriers. Second, we investigated if a gene dosage effect for IL6 exists when comparing biallelic with heterozygous *PRKN/PINK1* mutation carriers and idiopathic Parkinson's disease patients. Third, to explore a possible link between impaired mitophagy, the CGAS-STING pathway, and inflammatory signalling in Parkinson's disease, we quantified the abundance of ccf-mtDNA in serum in our cohort.

Phenotypic characterization of the study cohort

Table 1 shows demographic data for the five study groups investigated here. For patients with Parkinson's disease, mean age at onset, disease duration, MDS-UPDRS III, Hoehn and Yahr stage, and the frequency of olfactory dysfunction were additionally provided. The essential study groups, namely *PRKN/PINK1* biallelic, affected heterozygous, and healthy control subjects, were age-matched. By contrast, as idiopathic Parkinson's disease occurs at a later age than recessively inherited monogenic Parkinson's disease (Kasten *et al.*, 2018), patients with idiopathic Parkinson's disease were older at the time of examination and sampling of biomaterials.

In our cohorts, Parkinson's disease associated with biallelic mutations in *PRKN/PINK1* was characterized by an earlier age of onset, a longer disease duration, and reduced olfactory impairment, while affected heterozygotes exhibited similar clinical features as idiopathic Parkinson's disease patients. Accordingly, the clinical phenotypes of participants included in our study agreed with previously described characteristics (Alcalay *et al.*, 2011; Kasten *et al.*, 2018), indicating that we investigated a representative group of monogenic and idiopathic Parkinson's disease patients.

IL6 is elevated in the serum of Parkinson's disease patients with biallelic mutations in *PRKN* or *PINK1*

As a first step, we tested the distribution of serum IL6 levels in the German cohort (Table 2 and Fig. 1A and C). We hypothesized that patients with Parkinson's disease, due to biallelic mutations in *PRKN* or *PINK1*, exhibit higher IL6 levels compared to healthy control subjects and higher levels compared to affected and unaffected heterozygous mutation carriers, while heterozygous mutation carriers were expected to show higher IL6 levels than healthy control subjects. We confirmed the assumed order ($P = 0.0018$, Fig. 1A). Moreover, pairwise analyses of *PRKN/PINK1* biallelic patients compared to healthy controls showed a significant

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Table 1 Demographics and clinical characteristics of study participants

	PRKN/PINK1 mutation carrier	Sex, females (%)	Age at onset, years (*n*)	Age at examination, years (*n*)	Disease duration, years (*n*)	MDS-UPDRS III (*n*)	Hoehn and Yahr stage (*n*)	Olfactory impairment (%)
PRKN/PINK1 biallelic	23/11	21/34 (61.8)	33.2 ± 8.1 (34)	51.3 ± 11.7 (34)	18.1 ± 8.8 (34)	32.2 ± 16.9 (11)	2.6 ± 1.0 (11)	9/19 (47.4)
PRKN/PINK1 affected heterozygous	19/5	13/24 (54.2)	44.4 ± 11.8 (22)	53.8 ± 13.0 (24)	8.9 ± 8.9 (22)	24.8 ± 14.7 (6)	2.2 ± 0.41 (6)	9/13 (69.2)
PRKN/PINK1 unaffected heterozygous	15/11	10/24 (41.7)	NA	50.7 ± 13.4 (24)	NA	NA	NA	5/20 (25.0)
Idiopathic Parkinson's disease	NA	29/64 (45.3)	53.1 ± 11.9 (64)	61.6 ± 11.9 (64)	8.4 ± 6.1 (64)	31.7 ± 15.1 (40)	2.4 ± 0.6 (40)	32/39 (82.1)
Healthy controls	NA	57/99 (57.6)	NA	55.1 ± 10.9 (98)	NA	NA	NA	14/38 (36.8)

Sex and olfactory impairment are presented as part of the whole group with percentages in parentheses. Age at onset, age at examination, disease duration, Movement Disorder Society Unified Parkinson's Disease Rating Scale part III (MDS-UPDRS III), and Hoehn and Yahr stage are presented as mean ± standard deviation. NA = not applicable.

Table 2 IL6, CRP and mtDNA levels in the different study groups

	Cohort	IL6, pg/ml (*n*)	CRP, mg/l (*n*)	ccf-mtDNA, copy number (*n*)
PRKN/PINK1 biallelic	German	6.8 [5.6–8.5] (15)	2.7 [1.1–7.1] (15)	2774 [1315–5647] (13)
	Italian	3.0 [2.1–4.5] (19)	2.5 [0.8–4.3] (19)	NA
PRKN/PINK1 affected heterozygous	German	5.4 [4.7–6.5] (18)	1.1 [0.6–3.6] (18)	3608 [1724–5471] (17)
	Italian	2.3 [1.8–2.7] (5)	0.7 [0.3–6.9] (5)	NA
PRKN/PINK1 unaffected heterozygous	German	5.5 [4.6–6.4] (13)	0.9 [0.5–2.3] (13)	1487 [830–2194] (14)
	Italian	1.4 [1.4–2.2] (9)	1.0 [0.8–1.8] (9)	NA
Idiopathic Parkinson's disease	German	5.5 [4.7–7.1] (51)	1.2 [0.4–2.2] (51)	1226 [655–2015] (57)
	Italian	1.6 [1.5–2.5] (5)	1.0 [0.9–4.2] (5)	NA
Healthy controls	German	5.0 [4.5–6.0] (84)	1.0 [0.4–2.1] (85)	1434 [715–2688] (55)
	Italian	1.4 [1.4–1.7] (9)	0.7 [0.2–2.8] (9)	NA
All participants	German	5.3 [4.7–6.5] (181)	1.1 [0.5–2.4] (182)	1467 [752–3051] (156)
	Italian	2.1 [1.4–2.9] (47)	1.3 [0.7–3.0] (47)	NA

Distribution of IL6, CRP and ccf-mtDNA in the different study groups separated into the German and the Italian cohorts. Results are presented as median [IQR]. Statistics are shown in Figs 1 and 2 and Supplementary Fig. 3. NA = not applicable.

IL6 elevation in patients with Parkinson's disease due to biallelic *PRKN/PINK1* mutations ($P = 0.0006$, Fig. 1A). Investigating IL6 levels in the Italian cohort (Table 2 and Fig. 1B) showed similar differences (*PRKN/PINK1* biallelic > affected/unaffected heterozygotes > healthy controls; $P = 0.00008$). Pairwise analyses revealed significantly higher IL6 values in *PRKN/PINK1* biallelic patients ($P = 0.007$) and a trend towards elevated IL6 levels in affected heterozygous mutation carriers ($P = 0.065$) compared to healthy control subjects (Fig. 1B). By contrast, unaffected heterozygotes did not show an increase. Moreover, we found increased IL6 levels comparing *PRKN/PINK1* biallelic with unaffected heterozygous mutation carriers ($P = 0.0062$). A similar trend was also present in the German cohort ($P = 0.0302$, Fig. 1A).

Moreover, we hypothesized that *PRKN/PINK1* biallelic mutation carriers exhibit higher IL6 levels than patients with idiopathic Parkinson's disease, while we expected individuals in the idiopathic Parkinson's disease group to present with higher serum IL6 than healthy control subjects. Indeed, we confirmed the expected order (*PRKN/PINK1* biallelic > idiopathic Parkinson's disease > healthy controls; $P = 0.0005$, Fig. 1C). Performing pairwise analyses, we detected a trend towards higher IL6 levels in idiopathic Parkinson's disease patients than in healthy controls ($P = 0.0559$). As significance levels were adjusted to $P < 0.0167$ to account for multiple testing, differences in IL6 values between *PRKN/PINK1* biallelic mutation carriers and patients with idiopathic Parkinson's disease did not reach significance ($P = 0.0259$, Fig. 1C).

Analysing *PRKN* and *PINK1* mutation carriers separately, we did not detect any difference between IL6 levels, neither in biallelic nor in heterozygous mutation carriers (Supplementary Fig. 2). This was true for both the German (Supplementary Fig. 2A and C) and the Italian cohorts (Supplementary Fig. 2B and D).

IL6 triggers the release of CRP, which was also found to be upregulated in Parkinson's disease (Qiu *et al.*, 2019). Therefore, we investigated CRP levels (Table 2) exploring the same order as previously assumed for IL6. Investigating group differences in general, we saw no relevant differences (Supplementary Fig. 3A). However, we observed a trend

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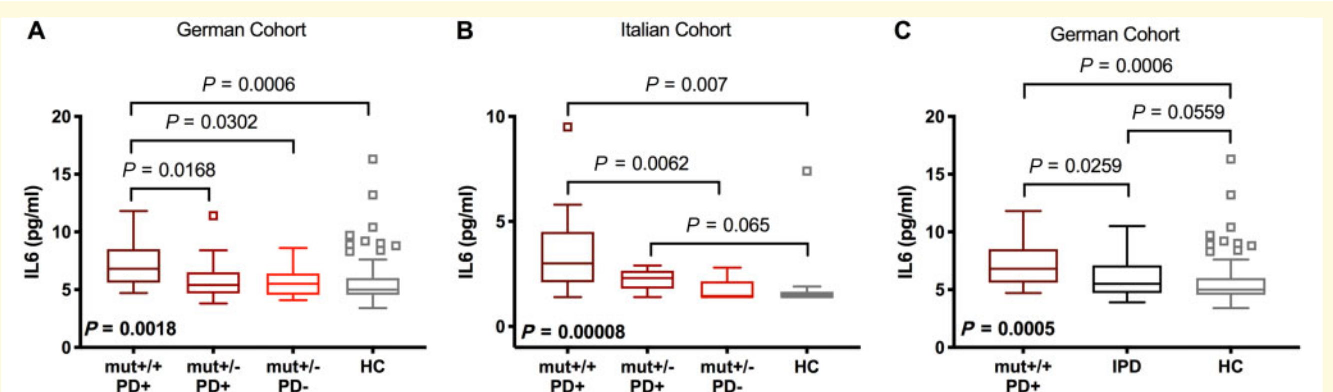

Figure 1 Interleukin 6 (IL6) is increased in serum from biallelic *PRKN/PINK1* mutation carriers compared to healthy controls. IL6 levels in serum samples from Parkinson's disease (PD) patients with mutations in *PRKN/PINK1*, unaffected mutation carriers, idiopathic Parkinson's disease (IPD) patients, and healthy control subjects (HC). (**A**) Patients with Parkinson's disease due to biallelic *PRKN/PINK1* mutations (mut+/+ PD+, *n* = 15) exhibited higher IL6 levels compared to healthy controls (*n* = 84). Unaffected heterozygous mutation carriers (mut+/– PD–, *n* = 13) showed similar IL6 levels compared to affected heterozygous mutation carriers (mut+/– PD+, *n* = 18). (**B**) Investigating an independent cohort from Italy yielded similar group differences as presented in **A**, while the total amount of IL6 among all participants was lower compared to the German cohort due to a batch effect (mut+/+ PD+, *n* = 19; mut+/– PD+, *n* = 5; mut+/– PD–, *n* = 9; HC, *n* = 9). Regarding the Italian cohort, biallelic *PRKN/PINK1* mutation carriers exhibited increased IL6 levels compared to unaffected heterozygotes. Moreover, there was a trend towards elevated IL6 levels in *PRKN/PINK1* affected heterozygotes compared to healthy control subjects. (**C**) In the German cohort, *PRKN/PINK1* biallelic patients showed a trend towards higher IL6 levels compared to patients with idiopathic Parkinson's disease (*n* = 51), while idiopathic Parkinson's disease patients exhibited a trend towards elevated IL6 release compared to healthy control subjects. As multiple testing was performed, the significance level was adjusted to $\alpha = 0.0167$. The assumed order among the different groups was tested with the Jonckheere-Terpstra test (bold, *lower left corner*). Pairwise differences between two groups were assessed using the Wilcoxon rank sum test. Data are presented as box and whisker plots. The box extends from the 25th to the 75th percentile. The line in the middle of the box represents the median. Tukey whiskers are used.

towards increased CRP levels in individuals with Parkinson's disease due to mutations in *PRKN/PINK1* compared to patients with idiopathic Parkinson's disease (exploratory *P* = 0.0319) and an elevation compared to healthy control subjects (exploratory *P* = 0.0122, Supplementary Fig. 3A). Moreover, CRP levels correlated with IL6 levels in affected biallelic and heterozygous *PRKN/PINK1* mutation carriers (Supplementary Fig. 3B), idiopathic Parkinson's disease patients (Supplementary Fig. 3C) and healthy control subjects (Supplementary Fig. 3D).

Circulating cell-free mtDNA is increased in Parkinson's disease patients with mutations in *PRKN* or *PINK1*

We expected affected heterozygous and biallelic *PRKN/PINK1* mutation carriers to exhibit higher serum levels of ccf-mtDNA compared to idiopathic Parkinson's disease patients and healthy controls. Statistical testing revealed group differences in general (*P* = 0.0006). Compared to patients with idiopathic Parkinson's disease, pairwise analyses showed increased ccf-mtDNA release in *PRKN/PINK1* biallelic mutation carriers (*P* = 0.0094) and affected heterozygous mutation carriers (*P* = 0.0002). Compared to healthy control subjects, we observed a trend towards elevated ccf-mtDNA levels in biallelic *PRKN/PINK1* mutation carriers (*P* = 0.0459) and an increase in affected heterozygous individuals (*P* = 0.0019). Furthermore, we found elevated ccf-mtDNA levels in affected compared to unaffected *PRKN/PINK1* heterozygotes (*P* = 0.0058, Table 2 and Fig. 2A). Moreover, assessing the utility of ccf-mtDNA levels to discriminate between idiopathic Parkinson's disease and Parkinson's disease associated with heterozygous *PRKN/PINK1* mutations, we found an area under the ROC curve of 0.81 (Fig. 2B).

IL6 levels are associated with disease duration in patients with *PRKN* or *PINK1* mutations

Focusing on idiopathic Parkinson's disease, we found a trend towards a positive correlation between IL6 levels and age (Fig. 3A). A similar association was absent in individuals with biallelic *PRKN/PINK1* mutations (r = 0.018, exploratory *P* = 0.949, *n* = 15) and in healthy control subjects (*r* = 0.076, exploratory *P* = 0.490, *n* = 84). Moreover, while in patients with Parkinson's disease due to *PRKN* or *PINK1* mutations IL6 levels correlated with disease duration (Fig. 3B), we found no such association in patients with idiopathic Parkinson's disease (*r* = 0.065, exploratory *P* = 0.65, *n* = 51).

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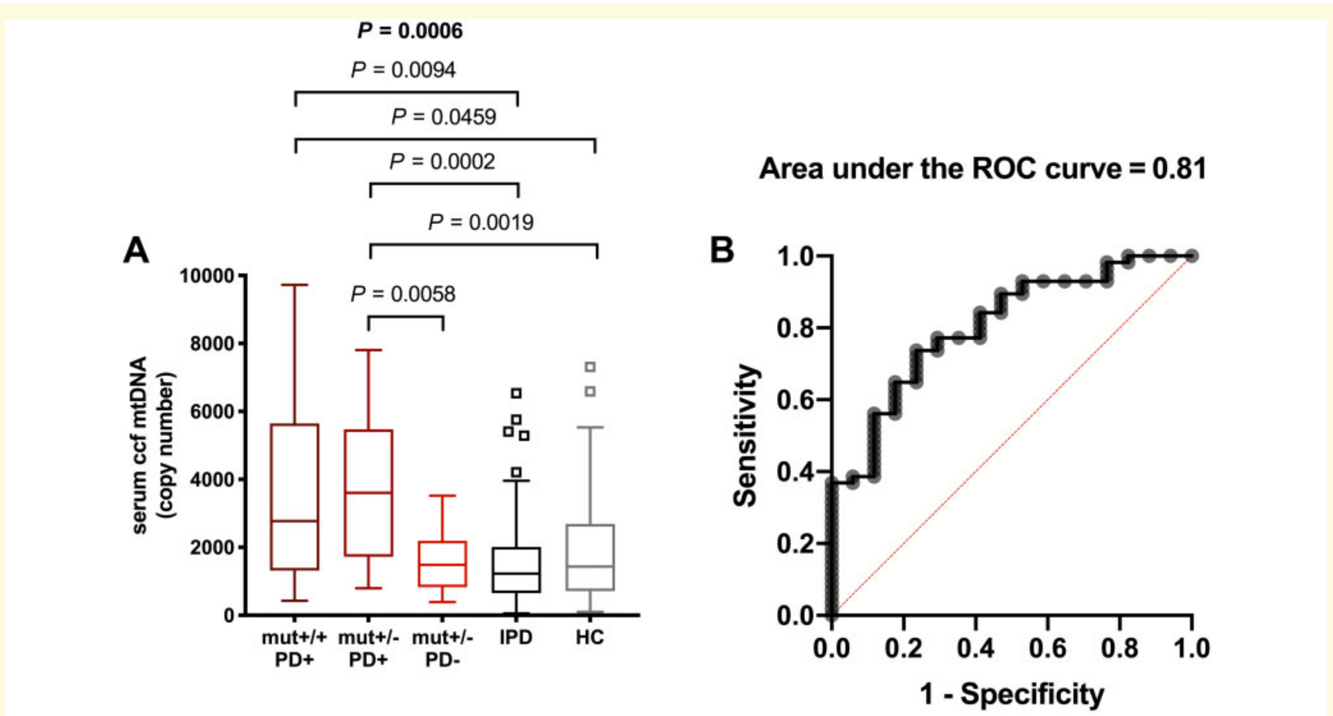

Figure 2 Serum ccf-mtDNA is increased in *PRKN/PINK1* mutation carriers and discriminates between affected heterozygotes and idiopathic Parkinson's disease patients. (**A**) Patients with Parkinson's disease (PD) due to biallelic *PRKN/PINK1* mutations (mut+/+ PD+, *n* = 13) and affected heterozygous individuals (mut+/– PD+, *n* = 17) exhibited elevated serum ccf-mtDNA levels compared to idiopathic Parkinson's disease patients (IPD, *n* = 57). Additionally, affected heterozygous patients showed higher ccf-mtDNA levels than healthy control subjects (HC, *n* = 55). Unaffected heterozygous mutation carriers (mut+/– PD–, *n* = 14) exhibited ccf-mtDNA levels between those of affected mutation carriers and idiopathic Parkinson's disease patients. As multiple testing was performed, the significance level was adjusted to $\alpha = 0.0167$. Group differences in general were tested via Kruskal-Wallis test (bold). Pairwise differences between two groups were analysed using the Wilcoxon rank sum test. Data are presented as box and whisker plots. The box extends from the 25th to the 75th percentile. The line in the middle of the box represents the median. Tukey whiskers are used. (**B**) Assessment of the utility of ccf-mtDNA levels to discriminate between idiopathic Parkinson's disease patients (*n* = 57) and affected heterozygous *PRKN/PINK1* mutation carriers (*n* = 17) investigated by a receiver operator characteristic (ROC) curve.

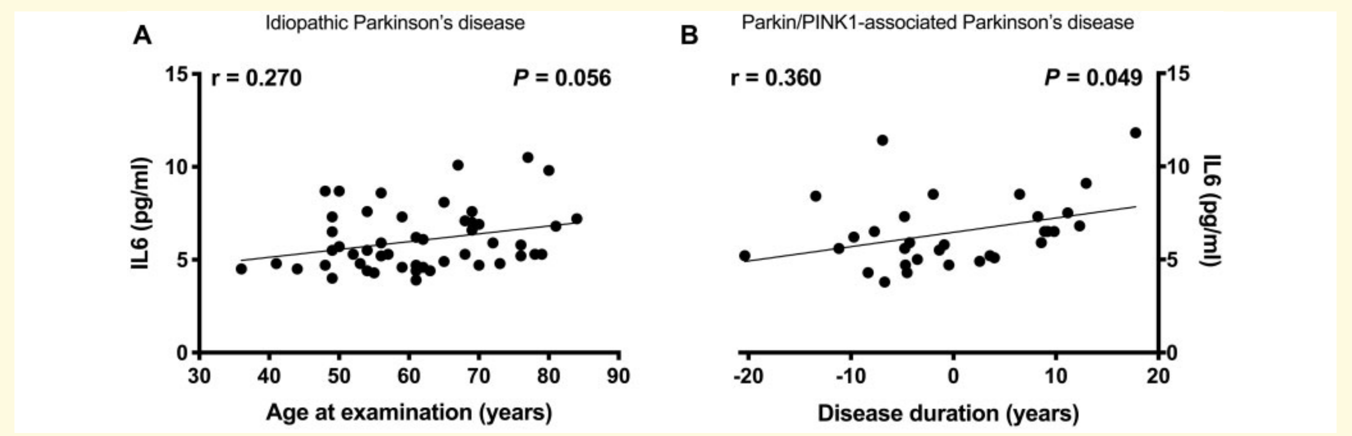

Figure 3 Association between age at examination, disease duration and IL6 release. (**A**) In patients with idiopathic Parkinson's disease (*n* = 51), we observed elevated IL6 levels with increasing age when determining the Spearman's correlation of both variables. (**B**) In *PRKN/PINK1* biallelic and heterozygous patients (*n* = 31), there was a positive correlation between disease duration and IL6 levels. The Spearman's correlation was calculated after adjusting for age at examination. Correlation coefficient is shown in the *top left*, exploratory *P*-values are presented in the *top right* corner. Lines represent linear regression.

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As a sensitivity analysis, we additionally performed a linear regression analysis to predict the influence of age at examination on IL6 and ccf-mtDNA levels and used the resulting residuals to test for differences between study groups while taking age into account. Thereby, group- and pairwise differences in ccf-mtDNA levels remained unchanged. In the context of IL6 levels, the assumed order was still present after the adjustment for age ($P = 0.0008$). However, this adjustment led to differences regarding pairwise analyses between *PRKN/PINK1* biallelic mutation carriers and affected heterozygotes ($P = 0.0133$), supporting the assumed gene dosage effect of *PRKN* and *PINK1* mutated alleles in the context of IL6. Furthermore, this regression analysis revealed a difference in IL6 levels between *PRKN/PINK1* biallelic mutation carriers and idiopathic Parkinson's disease patients ($P = 0.0129$).

Discussion

Our study further implicates inflammation due to impaired mitophagy and subsequent mtDNA release in the pathogenesis of *PRKN/PINK1*-associated Parkinson's disease. We extended our previous findings in a small group of biallelic *PRKN* mutation carriers to an additional 18 biallelic *PRKN*, and—for the first time—11 biallelic *PINK1* mutation carriers. By this, we showed that *PRKN* and *PINK1* mutation carriers exhibit similar IL6 levels when investigated separately. As only IL6 elevation in biallelic *PRKN* mutation carriers was previously described, our study provides first-time evidence that IL6 is similarly increased in patients with Parkinson's disease due to biallelic *PINK1* mutations in line with the notion of both proteins being involved in a common pathway during mitophagy (Narendra *et al.*, 2012).

PRKN/PINK1 biallelic mutation carriers showed higher IL6 levels compared to heterozygous mutation carriers, suggesting a gene dosage effect regarding IL6 release. However, this interpretation is limited. Although we confirmed the presence of the assumed order *PRKN/PINK1* biallelic > *PRKN/PINK1* heterozygous > healthy controls, pairwise analysis between heterozygotes and healthy controls did not reach significance, which would have been necessary to show a complete gene dosage effect.

In accordance with previous reports (Qin *et al.*, 2016), we also found a trend towards elevated IL6 levels in idiopathic Parkinson's disease compared to healthy controls. In addition, we detected a positive correlation between IL6 levels and disease duration in affected *PRKN/PINK1* mutation carriers that was absent in patients with idiopathic Parkinson's disease, indicating a specific influence of the genetic alterations on IL6 levels. Moreover, although *PRKN* and *PINK1* mutation carriers included in our study were younger than idiopathic Parkinson's disease patients, they exhibited higher IL6 values, arguing for an age-independent IL6 elevation in *PRKN/PINK1* mutation carriers. Our findings appear robust, as samples from two independent cohorts revealed similar results.

In line with previous research (Tanaka *et al.*, 2014), CRP levels increased with higher IL6 serum concentrations in *PRKN/PINK1* mutation carriers, as well as in patients with idiopathic Parkinson's disease and healthy control subjects. However, as we found no significant group differences when investigating CRP levels, our data support IL6 as a more specific marker in the context of (monogenic) Parkinson's disease.

Release of ccf-mtDNA represents a common feature in various systemic diseases (West and Shadel, 2017). However, an association between ccf-mtDNA accumulation and IL6 elevation has, so far, only been established in murine models in the context of Parkinson's disease (Sliter *et al.*, 2018). We observed increased ccf-mtDNA levels in the serum of *PRKN/PINK1* biallelic mutation carriers and affected heterozygotes compared to patients with idiopathic Parkinson's disease. In line with our previous experiments in *Prkn* knockout mice (Sliter *et al.*, 2018), this finding indicates that the release of damage-associated molecular patterns (DAMPs), such as mtDNA from mitochondria, is a consequence of impaired mitophagy in *PRKN/PINK1*-associated Parkinson's disease.

The question to what extent heterozygous mutations in autosomal recessively inherited Parkinson's disease may act in a dominant manner with highly reduced penetrance is still discussed controversially (Klein *et al.*, 2007; Reed *et al.*, 2019). However, a dominant-negative mechanism in the context of affected heterozygous *PINK1* mutation carriers has been reported recently, providing insights into how heterozygous mutations might increase the probability of developing Parkinson's disease (Puschmann *et al.*, 2017). In our study, we provide evidence that ccf-mtDNA serum levels are useful to discriminate affected heterozygous *PRKN/PINK1* mutation carriers from idiopathic Parkinson's disease patients with high accuracy. Thus, our observations support the idea of a role for heterozygous variants at least as a strong risk factor for Parkinson's disease. However, while we did not find an increase in ccf-mtDNA concentrations in unaffected heterozygous mutation carriers compared to healthy control subjects or patients with idiopathic Parkinson's disease, affected heterozygous mutation carriers exhibited higher ccf-mtDNA levels than unaffected heterozygotes. This difference might indicate that in unaffected carriers, mitochondrial clearance is operating effectively until currently unknown factors trigger a breakdown of the genetically vulnerable mitophagy machinery. Subsequent to this tipping point, clinical symptoms, as well as an increase in ccf-mtDNA (and IL6) levels occur. Accordingly, investigating serum ccf-mtDNA levels in *PRKN/PINK1*-assocciated Parkinson's disease may contribute to the elucidation of mechanisms mediating penetrance and may have the potential to serve as a marker of affection status for heterozygous *PRKN/PINK1* mutation carriers. A previous study quantifying ccf-mtDNA in CSF from idiopathic Parkinson's disease cases found decreased levels in early-stage patients (Pyle *et al.*, 2015). Contrary to this report, in our study, we investigated idiopathic Parkinson's disease patients in an

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advanced disease state and did not observe differences in serum ccf-mtDNA levels compared to healthy control subjects. Assuming that mtDNA levels differ over the course of the disease and vary between tissues, it will be necessary to investigate ccf-mtDNA levels in a longitudinal fashion using different patient-derived biomaterials.

Furthermore, the idiopathic Parkinson's disease group exhibited elevated serum IL6 despite low ccf-mtDNA levels. This finding can be interpreted in line with previous research providing evidence for inflammation due to impaired mitochondrial function and clearance independent of the mtDNA-cGAS-STING pathway. For example, neuromelanin-triggered NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling (Wilms *et al.*, 2003) and autophagy-mediated NLRP3 inflammasome activation (Zhong *et al.*, 2018) are established molecular mechanisms in Parkinson's disease. Moreover, mtDNA variants were recently described to act as immunogenic neoepitopes (Deuse *et al.*, 2019). Thus, somatic mtDNA mutations, which have been previously documented in idiopathic Parkinson's disease (Bender *et al.*, 2006), might constitute an additional parameter modulating cGAS-STING signalling and, in turn, IL6 levels. Together, these findings highlight the potential of patient stratification attempts in personalized medicine approaches for idiopathic Parkinson's disease.

In our previous study (Sliter *et al.*, 2018), we showed that mtDNA mutator mice genetically lacking parkin accumulate ccf-mtDNA in serum and display neurodegenerative features and motor impairment. Interestingly, both of these phenotypes could be rescued by genetic inactivation of STING. Thereby, a direct contribution of neuroinflammation to the Parkinson's disease-like phenotype associated with *PRKN* and *PINK1* mutations has been observed in murine models. In light of these findings, the presence of increased ccf-mtDNA in patients with *PRKN/PINK1* mutations also implicates cGAS-STING signalling in the human disease process. Assuming that neuroinflammation causes neurodegeneration in patients with *PRKN* or *PINK1* mutations, targeted anti-inflammatory treatment may present a strategy to slow disease progression specifically in this group of individuals, who are estimated to make up 12.3% of early-onset (<45 years) Parkinson's disease cases (Kilarski *et al.*, 2012). Several approaches to influence the ccf-mtDNA-CGAS-STING-IL6 pathway that could now be applied to Parkinson's disease have already been established in the past. For instance, a protective effect of acetylsalicylic acid has been discussed in STING-mediated disease (Elkon, 2019). This finding is in accordance with epidemiological results, indicating that non-steroid-anti-inflammatory-drugs (NSAIDs) might have a positive effect on Parkinson's disease in general (Noyce *et al.*, 2012). More specific, anti-IL6 (Emery *et al.*, 2008) and anti-JAK antibodies (Lee *et al.*, 2014) represent an established treatment approach in different inflammatory conditions such as for instance, rheumatoid arthritis, and might have therapeutic potential in monogenic Parkinson's disease.

Several limitations of our study should be mentioned. First, we performed a retrospective analysis that did not allow us to correct for influencing factors. In particular, inflammatory cytokines like IL6 (Tanaka *et al.*, 2014), but also ccf-mtDNA (Grazioli and Pugin, 2018) have previously been reported to be increased in a number of inflammatory and cardiovascular diseases as well as in cancer. Thus, the specificity of the markers investigated in this study might be reduced if applied in clinical testing. Second, 45 samples, which had already been analysed for IL6 serum levels in our pilot analysis (Sliter *et al.*, 2018), were reassessed in the current study to validate the original IL6 results in an independent laboratory and to additionally obtain data on CRP and ccf-mtDNA serum levels from these individuals. Statistical testing revealed that the key findings from our study hold up, whether or not these samples are included in the analysis (Supplementary Fig. 1). Third, despite measuring IL6 levels in a certified diagnostic laboratory, the concentrations differed between the two tested cohorts. This variability may be explained by batch effects or varying pre-analytic treatment of the blood samples. However, such experimental limitations can be addressed in further studies by implementing standardized sample processing and prospective study design, as well as by increasing the number of enrolled mutation carriers. Furthermore, the role of cGAS-STING signalling in the transition from health to disease state in heterozygous mutation carriers warrants further investigation in the context of reduced penetrance. Finally, additional molecular studies in patient-derived neurons and glia will be required to fully decipher the upstream processes and downstream targets of the ccf-mtDNA-cGAS-STING-IL6 pathway in Parkinson's disease.

In conclusion, we demonstrated for the first time an association between a *PRKN* or *PINK1*-mutant genotype, increased ccf-mtDNA release, and neuroinflammation in Parkinson's disease patients. Together, the combination of elevated ccf-mtDNA and IL6 levels in serum of *PRKN* or *PINK1* mutation carriers supports the concept of impaired mitophagy triggering IL6 release (most likely via cGAS-STING signalling) that was, thus far, only demonstrated in mouse models. Our study has significant translational impact as especially serum ccf-mtDNA levels could serve as a biomarker of disease state, directing therapeutic approaches, which target the innate immune response in *PRKN/PINK1*-associated Parkinson's disease in the near future.

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Competing interests

The authors report no competing interests. R.J.Y. did not supply patient materials and does not have a clinical protocol to report.

Supplementary material

Supplementary material is available at *Brain* online.

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