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## RESEARCH ARTICLE

# Platelet mitochondrial membrane potential in Parkinson's disease

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## Abstract

**Objective:** Mitochondrial dysfunction is a hallmark of idiopathic Parkinson's disease (IPD), which has been reported not to be restricted to striatal neurons. However, studies that analyzed mitochondrial function at the level of selected enzymatic activities in peripheral tissues have produced conflicting data. We considered the electron transport chain as a complex system with mitochondrial membrane potential as an integrative indicator for mitochondrial fitness. **Methods:** Twenty-five IPD patients (nine females; mean disease duration, 6.2 years) and 16 healthy age-matched controls (12 females) were recruited. Live platelets were purified using magnetic-activated cell sorting (MACS) and single-cell data on mitochondrial membrane potential ( $\Delta\psi$ ) were measured by cytometry and challenged with a protonophore agent. **Results:** Functional mitochondrial membrane potential was detected in all participants. The challenge test reduced the membrane potential in all IPD patients and controls ( $P < 0.001$ ). However, the response to the challenge was not significantly different between patients and controls. **Interpretation:** While the reported protonophore challenge assay is a valid marker of overall mitochondrial function in live platelets, intact mitochondrial membrane potential in platelets derived from IPD patients suggests that presumed mitochondrial enzymatic deficiencies are compensable in this cell type. In consequence, mitochondrial membrane potential in platelets cannot be used as a diagnostic biomarker for nonstratified IPD but should be further explored in potential Parkinson's disease subtypes and tissues with higher energy demands.

## Introduction

In idiopathic Parkinson's disease (IPD), cellular dysfunction is not limited to nigrostriatal cells. Mitochondrial dysfunction has been described in both neurons and non-neuronal cells.<sup>1</sup> It could potentially serve as biomarker for early diagnosis and patient stratification. However, translational studies on mitochondrial dysfunction are limited to readily available tissues such as blood components. Direct brain tissue measurements are excluded. Extensive reports have been published on PD-associated mitochondrial dysfunction in platelets.<sup>2–6</sup>

These reports used biochemical in vitro methods to compare the enzymatic activity of mitochondrial complexes between patients and controls. However, in vivo validation of these findings is missing and since the early 1990s the findings of defects in platelet mitochondrial activity remain controversial and without clinical application.

In this work, we investigate whether mitochondrial membrane potential ( $\Delta\psi$ ) in live platelets can be used as a biomarker for IPD. In contrast to complex-specific measurements of enzymatic activities,  $\Delta\psi$  provides functional assessment of overall mitochondrial fitness.

## Methods

### Subjects and clinical tests

The study was performed with written consent of all participants and after approval by the Luxembourg National Research Ethics Committee (CNER). Nondemented IPD patients and healthy age-matched controls without clinical evidence for neurodegenerative disorders were recruited within a prospective study on mitochondrial dynamics. IPD diagnosis was established following the London Brain Bank criteria.<sup>7</sup> All subjects underwent the following clinical tests, with the patients being on their usual antiparkinsonian medication: the Hoehn and Yahr scale,<sup>8</sup> the motor part of the Unified Parkinson Disease Rating Scale (UPDRSIII),<sup>9,10</sup> and the Mini-Mental State Examination (MMSE).<sup>11</sup> Fresh whole blood was collected in EDTA tubes and kept at room temperature. The samples were annotated with an anonymous but traceable barcode. Investigators performing downstream workflows such as platelet purification and measurements of mitochondrial membrane potential were blinded for the clinical identity of the samples.

### Purification of platelets

For the purification of platelets, 200  $\mu\text{L}$  of CD61 microbeads (130-051-101; Miltenyi Biotec, Leiden, The Netherlands) were mixed with 800  $\mu\text{L}$  of EDTA blood and incubated for 15 min at room temperature. The samples were diluted with 4 mL of 0.2  $\mu\text{m}$  filtered platelet buffer (PBS, 0.5% BSA, 2 mmol/L EDTA, pH 7.2) at room temperature. Magnetic sorting was run on an AutoMACS (Miltenyi Biotec), using AutoMACS columns (130-021-101; Miltenyi Biotec), running buffer (130-091-221; Miltenyi Biotec), and washing buffer (130-092-987; Miltenyi Biotec). The purified platelets were quantified on a CASY cell counter (Roche Applied Science, Penzberg, Germany), kept at room temperature, and prepared for the analysis of mitochondrial membrane potential.

### Mitochondrial staining and cytometry

Within the day of blood sampling mitochondrial membrane potential ( $\Delta\psi$ ) was analyzed by the nonquenching TMRM method.<sup>12</sup> One million platelets were collected in 4 mL DPBS (14190094; Invitrogen, Gent, Belgium) + 1% FBS (10500064; Invitrogen). From this master mix, three aliquots were prepared ( $3 \times 1 \text{ mL}$ ): one unstained control, and two samples stained with 10 or 20 nmol/L TMRM (T668; Invitrogen) as shown in the results. To equilibrate the level of TMRM in platelets and mitochondria,<sup>13</sup> the cells were incubated for 45 min. To remove

potential cell clumps, the purified platelets were filtered through a 40  $\mu\text{m}$  filter (352340; BD Falcon, Erembodegem, Belgium) before starting the measurements. One of the stained samples was used as a reference measurement and the other one was used for an FCCP challenge test as described in the next section. The intensity of TMRM fluorescence was measured via cytometry. Each data point corresponds to the total TMRM signal of one platelet.

Cytometry measurements were performed using a BD LSR Fortessa cytometer. The total number of events to be measured for each sample was set to 10,000. Forward scatter (FSC) and side scatter (SSC) signals were used to gate the platelets by size and complexity, respectively. For statistics the median forward scatter (MedFSC) and median side scatter (MedSSC) were used. TMRM fluorescence was excited with a 561-nm laser and detected behind a 582/15 bandwidth filter.

### Mitochondrial challenge

To dissipate mitochondrial membrane potential, cells were treated with the protonophore FCCP. Challenge with 5  $\mu\text{mol/L}$  FCCP started after 45 min TMRM staining.

### Statistical analysis

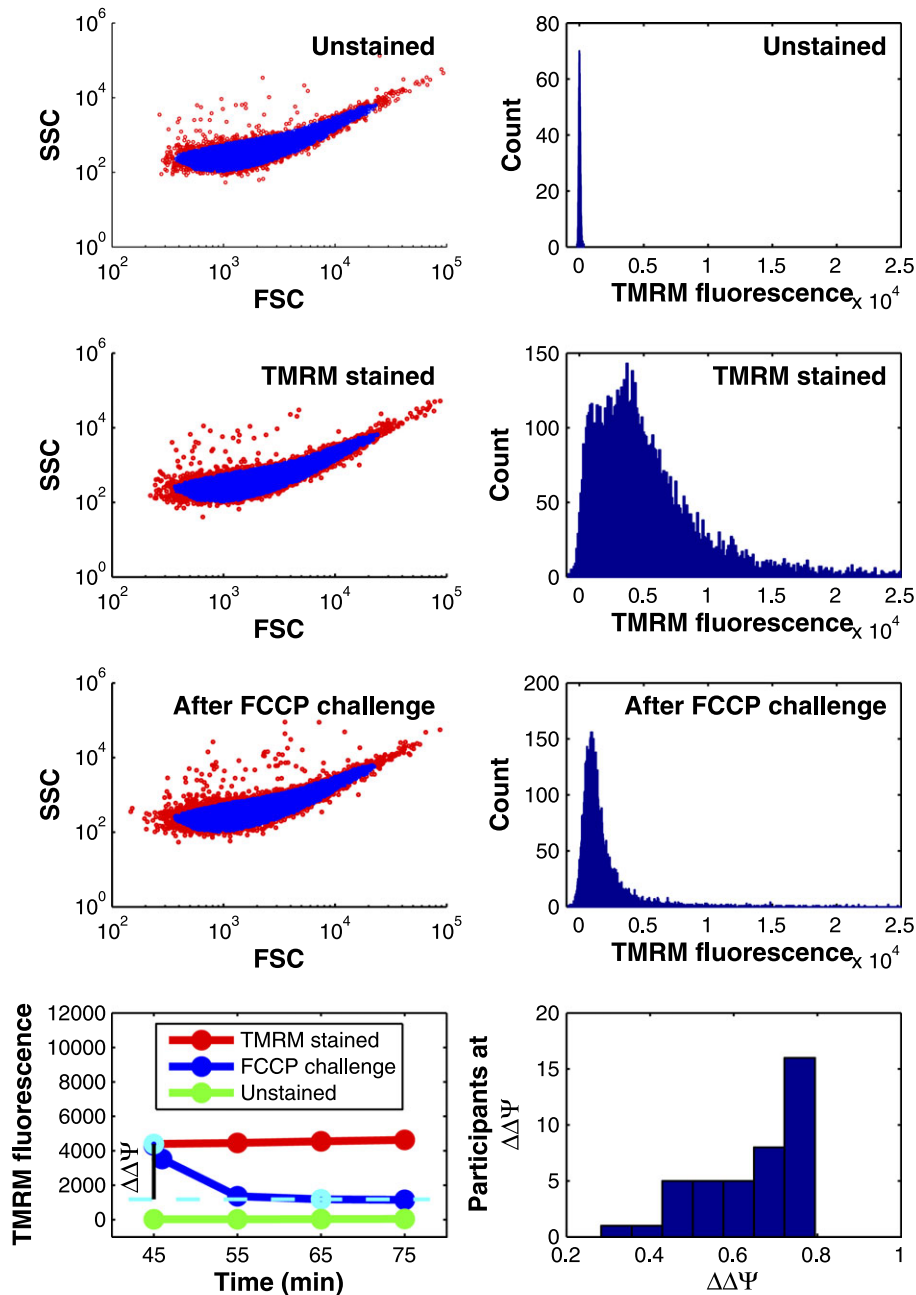
Single-cell data from cytometry were imported in fcs format to Matlab version R2013A (The MathWorks Inc.).<sup>14</sup> The analysis of fluorescence intensity was restricted to cells with defined size and complexity. To define coherent boundaries for SSC and FSC, all data points of the study were cumulated in a scatter plot and manually gated using polygonal selection (Fig. 1).

To quantify the drop in TMRM fluorescence induced by an FCCP challenge, we defined the following metric:

$$\Delta\Delta\psi = 1 - \frac{\text{TMRM}_{65}}{\text{TMRM}_{45}}$$

where  $\text{TMRM}_{45}$  is the median fluorescence intensity after 45 min of TMRM staining, minus the median background fluorescence in unstained samples at that timepoint.  $\text{TMRM}_{65}$  is the median fluorescence intensity after additional 20 min of treatment with FCCP, minus the median background fluorescence at that timepoint. Consequently,  $\Delta\Delta\psi$  corresponds to the FCCP-induced fractional loss of TMRM fluorescence. Mitochondria with high membrane potential present a higher  $\Delta\Delta\psi$  than mitochondria with low membrane potential. The ratio normalizes against changes in total TMRM fluorescence.

To evaluate the impact of FCCP treatments on the level of TMRM fluorescence in individual samples, the fluores-



**Figure 1.** Analysis of mitochondrial membrane potential in live platelets: Individual example data set: scatter plots highlight gated cells in blue and the corresponding histograms show counts of gated cells at TMRM fluorescence intensity. The line plot at the bottom left shows the dynamics of TMRM fluorescence in the given data set. The reference coordinates for the calculation of  $\Delta\Delta\psi$  are highlighted in cyan and  $\Delta\Delta\psi$  which represents the fractional loss of TMRM fluorescence due to FCCP challenge is shown in black. The histogram at the bottom right shows the cumulated count of study participants with given  $\Delta\Delta\psi$  values. SSC, FCS, and TMRM fluorescence are shown in arbitrary units. TMRM, tetramethylrhodamine, methyl ester; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; SSC, side scatter.

cence intensities of reference cell populations stained for 45 min with TMRM were compared to fluorescence levels in cell populations which underwent a subsequent FCCP challenge for 20 min. The two-sided Wilcoxon rank sum test was used to test for equality of population medians.<sup>15</sup>

To test if the means of clinical- and cytometry-based features are equal between patients and controls, two-tailed two-sample Student *t*-tests, with  $\alpha = 0.05$ , assuming sampling from populations with equal variances, were used.

Correlations between pair-wise features including cytometry-based data as well as clinical metadata were calculated using the Pearson's linear correlation coefficient ( $r$ ). Correlations of the type  $0.5 < |r| < 1$  are considered as strong correlations, where  $|r|$  is the absolute value of the coefficient of correlation.

## Results

Twenty-five IPD patients (nine females; age: 55–80 years) with IPD and 16 healthy controls (12 females; age: 60–83 years) were recruited. Demographics and cytometry data are presented in Table 1.

For the analysis of mitochondrial membrane potential, the fractional loss of TMRM fluorescence after FCCP challenge ( $\Delta\Delta\psi$ ) was quantified. To evaluate the sensitivity of the established  $\Delta\Delta\psi$  assay for live platelets, both staining efficiency and sensitivity for a loss in mitochondrial membrane potential were analyzed. The consistently and significantly increased fluorescence of TMRM-stained platelets compared to unstained cells, with  $P < 0.001$  for each participant, shows that MACS purified platelets are able to take up TMRM and hence present internal negatively charged compartments.<sup>13</sup> The consistent and significant drop of TMRM fluorescence after treatments with the protonophore FCCP, with  $P < 0.001$  and  $0.2 < \Delta\Delta\psi < 0.8$  for each study participant (Fig. 1), demonstrates the presence of detectable levels of mitochondrial membrane potential in all samples. Because treatment with 5  $\mu\text{mol/L}$  FCCP completely uncouples respiration, high  $\Delta\Delta\psi$  values indicate a high original mitochondrial membrane potential.

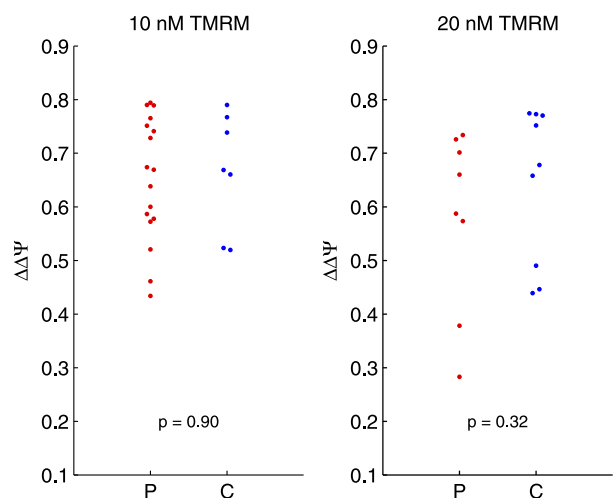
The results of FCCP challenge tests did not significantly differ between patients and controls (Fig. 2). Platelets from a first subcohort with 17 IPD patients and seven controls have been analyzed with a 10 nmol/L-TMRM protocol and no significant differences in  $\Delta\Delta\psi$  were found (Fig. 2). The negative skewness, a measure of distribution asymmetry, of  $-0.40$  for patients and  $-0.39$  for controls shows that the distributions are stretched toward low  $\Delta\Delta\psi$  values and most individuals present a high  $\Delta\Delta\psi$ . To validate this result, platelets from a second group of study participants including eight patients and nine controls were analyzed with a 20 nmol/L-TMRM protocol. Here, the absence of significant differences between patients and controls was confirmed. The skewness of  $-0.84$  for patients and  $-0.50$  for controls confirms the distribution asymmetry with a tail toward low  $\Delta\Delta\psi$  and higher density at high  $\Delta\Delta\psi$  (Fig. 2). The controls were matched for age but not for gender (Table 1). However, there was no significant difference in  $\Delta\Delta\psi$  between males and females ( $P = 0.51$ ). Interestingly, each subcohort presents an at least marginally stronger skewness in patients than in controls. Of note, the two participants with the lowest  $\Delta\Delta\psi$  in each subcohort were IPD patients (Fig. 2). The analysis of cell size and complexity showed no significant differences between patients and controls (Table 1).

The analysis of correlation within the whole study cohort shows strong correlations between the size and complexity of platelets, and, as expected, between disease progression and the progression of motor phenotypes (Hoehn Yahr and UPDRS). Strong negative correlations were found between these disease progression features

**Table 1.** Demographics and cytometry data.

	Parkinson's disease	Control	<i>P</i> value
Demographics			
Gender distribution (M:F)	25 (16:9)	16 (4:12)	0.025
Age	65.9 $\pm$ 5.8 (55–80)	66.9 $\pm$ 6.9 (60–83)	0.62
Clinical data			
Hoehn Yahr	1.5 $\pm$ 0.6 (1–2.5)	0 $\pm$ 0 (0–0)	3.80E-13
UPDRSIII	12.0 $\pm$ 6.1 (5–27)	0.9 $\pm$ 1.3 (0–3)	1.27E-08
MMSE	28.6 $\pm$ 1.4 (25–30)	28.9 $\pm$ 1.5 (26–30)	0.54
Schwab England (%)	85 $\pm$ 10% (60–100%)	100 $\pm$ 0% (100–100%)	1.02E-06
Disease duration (years)	6.2 $\pm$ 6.0 (1–25)	NA	NA
Cytometry data			
Duration (min)	352 $\pm$ 49 (257–446)	343 $\pm$ 48 (276–438)	0.57
MedFSC	1787 $\pm$ 238 (1446–2297)	1769 $\pm$ 200 (1294–2107)	0.81
MedSSC	337 $\pm$ 24 (300–398)	347 $\pm$ 24 (298–391)	0.19
$\Delta\Delta\psi$	0.63 $\pm$ 0.14 (0.28–0.79)	0.65 $\pm$ 0.13 (0.44–0.79)	0.58

The demographics on gender distribution are given in the format "participants (males: females)." The remaining statistics are presented in the format "mean  $\pm$  standard deviation (minimum–maximum)." The *P* value for gender distribution was computed via Fisher's exact test. All other *P* values were calculated using Student *t*-tests. The duration in the cytometry section corresponds to the duration between blood sampling and cytometry measurement. NA, not applicable; MedSSC, median side scatter; MedFSC, median forward scatter.

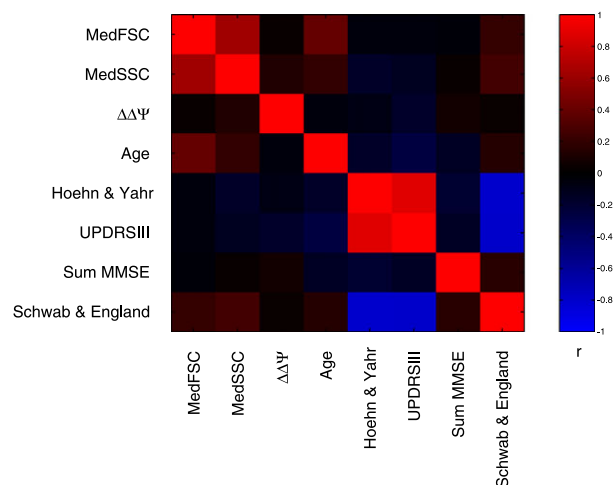


**Figure 2.** Mitochondrial membrane potential in platelets is intact in both, Parkinson's disease patients and controls: The study cohort was split in two groups. In a first group, platelets were stained with 10 nmol/L-TMRM (left plot) and no significant changes between patients (red, P) and controls (blue, C) were found. In the second group of participants (right plot), this result was validated using 20 nmol/L-TMRM. The *P* values from two-sided Wilcoxon rank sum tests are shown at the bottom of each plot. Furthermore, there were no significant differences between the 10 and 20 nmol/L-TMRM subcohorts ( $P_{\text{patients}} = 0.28$  and  $P_{\text{controls}} = 0.84$ ). TMRM, tetramethylrhodamine, methyl ester.

and individual levels of independence (Schwab & England).  $\Delta\Delta\psi$  does not correlate strongly with any clinical parameter (Fig. 3).

## Discussion

Since the 1980s, mitochondrial dysfunction in platelets has been vividly debated as a potential biomarker for IPD.<sup>16–18</sup> Parker et al. reported a 55% reduced complex I activity in enriched mitochondria preparations from parkinsonian platelets.<sup>2</sup> Benecke et al. concluded on a similar downregulation of complex I activity by about 50%,<sup>5</sup> via the analysis of NADH oxidation, which is catalyzed by complex I. Krige et al.<sup>3</sup> who worked with a duration-optimized protocol for the enrichment of platelet mitochondria reported a 16% reduced activity of complex I in IPD patients. Other authors did not find differences in complex I activity between patients and controls by analyzing platelet homogenates or platelets obtained by apheresis.<sup>6,18</sup> These contradictory results were probably due to differences in sample preparation and analytical methodology. The quantification of enzymatic activity within the electron transport chain was based on biochemical assays requiring prior isolation of intact enzymes extracted from blood, isolated platelets, or isolated platelet mitochondria.



**Figure 3.** Analysis of correlation: no strong correlations were found between  $\Delta\Delta\psi$  and demographic-, clinical-, or cytometry-based features. Coefficients of correlation (*r*) are color-coded according to the colormap at the right.

Resulting differences in the level of platelet and enzyme purity, and in the degree of enzymatic activity loss, due to the use of different methods have a high impact on obtained results. While the biochemical finding of reduced complex I activity in platelets was reported in the studies with the most efficient extraction methods for functionally active enzymes,<sup>2–4</sup> significance levels were not reached with less sophisticated protocols.<sup>18,19</sup>

In comparison with former studies focusing on enzymatic activities, the present approach has several advantages: the cellular environment of the mitochondria is maintained, the volume of the required blood samples is reduced, and cytometric single-cell data are available. Furthermore, MACS reduces the risk for inadvertently induced platelet activation as it does not require sample centrifugation such as apheresis. MACS also involves several washing steps, separates the platelets from potential blood-derived coagulation-promoting factors, and hence further reduces the risk for platelet activation during cell sorting. This centrifugation-free purification method provides sufficient amounts of intact platelets for the cytometry-based analysis of  $\Delta\psi$ . The reproducible response to FCCP challenge tests both in patients and controls shows that the MACS purified platelets carry active mitochondria and that the introduced metric  $\Delta\Delta\psi$  permits the study of mitochondrial activity in live platelets. While measurements of mitochondrial membrane potential in live platelets have already been reported in the context of a platelet activation study.<sup>20</sup> A novelty of the present study is the use of an FCCP challenge test in TMRM-stained live platelets. This method allows to study the dynamics of  $\Delta\psi$  under normalized conditions. Based on

the findings presented above, our study demonstrates the presence of intact mitochondrial membrane potential in platelets from IPD patients and excludes quantitative measurements of mitochondrial membrane potential in live platelets as diagnostic biomarker for IPD in general. However, different subtypes of IPD might still present different levels of mitochondrial dysfunction. Thus, the observation that those participants with the lowest  $\Delta\Delta\psi$  values have been IPD patients allows to speculate on a yet undefined IPD subtype with an especially severe mitochondrial phenotype.

As our findings are at first look in contradiction with previous findings on enzymatic defects in oxidative phosphorylation, compensatory mechanisms, able to re-establish intact  $\Delta\psi$ , have to be discussed. Reduced activity of complex I could be compensated by an increased activity of complex II.<sup>21</sup> Compensation of complex I dysfunction through increased activity in more distal complexes has also been seen in OPA1-linked autosomal dominant optic atrophy.<sup>21</sup> Increased mitochondrial NADH levels could also compensate complex I deficiency.<sup>22</sup> Finally, it should be emphasized that IPD patients lack any clinical signs suggestive of platelet dysfunction, an observation well in line with the present findings.

Cell types with higher energetic needs such as dopaminergic neurons of the substantia nigra might fail to compensate mitochondrial deficits. First, it has been reported that complex I and I + III activity in platelets does not correlate with cerebral oxidative mitochondrial metabolism.<sup>23</sup> Second, there are major physiological differences between platelets and neuronal cell types, which are typically affected in IPD. While both, platelets and neurons are post-mitotic cells, platelets have a life expectancy of a week and neurons a life expectancy comparable to the life expectancy of humans. Energetic activity patterns are also different: platelets are small cells with a diameter in the order of a few micrometers, permitting only a few mitochondria per cell. Increased energetic needs in platelets have been described exclusively in the context of platelet activation,<sup>20</sup> which has been carefully avoided in the present setting. In contrast, neurons affected in IPD have substantially higher energetic needs. Cellular features requiring high energy demands include the generation of autonomous action potentials, poor myelination, and long and highly branched axons.<sup>24</sup> Finally, the reported levels of complex I deficiency in substantia nigra outreach the reported levels of complex I deficiency in platelets.<sup>16,18,25</sup>

The study has several limitations. We have recruited a relatively small number of patients, mostly at a middle stage of the disease. Thus, the observed difference in skewness between patient and control groups might be coincidental and we cannot exclude that significant deficits in  $\Delta\Delta\psi$  may be seen in more advanced stages of

the disease. All samples were derived from treated IPD patients and a compensatory effect of the dopaminergic treatment on  $\Delta\Delta\psi$  integrity cannot be excluded. Furthermore, flow cytometry quantifies whole-cell fluorescence ( $F_{WC}$ ), corresponding to the sum of mitochondrial fluorescence ( $F_M$ ) and cytoplasmic fluorescence ( $F_C$ ). In cells with polarized mitochondria, the ratio of  $F_M/F_C$  is very high, at least in nonquenching mode, and therefore,  $F_{WC}$  is accepted as satisfactory surrogate for  $F_M$ .<sup>12</sup> After release from mitochondria TMRM rapidly redistributes across the plasma membrane, especially in cells with a high surface-to-volume ratio.<sup>12,26</sup> The fluorescence remaining after equilibration is in dependence of the plasma membrane potential (Fig. 1). This cytoplasmic background according to the Nernst equation is perfectly compatible with the  $\Delta\Delta\psi$  method when assumed to be constant. However, possible changes in plasma membrane potential due to FCCP-induced effects including potential shortage in ATP are ignored. The ceiling effect observed in the  $\Delta\Delta\psi$  histogram (Fig. 1) indicates that the dynamic range of the assay corresponds to 80% of  $F_{WC}$ . Filtering during sample preparation could have removed very large platelet clumps and thereby mask potential differences in platelets that tend to clump. Finally, we did not evaluate complex I deficits in study participants. In consequence, the study cannot analyze the correlation between complex I activity and mitochondrial membrane potential.

In summary, this study introduces a new easily applicable and reproducible method to probe mitochondrial membrane potential in live platelets. While the present study excludes  $\Delta\Delta\psi$  in platelets as a diagnostic biomarker for IPD, it does not exclude the existence of IPD subtypes with characteristic  $\Delta\Delta\psi$  boundaries. Future applications of the  $\Delta\Delta\psi$  method in larger cohorts, possibly at a later stage of the disease or before and during medication trials should be envisioned. In order to gain full understanding of the hypothesized cell type-specific mitochondrial robustness in IPD, studies on other cell types such as fibroblasts and neurons derived from autonomic ganglia in skin or colon are also needed.<sup>27</sup>

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## Author Contributions

Paul M. A. Antony supervised the experimental work and wrote this manuscript. Olga Boyd performed cytometry experiments. Christophe Trefois and Paul M. A. Antony developed the Matlab script for the statistical analysis. Wim Ammerlaan was responsible for MACS. Marek

Ostaszewski, Aidos S. Baumuratov, and Laurent Antunes coordinated the cooperation between the involved institutions. Laura Longhino performed the clinical tests. Werner Koopman consulted the interpretation of mitochondria-related data and revised the manuscript. Rudi Balling enabled the interinstitutional cooperation and revised the manuscript. Nico Diederich designed the project and revised the manuscript.

## Conflict of Interest

None declared.

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