

Discordant Monozygotic Parkinson Disease Twins: Role of Mitochondrial Integrity

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Objective: Even though genetic predisposition has proven to be an important element in Parkinson's disease (PD) etiology, monozygotic (MZ) twins with PD displayed a concordance rate of only about 20% despite their shared identical genetic background.

Methods: We recruited 5 pairs of MZ twins discordant for idiopathic PD and established skin fibroblast cultures to investigate mitochondrial phenotypes in these cellular models against the background of a presumably identical genome. To test for genetic differences, we performed whole genome sequencing, deep mitochondrial DNA (mtDNA) sequencing, and tested for mitochondrial deletions by multiplex real-time polymerase chain reaction (PCR) in the fibroblast cultures. Further, the fibroblast cultures were tested for mitochondrial integrity by immunocytochemistry, immunoblotting, flow cytometry, and real-time PCR to quantify gene expression.

Results: Genome sequencing did not identify any genetic difference. We found decreased mitochondrial functionality with reduced cellular adenosine triphosphate (ATP) levels, altered mitochondrial morphology, elevated protein levels of superoxide dismutase 2 (SOD2), and increased levels of peroxisome proliferator-activated receptor-gamma coactivator- α (PPARGC1A) messenger RNA (mRNA) in skin fibroblast cultures from the affected compared to the unaffected twins. Further, there was a tendency for a higher number of somatic mtDNA variants among the affected twins.

Interpretation: We demonstrate disease-related differences in mitochondrial integrity in the genetically identical twins. Of note, the clinical expression matches functional alterations of the mitochondria.

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Overall, monozygotic (MZ) twins exhibit marked phenotypic similarities for many traits due to their shared genetic makeup and, therefore, are considered a valuable model for dissecting complex genetic-environmental interactions and molecular mechanisms.

Parkinson's disease (PD) is widely accepted as a multifactorial disease with both genetic and environmental contributions. Interestingly, despite their shared identical genetic background, a concordance rate of only about 20% has been shown in MZ twins with PD.¹ Follow-up clinical examinations revealed that subtle motor signs were often present in putatively unaffected twins (UTs)^{1–3} suggesting that the “discordance” rate may be considerably lower than previously reported. Nevertheless, the true nature of the phenotypic variability in MZ twins remains poorly understood.

Mitochondrial dysfunction has been previously described in idiopathic PD, including altered mitochondrial morphology, increased oxidative stress, and defects in mitochondrial membrane potential and different complexes of the respiratory chain.^{4–6} Further, mitochondrial DNA (mtDNA) impacts on the functionality of mitochondria. Variability in mtDNA comprises both inherited variants from the mother and somatic changes. Of note, somatic substitutions as well as deletions in the mtDNA have been detected in idiopathic patients with PD.⁷ Due to lack of effective mtDNA repair mechanisms, somatic mtDNA mutations accumulate and are usually compensated for by a large number of mtDNA copies per mitochondrion and per cell, respectively. Additionally, mtDNA molecules are randomly distributed during cell division leading to different degrees of (inherited) heteroplasmy.⁸ In MZ twins, the uneven division and somatic changes may result in a different repertoire of mtDNA variants suggesting them as a potential genetic modifier of phenotypes.

In this study, we unravel various mitochondrial changes that match the clinical discordance of PD in MZ twins.

Patients and Methods

Subjects

We included 5 MZ twin pairs, with 1 twin each meeting the Movement Disorder Society clinical diagnostic criteria for PD⁹ (affected twin [AT]), and the other one being unaffected (UT;) but not necessarily asymptomatic. The clinical status was determined at inclusion and can change over time (ie, the UT becoming affected). Comprehensive clinical phenotyping for 4 of the twin pairs (AT/UT 1–4) has been reported previously.³ Pathogenic variants in established PD-causing genes were previously excluded in

these twins by panel sequencing.³ In this study, we included an additional pair of MZ female twins discordant for PD (AT/UT 5). The affected sister has been suffering from tremor-dominant PD for > 20 years, whereas her sister remained unaffected throughout life (Supplementary Video S1). Gene panel analysis excluded pathogenic variants in known PD genes but revealed a common *PINK1* variant in the heterozygous state in both sisters (c.344A > T; p. Q115L; minor allele frequency among non-Finnish Europeans: 5.5% [https://gnomad.broadinstitute.org/variant/1-20960385-A-T?dataset=gnomad_r2_1]). The study was approved by the local ethics committee of the University of Lübeck (16–039), and all participants gave written informed consent prior to the study.

Cell Culture

Skin biopsies were used to establish fibroblast cultures, which were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Life Technologies).

Genome Sequencing

Whole genome sequencing was performed in all 5 twin pairs at BGI Hong Kong (mean coverage: 30 times). We used the genome data to look for sequence differences within each twin pair. For this, we compared single nucleotide variants (SNVs) and further filtered for rare variants (minor allele frequency ≤ 0.005 in Exome Sequencing Project [ESP; <https://evs.gs.washington.edu/EVS/>] and 1,000 Genomes Project [<https://www.internationalgenome.org/home>]) and for variants with a Combined Annotation Dependent Depletion (CADD) score > 20 (<https://cadd.gs.washington.edu/>). The respective candidate regions were then visualized by the Integrative Genomics Viewer (IGV) or Sanger sequenced for validation.

MtDNA Mutational Burden and 7S DNA Analysis

MtDNA was extracted from fibroblast cultures (passages 5 to 10). For deep mtDNA sequencing, samples were amplified with 2 rounds of overlapping long-range polymerase chain reactions (PCRs), which were subsequently tagged and sequenced on an Illumina NextSeq, as previously described (mean coverage: > 10,000 times).¹⁰ The heteroplasmic frequency of the variants was used as a measure for mtDNA heteroplasmy, which was estimated as base heteroplasmy (mutant allele frequency over the total coverage of all alleles). We defined 3 groups: low-level heteroplasmic (1–15%), medium-level heteroplasmic (15–70%), and high-level heteroplasmic and homoplasmic (> 70%) variants.

TABLE. Demographic Data and Experimental Results of the Probands

ID number (primary skin fibroblasts)					SOD2 protein levels ^a	Form factor ^b [x 10 ⁵]	ATP levels ^c [x 10 ⁻¹⁰]	MMP levels ^d [x 10 ⁴]	<i>PPARGCIA</i> mRNA levels ^e	mtDNA mutational burden (number of mtDNA variants)		
	AAB	PD	AAO	Code						High-level heteroplasmy/ Homoplasmy	Medium- level heteroplasmy	Low-level heteroplasmy
L-11167	60	Negative	-	UT1	0.6 + 0.5	12.7 + 8.0	18.5 + 3.1	2.1 + 0.1	1.1 + 0.6	19	2	0
L-11166	60	Positive	43	AT1	3.6 + 2.5	7.7 + 3.5	2.9 + 5.5	1.2 + 0.4	1.4 + 1.3	20	4	8
L-11148	74	Negative	-	UT2	2.0 + 2.3	11.1 + 5.9	22.1 + 6.2	1.4 + 3.5	0.1 + 1.0	32	5	13
L-11147	74	Positive	71	AT2	2.5 + 2.2	11.0 + 9.8	18.1 + 2.9	1.5 + 1.5	1.6 + 0.1	32	6	7
L-11156	59	Negative	-	UT3	7.0 + 4.6	6.4 + 5.4	16.4 + 9.9	1.5 + 0.4	0.1 + 0.9	16	5	8
L-11155	59	Positive	57	AT3	7.2 + 3.3	3.2 + 6.4	20.0 + 1.1	1.2 + 2.1	0.3 + 0.7	16	2	18
L-10886	76	Negative	-	UT4	4.2 + 0.9	10.0 + 1.1	20.0 + 9.7	2.3 + 4.9	1.2 + 0.3	15	2	11
L-10887	76	Positive	74	AT4	12.1 + 6.6	6.2 + 5.3	14.5 + 1.2	1.4 + 5.4	1.3 + 0.2	15	3	21
L-3857	82	Negative	-	UT5	0.7 + 0.2	10.0 + 7.4	33.8 + 8.2	1.3 + 0.0	0.9 + 0.4	19	0	4
L-3856	82	Positive	59	AT5	3.8 + 1.4	3.9 + 1.2	14.5 + 3.5	1.2 + 0.4	1.2 + 0.1	19	4	11

Median and interquartile range (IQR) for different mitochondrial phenotypes in individual twin pairs (UT/AT 1–5) are presented.

^aSOD2 protein levels relative to β -actin (loading control) from $n = 4$ independent experiments.

^bThe degree of mitochondrial branching (calculated as form factor in 10 cells per each individual from $n = 2$ independent experiments) presented in arbitrary units.

^cIntracellular ATP levels presented in mol ATP/min/mg protein from $n = 3$ different experiments.

^dMMP presented in arbitrary units from $n = 3$ independent experiments.

^e*PPARGCIA* mRNA levels relative to *ACTB* and *G6PD* (reference genes) from $n = 5$ independent experiments.

AAB = age at biopsy; AAO = age at onset; AT = affected twin; ATP = adenosine triphosphate; MMP = mitochondrial membrane potential; mRNA = messenger RNA; mtDNA = mitochondrial DNA; PD = Parkinson's disease; *PPARGCIA* = peroxisome proliferator-activated receptor-gamma coactivator- α ; SOD2 = superoxide dismutase 2; UT = unaffected twin.

We additionally quantified mtDNA deletions using an established multiplex real-time PCR assay, which compares the cellular concentration of *MT-ND4* (situated within the common major arc deletion) and *MT-ND1* (situated in the minor arc; *MT-ND4:MT-ND1*).¹¹ Moreover, we assessed the proportion of transcription-active mtDNA molecules with a triple-stranded non-coding region (NCR). During transcription, 7S DNA is incorporated in the NRC region to form a D-loop that can be targeted by real-time PCR (7S DNA: *MT-ND1*).¹¹

Analysis of Mitochondrial Integrity

For Western blot analyses, proteins were extracted using SDS extraction buffer and gels were blotted onto nitrocellulose membranes. Antibodies against β -actin (1:1,000,000; Sigma) and anti-superoxide dismutase 2 (SOD2) (1:1,000; Abcam) were used. Signal intensities on immunoblots were measured with Image Lab software (Bio-Rad Laboratories). SOD2 protein levels were quantified in all 10 individuals from 4 different blots.

For immunocytochemical analysis, cells were stained with primary anti-GRP75 antibody (1:1,000; Abcam). Form factor was calculated from 10 cells per individual as

a measure for the mitochondrial network, as described previously.¹² The culturing on coverslips and staining of the cells was done twice for all 10 individuals.

Cellular adenosine triphosphate (ATP) levels were determined based on luminescence using the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics).¹³ We measured intracellular ATP levels in all 10 individuals 3 times.

Mitochondrial membrane potential (MMP) was analyzed in tetramethylrhodamine methyl ester perchlorate (TMRM)-stained dermal fibroblasts using a BD LSR II flow cytometer. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was used to suppress MMP. The mean fluorescence intensity (MFI) of TMRM-stained cells was calculated using the following formula: $MFI\ TMRM^{untreated} - MFI\ TMRM^{FCCP} = MMP$. The analysis was performed 3 times in all 10 individuals.

For expression analysis of peroxisome proliferator-activated receptor-gamma coactivator- α (*PPARGCIA*), total RNA from fibroblast cultures was extracted using the RNeasy kit (Qiagen) and reverse-transcribed into cDNA with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR analysis was performed with SYBR Green (Roche Diagnostics) on a LightCycler480 (Roche Diagnostics). *ACTB* and *G6PD*

served as reference genes. Primer sequences are available upon request. *PPARGCIA* levels were determined 5 times in duplicate for all 10 individuals.

Statistical Analysis

We evaluated differences between the group of ATs and the group of UTs. Data on mitochondrial integrity were generated for 5 parameters (SOD2 protein levels, form factor, cellular ATP levels, MMP, and *PPARGCIA* expression) in 2 to 5 replications of the experiments based on newly extracted samples. All twins had the same number of experiments per parameter. To compare the group of ATs with the group of UTs, we performed nonparametric analyses of variance (ANOVA) with repeated measure effects for affection status, experiments, and runs where applicable.

Analyses of mtDNA changes were based on a single sample collection. To compare the group of ATs with the group of UTs, we applied a Wilcoxon signed rank test.

Descriptive statistical values (uncorrected p values) as well as a relative treatment effect (RTE) from the ANOVA with the corresponding 95% confidence interval (95% CI) are provided. The RTE for a group reflects the probability that a random observation from the entire data set has a lower value than a random observation of the specific group. Therefore, the RTE under the null hypothesis is 0.5.

Results

Genomic and mtDNA Variants in the Twin Pairs

Assuming that the phenotypic discordance of the twins has a genetic background, we hypothesized that potential genetic differences could have 2 origins (ie, in the nuclear DNA due to heterozygous de novo changes or in the

mtDNA due to heteroplasmic variants with different levels of heteroplasmy).

Genome data analysis revealed 303,219 to 312,687 SNVs per twin pair, which were called in either the ATs or the UTs. After filtering for rare variants with an in silico predicted deleterious effect (based on CADD score), we evaluated 152 to 243 candidate variants per pair by visualization with IGV and/or Sanger sequencing. Surprisingly, none of the evaluated variants was exclusive to one twin, and thus cannot be implicated as a potential disease-related genomic variant in these twin pairs.

Deep mtDNA sequencing revealed 23 to 88 (median = 50) variants per individual (Fig 1A). Most of these variants were called in both twins and were homoplasmic. The number of low-level heteroplasmic variants ranged from 0 to 21 (median = 4;). The number of low-level heteroplasmic variants was higher in the AT compared with the respective UT in all pairs except pair #2. The group comparison suggests a possible trend for a higher number of low-level heteroplasmic mtDNA variants in the group of ATs compared with the group of UTs (Fig 1B; descriptive $p = 0.10$ [Wilcoxon signed rank test]).

In addition, we assessed the presence of mtDNA major arc deletions by determining the *MT-ND4:MT-ND1* ratio and the proportion of transcriptionally active mtDNA molecules by determining the *7S DNA:MT-ND1* ratio with a triple-stranded NCR. Both analyses revealed no differences between the ATs and the UTs (data not shown).

Impaired Mitochondrial Integrity in the Affected Twins

We next examined mitochondrial morphology and function in the twin pairs. Fibroblasts from ATs exhibit elevated

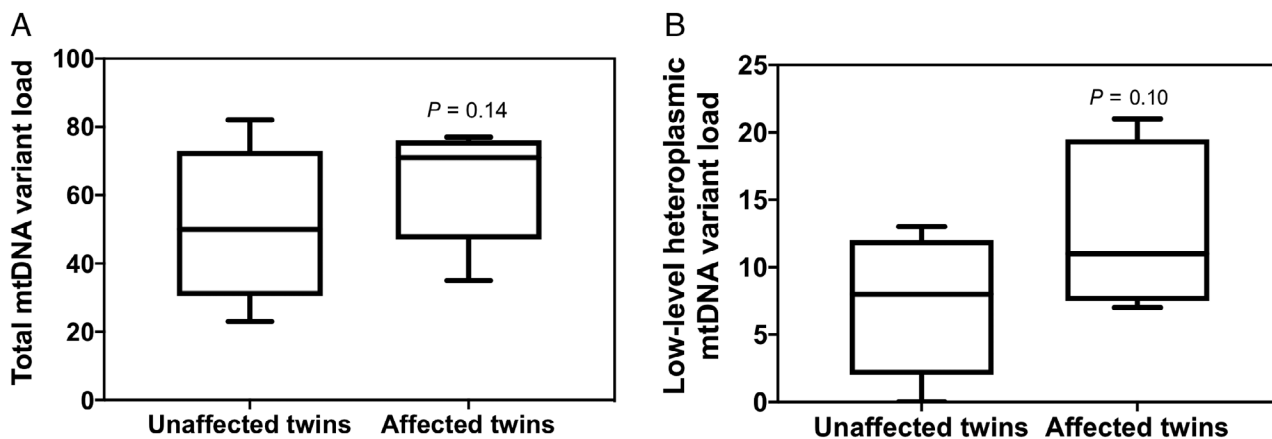


FIGURE 1: Mitochondrial DNA (mtDNA) variants in the affected vs. unaffected twins. The number of total (homoplasmic and heteroplasmic) (A) and low-level heteroplasmic (frequency 1–15%) variants (B) mtDNA variants in the groups of unaffected and affected twins is shown. The p value refers to Wilcoxon signed rank test.

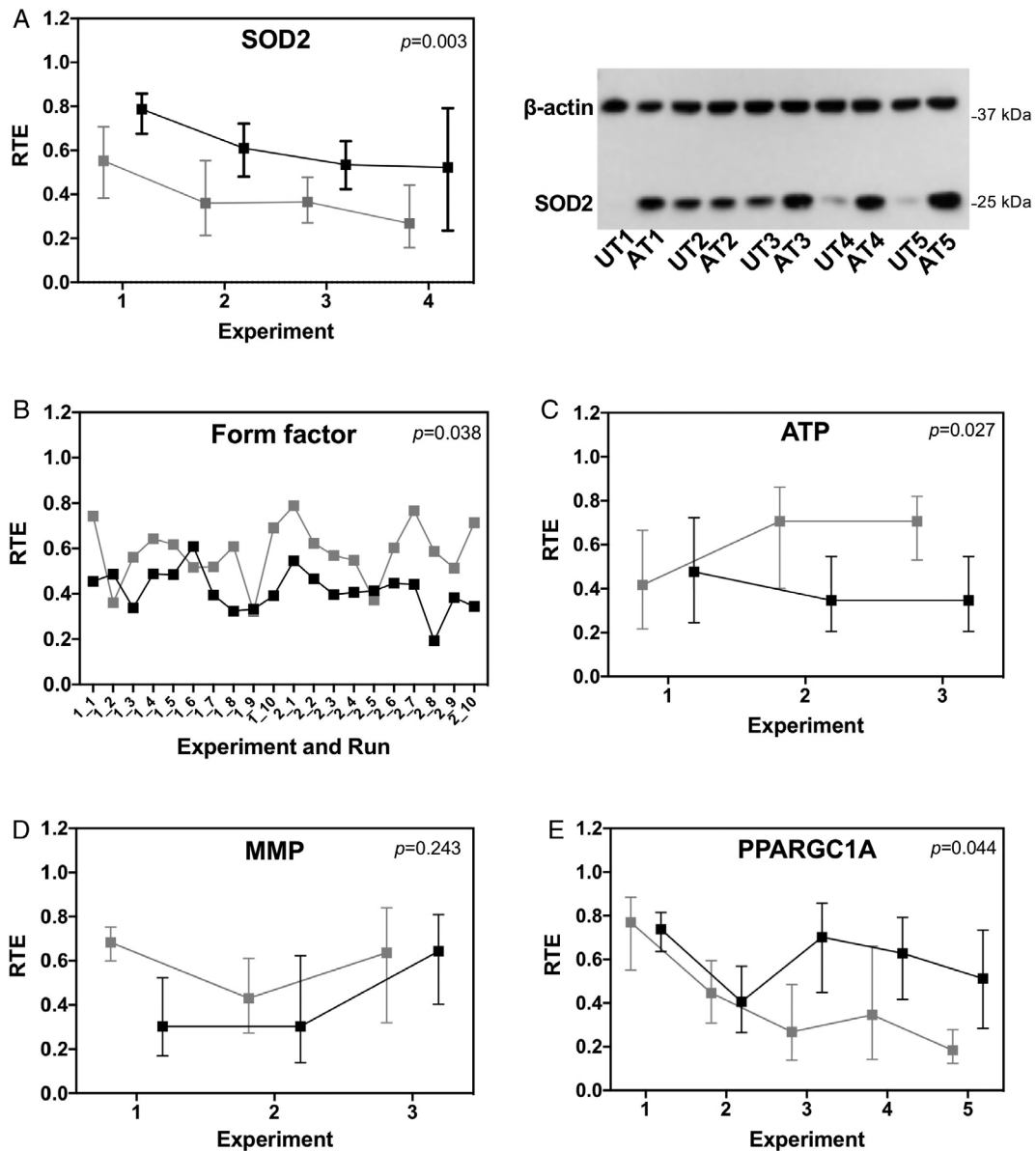


FIGURE 2: Impaired mitochondrial integrity in the affected twins. The diagrams show the relative treatment effect (RTE) value and corresponding 95% confidence intervals for the affected (gray line) and unaffected (black line) twins for the different experiments/runs. Indicated p values refer to analysis of variance (ANOVA) test. (A) Increased levels of superoxide dismutase 2 (SOD2) in fibroblasts from affected monozygotic (MZ) twins were noticed in all 4 respective investigations (left panel). Representative Western blot of SOD2 protein levels in the unaffected twin (UT) and affected twin (AT) pairs; β -actin served as loading control and was used for normalization (right panel). (B) Altered mitochondrial network in fibroblasts from affected MZ twins. The form factor as a measure for mitochondrial network was calculated for the groups of unaffected and affected twins (RTE values for 10 cells [Experiment] from two independent stainings [Run] are indicated). (C) Decreased intracellular adenosine triphosphate (ATP) levels in fibroblasts from affected MZ twins. ATP concentration (μ moles) was calculated per minute (min) per milligram (mg) of protein in the groups of unaffected and affected twins in 3 independent experiments. (D) Mitochondrial membrane potential (MMP) analysis in fibroblasts from MZ twins discordant for PD. MMP was quantified by flow cytometry as a number of tetramethylrhodamine methyl ester perchlorate (TMRM)-positive cells normalized to Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)-treated cells in the groups of unaffected and affected twins in 3 independent experiments. (E) Increased levels of Peroxisome proliferator-activated receptor-gamma coactivator- α (PPARGC1A) messenger RNA (mRNA) in fibroblasts from affected MZ twins. PPARGC1A mRNA levels were quantified by real-time polymerase chain reaction (PCR) in fibroblasts from unaffected and affected twins. The results are based on the mean ratios of PPARGC1A expression compared to 2 reference genes ACTB and G6PD in 5 independent experiments.

protein levels of mitochondrial antioxidant enzyme SOD2 in each AT compared to the UT (). We observed an increase in protein levels for the group comparison (descriptive $p = 0.003$

[ANOVA]; RTE [95% CI]: affected = 0.61 [0.45–0.75]; unaffected = 0.39 [0.26–0.55]; Fig 2A) underlining ongoing mitochondrial stress in the ATs. Additionally, we observed a

decreased mitochondrial form factor in all ATs compared with the UTs indicating a diminished mitochondrial network (; descriptive $p = 0.038$ [ANOVA]; RTE [95% CI]: affected = 0.42 [0.22–0.64]; unaffected = 0.58 [0.37–0.76]; Fig 2B). Further, we detected reduced cellular ATP levels (descriptive $p = 0.027$ [ANOVA]; RTE [95% CI]: affected = 0.39 [0.22–0.61]; unaffected = 0.61 [0.38–0.78]) in the group of ATs compared to the group of UTs (Fig 2C), which was found in all twin pairs but #3 (). The decrease in ATP levels was accompanied by a seemingly decreased MMP in all twin pairs but #2 (); although there was no visible difference for the group of ATs vs. UTs (descriptive $p = 0.243$ [ANOVA]; RTE [95%CI]: affected = 0.42 [0.24–0.65]; unaffected = 0.58 [0.40–0.73]; Fig 2D). Reduced ATP levels and decreased MMP may correspond to impaired mitochondrial function. Finally, gene expression levels of *PPARGCIA* were higher in all ATs compared with the UTs (; descriptive $p = 0.044$ [ANOVA]; RTE [95% CI]: affected = 0.60 [0.41–0.75]; unaffected = 0.40 [0.25–0.58]; Fig 2E) indicating induced mitochondrial biogenesis.

Discussion

Genetic predisposition has proven to be an important element in PD etiology.^{14,15} Nonetheless, MZ twins seemingly displayed a discordance of 80% in developing full-blown PD.¹ This demonstrates that PD is moderately heritable, in agreement with observations that familial PD cases are relatively uncommon and that monogenic forms of PD have reduced penetrance.¹⁶

This study examined skin fibroblast cultures from 5 pairs of MZ twins discordant for PD to elucidate molecular reflections of the discordance. The value of fibroblast cultures in PD research has been demonstrated in previous reports showing that familial or idiopathic PD patient-derived fibroblast cultures have impaired mitochondrial function, altered morphology, and decreased mitophagy.^{6,17–19} In addition, analysis of whole-transcriptome changes in fibroblast cultures obtained from 3 MZ twin pairs with PD suggests that fibroblasts can be considered as a cellular model to study PD pathogenesis in this specific cellular model.²⁰ Moreover, a recently published study showed differential accumulation of α -synuclein oligomers in skin samples of MZ twins discordant for PD.²¹

In this study, we searched for genetic differences in the nuclear and mitochondrial genome and comprehensively investigated mitochondrial integrity and function. Despite panel and nuclear genome sequencing, a genetic cause of PD could not be identified in any of our patients, neither in the form of a pathogenic variant being present in both twins (ie, showing reduced penetrance in the UT) nor as a variant exclusively being present in the AT (ie, *de*

novo variant). To unravel alternative differences in the genetic makeup within the twin pairs, we also looked for mtDNA variants. Although we did not detect any high frequency (inherited) differences, we may have observed a trend for an increased number of low-frequency mtDNA variants in the twins with PD compared with the UTs. Such low-frequency variants are most likely the result of somatic mutations. Unfortunately, parents of the twins were not available for any genetic investigation. In contrast, a study on mtDNA-encoded genes of complex I and tRNAs in 4 pairs of MZ twins discordant for PD did not report mtDNA sequence differences.²² However, the authors did not perform deep-sequencing analyses and thus were not able to detect low-frequency variants.

To our knowledge, we demonstrated for the first time that the degree of mitochondrial functionality matches the affected status in the discordant MZ twins. We observed reduced cellular ATP levels, altered mitochondrial network, and elevated SOD2 protein levels in fibroblast cultures from the ATs compared with the UTs. Additionally, increased expression of *PPARGCIA* encoding the mitochondrial biogenesis master regulator PGC-1 α may suggest a compensatory mechanism to counteract the functional deficit.

Although our results show an association between mitochondrial phenotype and current clinical status of MZ twins discordant for PD, the answer to the question whether the observed mitochondrial alterations are the cause of the disease or whether it is itself a consequence of dysfunction in other pathways remains elusive. In the absence of identifiable genetic causes/differences, the discordance may be either based on a random process (ie, spontaneously occurring mitochondrial dysfunction leading to self-enhancing alterations and disease, potentially linked to somatic variants in respective brain regions) or be related to other nongenetic factors, such as environmental insults. However, in our 5 twin pairs, discordance cannot easily be explained by known environmental risk factors. None of the twins were known to have worked with pesticides and 4 twin pairs were concordant for nonsmoking (#2, #4, and #5) or smoking at comparable intensities (#3). Only in one twin pair, the AT did smoke whereas the unaffected co-twin was not exposed to smoking (#1). Although a definitive answer to the question if mitochondrial dysfunction is cause or consequence remains elusive, it is clear that mitochondrial dysfunction has an important role in the cascade of changes that leads to PD.

In conclusion, we here demonstrate that subtle molecular changes of the mitochondrial integrity reflect the clinical status and that somatic mtDNA variants might act as modifiers or biomarkers of disease manifestation.

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Author Contributions

M.D.M., I.R.K., J.T., C.K., and K.L. contributed to the conception and design of the study. M.D.M., I.R.K., J.T., S.H.D., P.U., E.K., N.K., L.C.I., F.H., J.W., P.K., A.B., S.D., T.V., A.G., C.K., P.S., and K.L. contributed to the acquisition and analysis of data. M.D.M., I.R.K., J.T., and K.L. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interests

The authors declared no conflict of interest.

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