RESEARCH ARTICLE

Peripheral Mitochondrial Function Correlates With Clinical Severity in Idiopathic Parkinson's Disease

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ABSTRACT: Background: Parkinson's disease is an intractable disorder with heterogeneous clinical presentation that may reflect different underlying pathogenic mechanisms. Surrogate indicators of pathogenic processes correlating with clinical measures may assist in better patient stratification. Mitochondrial function, which is impaired in and central to PD pathogenesis, may represent one such surrogate indicator.

Methods: Mitochondrial function was assessed by respirometry experiment in fibroblasts derived from idiopathic patients (n = 47) in normal conditions and in experimental settings that do not permit glycolysis and therefore force energy production through mitochondrial function. Respiratory parameters and clinical measures were correlated with bivariate analysis. Machine-learning-based classification and regression trees were used to classify patients on the basis of biochemical and clinical measures. The effects of mitochondrial respiration on α-synuclein stress were assessed monitoring the protein phosphorylation in permitting versus restrictive glycolysis conditions.

Results: Bioenergetic properties in peripheral fibroblasts correlate with clinical measures in idiopathic patients, and the correlation is stronger with predominantly non-dopaminergic signs. Bioenergetic analysis under metabolic stress, in which energy is produced solely by mitochondria, shows that patients' fibroblasts can augment respiration, therefore indicating that mitochondrial defects are reversible. Forcing energy production through mitochondria, however, favors α-synuclein stress in different cellular experimental systems. Machine-learning-based classification identified different groups of patients in which increasing disease severity parallels higher mitochondrial respiration.

Conclusion: The suppression of mitochondrial activity in PD may be an adaptive strategy to cope with concomitant pathogenic factors. Moreover, mitochondrial measures in fibroblasts are potential peripheral biomarkers to follow disease progression. © 2019 The Authors. *Movement Disorders* published by Wiley Periodicals, Inc. on behalf of International Parkinson and Movement Disorder Society.

Key Words: clinical phenotyping; mitochondria; Parkinson's disease; α-synuclein

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Parkinson's disease (PD) is a multisystem disorder characterized by a broad spectrum of motor (stiffness, slowness, tremor, gait and balance difficulties) and nonmotor (cognitive, psychiatric, sleep, alertness, autonomic) disturbances. The latter may antedate the motor symptoms, worsen as the disease advances, and predominate in causing disability during the later stages of the disease.[1] PD patients do not follow a uniform disease course, but exhibit conspicuous differences in primary disease-related and medication-induced complications as well as in the rate of progression of the disease, reflecting the existence of subtypes. In early PD, clinical characteristics are insufficient to identify subtypes.[2] There is, however, consensus that the age at onset of manifest disease is a major determinant of progression, with a more progressive course being associated with a higher age at onset.[3]

Better patient stratification is essential in designing clinical trials and may be achieved by integrating clinical data with quantitative biomarkers to best reflect the progression of the disease and its underlying biological pathophysiology. Furthermore, these biomarkers may have the potential to identify systems or persons at-risk before overt expression of the disorder and will allow earlier diagnosis and faster evaluation of clinical trials outcomes. Ideal surrogate measures reflect processes in a crucial causal pathway of the disease and correlate with the true clinical outcome.[4]

Dysfunction in mitochondrial oxidative phosphorylation (OXPHOS) has been linked to PD by multiple sources of converging evidence including genetic, toxicological, and epidemiological studies.[5-7] Mitochondrial complex I damage induces parkinsonism in humans and models the diseases in laboratory animals; moreover, mitochondrial defects are detectable in peripheral cells of genetic and idiopathic PD cases.[6,8-10] In addition, interventions targeting mitochondria ameliorate pathology in multiple animal models and improve respiration efficiency in patients' fibroblasts.[11-13] Collectively, these elements indicate that mitochondrial parameters might serve as an alternative outcome to complement clinical measures.

We performed bioenergetic characterization in primary fibroblasts from a highly characterized cohort of 47 PD patients to test the hypothesis that mitochondrial parameters correlate with clinical features and may therefore be informative of the clinical outcome. We applied statistical models and machine-learning procedures to describe the complex relationship between the different analyzed parameters and achieved unbiased grouping of patients on the basis of both clinical and laboratory measures. To fully expose the mitochondrial defects, we performed biochemical experiments under conditions of metabolic stress where the function of glycolysis—which could compensate and hide mitochondrial anomalies—is minimized. Finally, we explored detrimental synergies between bioenergetics and α-synuclein (α-syn) pathology in primary fibroblasts and differentiated neurons.

Materials and Methods

Patients

This cross-sectional study in PD patients is part of the Profiling Parkinson's Disease study. Patients were recruited from the outpatient clinic for Movement Disorders of the Department of Neurology of the Leiden University Medical Center (Leiden, the Netherlands) and nearby university and regional hospitals. All participants fulfilled the U.K. Parkinson's Disease Society Brain Bank criteria for idiopathic PD.[14] Evaluations occurred between January 2013 and January 2016. Exclusion criteria were previous or other disorders of the central nervous system, peripheral nerve disorders influencing motor and/or autonomic functioning, and psychiatric comorbidity not related to PD.

All patients except for 18 dopaminergic drug-naïve patients were tested while on dopaminergic medication. The severity of motor symptoms was quantified using the Movement Disorder Society version of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS) motor examination (part III).[15] In Addition, the Severity of predominantly Non-dopaminergic Symptoms in PD (SENS-PD) scale was administered, which is a composite score comprising 3 items with 4 response options (0-3) from each of the following six domains: postural instability and gait difficulty, psychotic symptoms, excessive daytime sleepiness, autonomic dysfunction, cognitive impairment, and depressive symptoms (total range 0-54).[1] These 6 domains represent a coherent complex of symptoms that largely do not improve with dopaminergic medication that is already present in the early disease stages and increases in severity when the disease advances.[16] Higher scores on both scales reflect more severe impairment. Cognitive performance was assessed using the Scales for Outcomes in Parkinson's Disease–Cognition (range 0-43), a valid and reliable instrument examining the following domains: memory, attention, executive functioning, and visuospatial functioning[17]; lower scores reflect more severe impairment. A levodopa dose equivalent of daily levodopa, dopamine agonists, and a total levodopa dose equivalent was calculated according to the formula developed by Tomlinson and colleagues.[18]

The study was approved by the medical ethics committee of the Leiden University Medical Center, and written informed consent was obtained from all PD patients.

Fibroblasts Cultures

PD patients' fibroblasts were prepared isolated at Leiden University Medical Center from skin biopsies derived from the ventral side of the upper leg and cultured under highly standardized conditions at 37°C and 5% Carbon dioxide (CO_2) up to a maximum of 10 passages. The number of passages was kept consistent within groups. Fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (P4333, Sigma-Aldrich) until

reaching confluency. Prior to Seahorse analysis, fibroblasts were rinsed with Phosphate-Buffered Saline (PBS) and cultured with glucose (glucose 10 mM, 10% FBS [F6178, Sigma-Aldrich], 2 mM glutamine, 5 mM Hepes, and 1% penicillin-streptomycin (P4333, Sigma-Aldrich, St. Louis, MI, USA) or galactose (galactose 10 mM, 10% FBS, 2 mM glutamine, 5 mM Hepes, and 1% penicillin-streptomycin) medium for 3 days. Fibroblasts from sex-matched controls of comparable age were either obtained from the Coriell biorepository (identification codes AG04659, AG05266, AG06283, AG07936, AG08152, AG08268, AG08269, AG08543, AG09162, AG09271, AG09879, AG12428, AG12951, AG13077, AG13348), Coriell Institute for Medical Research (Camden, New Jersey), or from the biorepository available in the Department of Molecular Genetics of the Erasmus Medical Center (MC), Rotterdam, The Netherlands.

Mitochondrial Respiration and Glycolysis Determination

Bioenergetics profiles of human primary skin fibroblasts were generated in real time with a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, California) as previously described.[8,12] Fibroblasts were seeded on a Seahorse XF-24 plate at a density of 6×10^4 cells per well and grown overnight in DMEM (10% of FBS and 1% Pen-Strep) at 37°C, 5% CO_2. This density ensures a proportional response to the uncoupler Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Carbonyl cyanide 4-[trifluoromethoxy]phenylhydrazone) with the cell number[8,12] and resulted in confluent cultures in which cell replication was further prevented by contact inhibition. In addition, no significant cell death occurred during the experiment, as indicated by the uniform, dense layer of adherent cells (Supplementary Fig. 1A). On the experimental day, the medium was changed to unbuffered DMEM (XF Assay Medium; Agilent Technologies) supplemented with 5 mM glucose and 1 mM sodium pyruvate and incubated for 1 hour at 37°C in the absence of CO_2. Medium and reagent acidity were adjusted to pH 7.4 on the day of the assay, according to manufacturer's procedure. After 4 baseline measurements for the oxygen consumption ratio, the cells were sequentially challenged with injections of mitochondrial toxins: 0.5 μM oligomycin (Adenosine triphosphate (ATP) synthase inhibitor), 1 μM FCCP (mitochondrial respiration uncoupler), 0.5 μM rotenone (complex I inhibitor), and 0.5 μM antimycin (complex III inhibitor).

A total of 3 Seahorse replicates were performed for each fibroblast line. In each replicate, we used 7 wells for each line. In each run, 6 wells were always used for a reference primary fibroblast line with highly characterized bioenergetics behavior. Because the behavior of this cell line in the Seahorse run is reproducible and ascertained, this additional precaution allows for double checking the quality of the Seahorse run. Data represent the mean of the different replicates.

Statistical Analysis

Statistical associations were determined using classical bivariate analysis: the Kruskall–Wallis test was used for comparisons of quantitative against categorical variables, the chi-square test was used for comparisons of categorical versus categorical variables, and the Spearman correlation coefficient was used for the comparison of quantitative versus quantitative variables. The significance level was set at .05.

Individual mitochondrial parameters were compared with the group means using 1-way ANOVA analysis of variance and Dunnett's test. A modified t test as described in Crawford and Howell[19] provided conceptually comparable results (data not shown).

Stratification was achieved using applied classification and regression trees (CART).[20] The rpart package[21] in R software[22] was used to fit data into CART, and the function rpart was used with the analysis of variance. All statistical analyses were performed in R version 3.3.2 (see also the Supporting Methods).

Results

Characterization of Mitochondrial Function in Permitting Versus Nonpermitting Glycolysis Conditions

Bioenergetics Analysis

PD presentation is highly heterogeneous and to be a realistic candidate as surrogate measure, mitochondrial function must consistently reflect such variability. We therefore assessed the extent of heterogeneity in peripheral mitochondrial activity by performing extensive bioenergetics analysis using a Seahorse Extracellular Flux Analyzer. We compared individual data of patients' fibroblasts to the average of a group of controls with comparable age and gender distribution (N = 21). To unravel the defects that are eventually masked in glycolysis-permitting conditions, we also performed the experiments in conditions in which glucose was replaced with galactose because the latter forces cells to rely on mitochondria for ATP production. Of note, these culturing conditions are lethal for fibroblasts from patients with mitochondrial pathologies.[23] Surprisingly, however, PD patients' fibroblasts perfectly survived in galactose medium, and no cell death was observed (data not shown).

The results exposed significant variability in both glucose and galactose-cultured specimens (Fig. 1B,C). As expected, forcing bioenergetics through oxidative metabolism (ie, galactose medium) unmasked anomalies, and several lines that did not exhibit alterations in glucose medium revealed differences in basal respiration when cultured with galactose (Fig. 1D,E). However, no changes were detected in reserve capacity (Fig. 1E). Galactose

ability to amplify bioenergetics differences was also confirmed when a more general analysis was performed at the group level, that is, pooled PD versus controls. Here, the reserve capacity in the galactose medium was the only significant difference detected (Supplementary Fig. 1B-D). Consistent with previous analyses on idiopathic PD[8] or Parkin mutant fibroblasts,[24] we did not observe any difference in basal or stimulated extracellular acidification rate in both glucose- or galactose-culturing conditions (Supplementary Fig. 1E).

Heterogeneity among PD specimens was also observed in parameters related to mitochondrial function such as mitochondrial superoxide production and ATP/ADP ratio (Fig. 1F).

PD Fibroblasts Can Augment Respiration in Conditions Forcing Metabolism Through Oxidative Phosphorylation

The adaptation of mitochondrial function to conditions that force bioenergetics through oxidative metabolism (ie, galactose medium) is reflected in the ratio between respiration parameters obtained in galactose and glucose conditions (galactose-to-glucose ratio).

In cells from healthy controls, the galactose medium mostly augmented respiration (ie, galactose-to-glucose ratio >1) and only 5 of 21 tested lines displayed reduced reserve capacity (Fig. 2B).

The galactose medium also altered the mitochondrial function in PD fibroblasts, albeit the magnitude of response was variable among the different lines (Fig. 2C). The direction of the changes observed in the patient specimens, however, was unexpected. In fact, given the mitochondrial defects intrinsic to PD, which are observed also peripherally, one would predict an inability to augment respiration and even lethality—that is, the opposite outcome than in control cells—as reported for typical mitochondrial disorders.[23] However, basal respiration, reserve capacity, and rotenone-sensitive respiration significantly increased in several PD fibroblast lines, whereas only 1 of the analyzed PD specimens displayed a reduction, exclusively in reserve capacity (Fig. 2E). This evidence indicates that, rather than being irreversibly compromised, mitochondrial dysfunction in the fibroblasts of PD patients can be restored under certain metabolic conditions.

Overall, these results indicate that analysis under metabolic challenge may amplify PD distinctive biochemical features and also demonstrate heterogeneity in peripheral PD specimens. The next logical question is whether variability in these parameters reflects and correlates with clinical manifestations.

Correlation Between Laboratory and Clinical Measures

We next examined whether the variability observed in respiration parameters might reflect clinical characteristics.

No significant correlations (r_s) were found between respirometry parameters and age, age at onset, disease duration, or levodopa equivalent doses; in addition, the severity of motor symptoms did not correlate with any of the laboratory parameters (Fig. 3A). However, the SENS-PD scores correlated with glucose reserve capacity ($r_s = 0.342$, $P = .026$), whereas the Scales for Outcomes in Parkinson's Disease–Cognition score displayed significant correlations with reserve capacity in the glucose medium ($r_s = -0.370$, $P = .017$) and rotenone-sensitive respiration ($r_s = -0.320$, $P = .041$) in the glucose medium (Fig. 4A,B). In addition, a correlation was found between the MDS UPDRS III and both mitochondrial superoxide and ATP/ADP levels determined in galactose ($r_s = 0.344$, $P = .026$ and $r_s = -0.337$, $P = .0292$, respectively); these correlation coefficients indicate that the higher symptom severity is associated with higher superoxide production and lower ATP/ADP levels. Association was not found when the cells were cultured in the glucose medium, further confirming the higher ability of galactose conditions to reveal PD-related differences.

Unbiased Grouping of Patients on the Basis of Laboratory and Clinical Measures

The heterogeneity in clinical presentation, the dispersion we observed in mitochondrial physiology, and their correlation lend support to the hypothesis that there may be subgroups of different mitochondrial phenotypes correlating with the symptomatology in the general population of idiopathic PD patients. To test this possibility, we used machine-learning methodology and recursive partitioning to build a CART,[25] which have been successfully used in applications such as clinical subtypes classification[26] and neuroimaging data analysis to predict Alzheimer's disease.[27]

All available parameters—that is, demographic variables (ie, age, age at onset, duration of the disease, and gender), the equivalent of levodopa medication, respirometry, and acidification parameters—were used in the CART process as input variables to predict either the SENS-PD or the MDS-UPDRS III (ie, response variables). Intrinsic to CART modeling is a selection step that eliminates in an unbiased fashion redundancy among the input variables to identify the most significant parameters.

When the CART analysis was applied using SENS-PD as a response variable, 3 rules—nodes 1, 2, and 3—grouped the cases in 4 classes (Fig. 3D). The first rule, node 1, identifies disease duration as a classifying variable and identifies a class of patients (class 4) with disease duration longer than 9.45 years presenting with the most severe symptoms. The second and third rules—nodes 2 and 3—respectively identify basal respiration in galactose and reserve capacity in a glucose

(A)

	Controls (mean±SD)	PD patients (mean±SD)
N (M/F)	21 (10/11)	47 (30/17)
Age (years)	68.8 ±9.9	65.3 ± 6.9
Disease duration (years)		5.9 ± 4.1
Levodopa equivalent doses (mg/day) n=29		975.2 ± 483.7
SENS PD		13.2 ± 8.0
MDSUPDRS III		32.8 ± 13.8
SCOPA-COG		28.2 ± 6.5

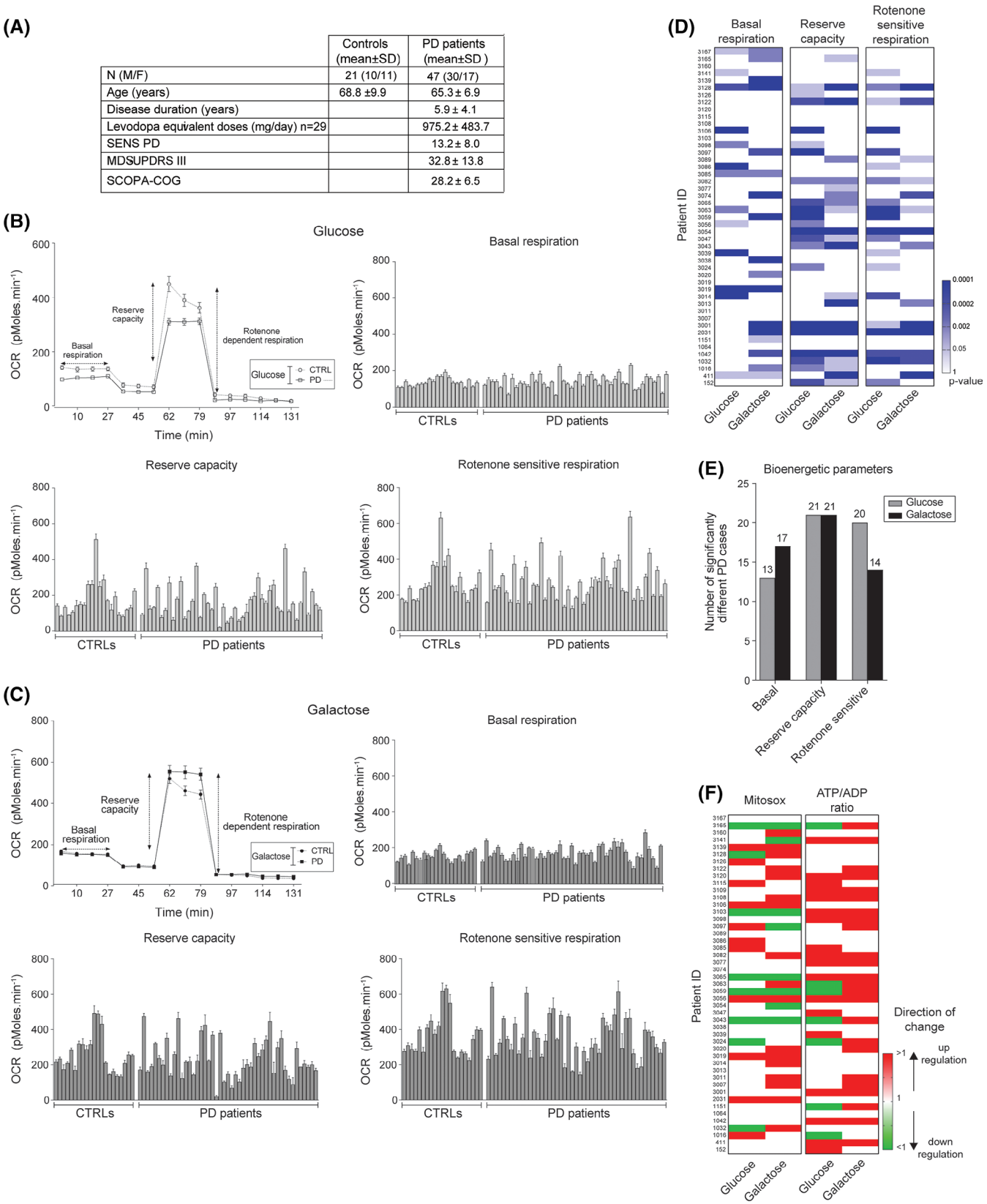


FIG. 1. (**A**) Clinical description of patients and controls included in this research. (**B**, **C**) Analysis of individual bioenergetics data highlights high variability in reserve capacity and in rotenone sensitive respiration in both glucose (**B**) and galactose (**C**) medium conditions. (**D**) Heat map plotting statistical significance values of the differences between individual patients' respiration data and the mean of healthy controls. Significance was determined using one-way analysis of variance and Dunnett's test. (**E**) Bar graph showing the number of patients with statistically significant difference in respiratory parameters. (**F**) Bar graphs and heat map illustrating variability in mitochondrial superoxide (Mitosox) production and ATP/ADP ratio in individual PD specimens when compared with the average of the control group (green = downregulation, red = upregulation; $P < .05$). CTRL, controls; ID, identification; f, female; m, male; OCR, oxygen consumption rate ; SCOPA-COG, Scales for Outcomes in Parkinson's Disease–Cognition.

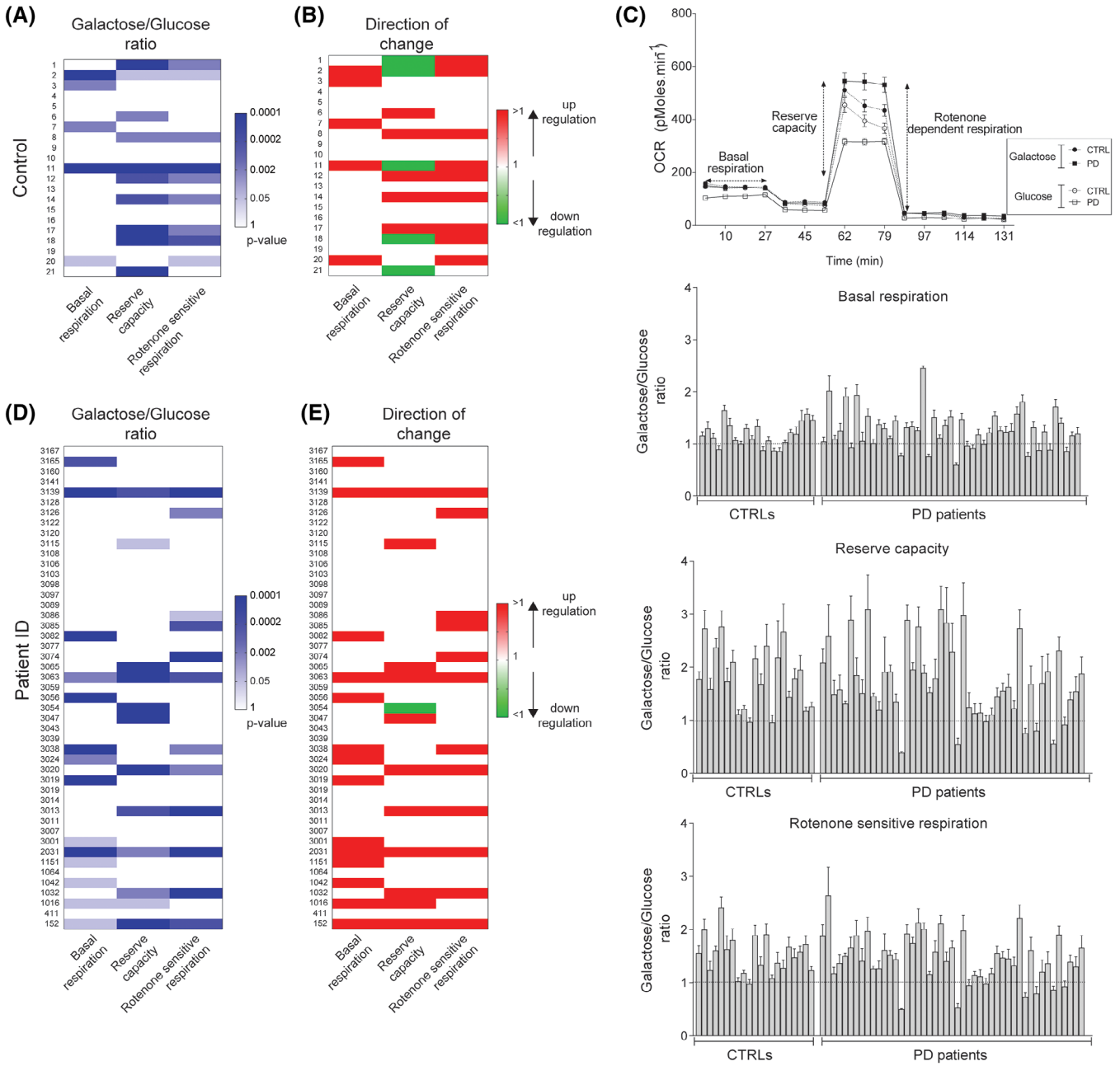


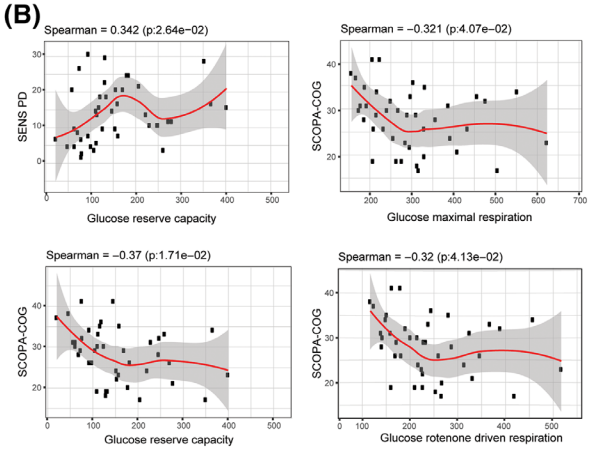
FIG. 2. Analysis of the galactose-to-glucose respiration ratio as an index of mitochondria alterations induced by conditions that do not permit glycolysis. (**A**) Replacing glucose with galactose alters respiration parameters in control cells. The heat map illustrates statistical significance values obtained testing the hypothesis that in control specimens the galactose-to-glucose respiration ratios are different than one, which would indicate altered respiration in non-permitting versus permitting glycolysis conditions. (**B**) Heat map illustrating the direction of the changes in the galactose-to-glucose ratios; red indicates a ratio higher than one, i.e. upregulation of respiration in galactose. The vast majority of control lines potentiates respiration when bioenergetics is forced through OXPHOS. (**C**) Representative Seahorse trace and histograms of respiratory parameters of individual bioenergetics data expressed as galactose over glucose ratio. (**D**) Heat map displaying statistical significance values obtained testing the hypothesis that in PD specimens the galactose-to-glucose respiration ratios are different than one. (**E**) Heat map illustrating the direction of the changes in the galactose-to-glucose ratios; red indicates a ratio higher than one, i.e. upregulation of respiration in galactose. The vast majority of PD lines augments respiration when bioenergetics is forced through OXPHOS. Significance was determined using one-way analysis of variance and Dunnett's tests. CTRL, controls; ID, identification; OCR, oxygen consumption rate.

medium as classifying variables and divide patients with disease duration shorter than 9.45 years into 3 further classes. Class 1 is defined by lower galactose basal respiration (<175.5 pmol/min^{-1}) and lower glucose reserve capacity (<112.5 pmol/min^{-1}) and includes patients with the least severe clinical presentation. Class 2 includes patients with lower galactose basal respiration, but higher glucose reserve capacity, and in class 3 both respiration parameters are higher. Presentation in these 2 classes is more severe than in class 1, with class 3 encompassing patients with worse symptoms. Overall, these data indicate that in patients with shorter

(A)

Multivariate analysis of variance		Age	Age at onset	Disease duration	Levodopa doses	MDS UPDRS	MDS UPDRS III	SENS PD	SCOPA COG
Glucose basal respiration	Correlation coefficient	-0.13	-0.15	0.03	0.06	-0.06	-0.06	0.03	0.03
	Sig (2-tailed)	0.413	0.331	0.862	0.718	0.734	0.696	0.846	0.829
Galactose basal respiration	Correlation coefficient	-0.04	-0.09	0.04	0.08	0.12	-0.08	0.18	-0.10
	Sig (2-tailed)	0.812	0.578	0.818	0.604	0.475	0.622	0.241	0.540
Glucose maximal respiration	Correlation coefficient	0.08	0.06	-0.04	-0.04	0.10	0.07	0.28	-0.32
	Sig (2-tailed)	0.611	0.702	0.813	0.800	0.562	0.657	0.070	0.041
Galactose maximal respiration	Correlation coefficient	0.06	0.10	-0.20	-0.08	0.25	0.20	0.18	-0.26
	Sig (2-tailed)	0.711	0.545	0.199	0.630	0.132	0.213	0.256	0.101
Glucose reserve capacity	Correlation coefficient	0.11	0.13	-0.10	-0.12	0.14	0.07	0.34	-0.37
	Sig (2-tailed)	0.477	0.415	0.512	0.448	0.415	0.644	0.026	0.017
Galactose reserve capacity	Correlation coefficient	0.02	0.10	-0.29	-0.15	0.23	0.23	0.09	-0.21
	Sig (2-tailed)	0.898	0.514	0.063	0.331	0.163	0.142	0.568	0.181
Glucose rotenone sensitive respiration	Correlation coefficient	0.04	0.03	-0.05	-0.04	0.09	0.05	0.27	-0.32
	Sig (2-tailed)	0.805	0.868	0.757	0.818	0.594	0.746	0.083	0.041
Galactose rotenone dependent respiration	Correlation coefficient	0.04	0.08	-0.20	-0.05	0.26	0.22	0.18	-0.26
	Sig (2-tailed)	0.810	0.620	0.201	0.744	0.111	0.166	0.255	0.107
Glucose basal ECAR	Correlation coefficient	-0.06	0.08	-0.25	-0.14	0.10	0.06	0.11	-0.12
	Sig (2-tailed)	0.688	0.613	0.110	0.373	0.542	0.729	0.491	0.468
Galactose basal ECAR	Correlation coefficient	-0.08	0.14	-0.22	-0.06	0.20	0.14	0.07	-0.09
	Sig (2-tailed)	0.610	0.367	0.169	0.713	0.234	0.381	0.674	0.570
Glucose maximal ECAR	Correlation coefficient	0.11	0.17	-0.18	0.00	0.05	0.05	0.30	-0.29
	Sig (2-tailed)	0.472	0.294	0.259	0.990	0.754	0.754	0.050	0.066
Galactose maximal ECAR	Correlation coefficient	-0.10	0.05	-0.15	0.04	0.21	0.10	0.12	-0.18
	Sig (2-tailed)	0.510	0.776	0.334	0.816	0.209	0.514	0.435	0.248

(B)

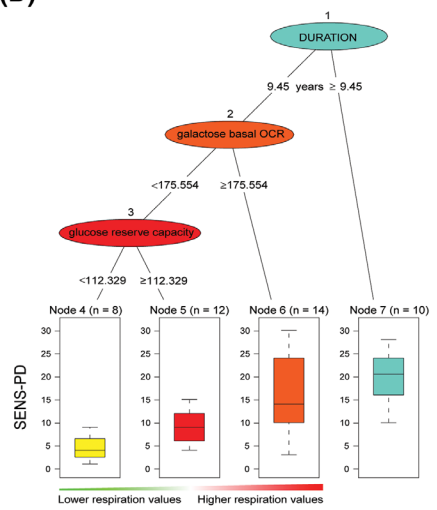


(C)

Analysis of Variance Table

	Df	Pr(>F)
(Intercept)	11	<2e-16 ***
SEX	11	0.5199
Duration	11	0.5982
AgeOnset	11	0.7749
LEVDOSEQ0Z2	11	0.8552
SENSPD_tot	11	0.8531
SEX:Duration	11	0.6897
SEX:AgeOnset	11	0.2073
SEX:LEVDOSEQ0Z2	11	0.2709

(D)



(E)

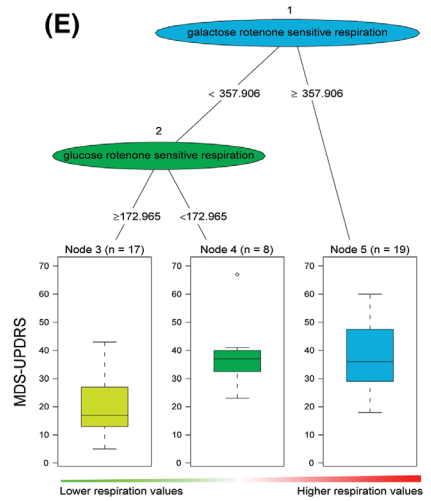


FIG. 3. Correlation between raw respiration data and clinical measures. (**A**) Multivariate analysis of variance showing Spearman's correlation coefficients between laboratory and clinical measures and related significance. (**B**) Graphs of clinical and raw laboratory variables displaying statistically significant correlations. (**C**) Linear regression with interactions and analysis of variance indicates that correlation between the clinical and laboratory measures is independent from gender, age, age at onset, duration of the disease, and medication. (**D**) Grouping of patients using unbiased classification and regression tree analysis using the SENS-PD as a response variable. (**E**) Classification and regression trees analysis using the MDS-UPDRS score as response variable. ECAR, extracellular acidification rate; SCOPA-COG, Scales for Outcomes in Parkinson's Disease–Cognition.

disease duration (ie, less than 9.45 years) higher respiration is associated with increasing symptom severity.

When the MDS-UPDRS was used as response variable, CART analysis returned only 2 rules (node 1, rotenone-sensitive respiration in galactose; node 2, rotenone-sensitive respiration in glucose), dividing the patients in 3 classes (Fig. 3E). Also, in this case higher values in respiration parameters are associated with more severe symptoms.

Increased Mitochondrial Function Promotes α-Syn Stress In Vitro

Taken together, our combined biochemical and clinical data indicate that mitochondrial function is suppressed in PD patients' fibroblasts and—given that lower respiration correlates with less severe symptoms—this alteration may reflect a protective adaptation to counterbalance PD pathogenesis. As a corollary, high mitochondrial activity may synergize with other pathogenic factors to elicit deterioration. Given that α-syn aggregation and stress are hallmarks of PD, we hypothesized that forcing mitochondrial activity in a galactose medium could favor synucleinopathy. Investigating these aspects in fibroblasts, however, poses critical issues because α-syn expression is extremely low in this cell type.[28] We therefore took advantage of lentiviral technology to engineer fibroblasts to express Green Fluorescent Protein (GFP)-tagged human α-syn in 3 control lines (AG08268,

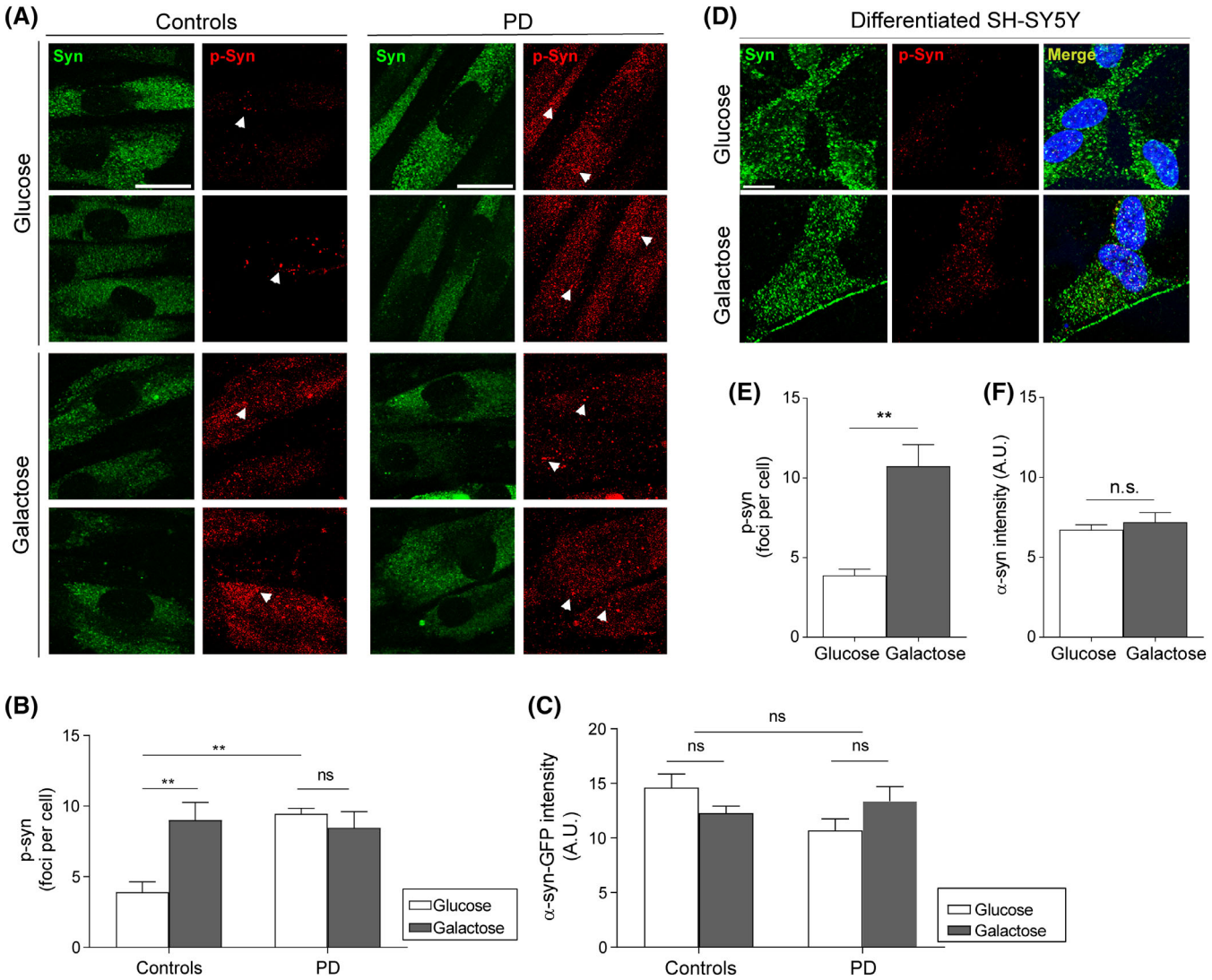


FIG. 4. Increased mitochondrial function in galactose medium favors α-syn stress. (**A**) Representative laser scanning confocal microscopy imaging showing GFP-tagged α-syn (green) and p-syn (red) levels. In healthy controls (N = 3), galactose significantly increases the number of intracellular p-syn foci (arrowheads) pointing to α-syn stress. In PD cells (N = 3), p-syn levels are elevated also in glucose conditions and do not increase in galactose medium. (**B**) Quantification of intracellular p-syn foci. (**C**) Quantification of α-syn GFP levels indicating comparable levels in control and PD specimens. (**E**) Representative laser scanning confocal microscopy imaging of differentiated SH-SY5Y cells showing endogenous α-syn (green) and p-syn (red) levels in glucose- or galactose-culturing conditions. (**F**) Quantification of intracellular p-syn foci showing increased α-syn stress in galactose medium. (**G**) Quantification of endogenous α-syn shows no differences between the 2 culturing conditions. $^{**}P < .0021$, Kruskal–Wallis nonparametric test. Scale bar = 10 μm. α-syn, α-synuclein; A.U., Arbitrary Unit; n.s., not significant; p-syn, phosphorylated α-synuclein.

AG08543, and AG13077) and 3 PD lines able to upregulate mitochondrial function in galactose (3020, 3039, and 3086). We evaluated protein stress by measuring the number of foci of phosphorylated α-syn (p-syn), which is the principal modified species of a-syn within PD pathological inclusions[29]; intracellular foci therefore reflect early forms of aggregation.

In control cells, the galactose medium conditions caused a significant increase in the number of p-syn foci—which were quantified by an unbiased semiautomated procedure—therefore indicating that increased mitochondrial function may indeed favor synucleinopathy. In PD fibroblasts, p-syn levels were also elevated in glucose conditions and did not show any further increase in galactose (Fig. 4A,B). These effects were not caused by different levels of α-syn-GFP because the exogenous protein was expressed at comparable levels in the control and PD specimens (Fig. 4C, green signal). Of note, the observed effect cannot be attributed—at least unambiguously—to increased production of Reactive Oxygen Species (ROS) because 2 (3039 and 3086) of the 3 tested PD lines did not exhibit a significant increase in mitochondrial superoxide production (Fig. 1F).

To determine whether forcing bioenergetics metabolism through mitochondrial function also aggravates endogenous α-syn stress in neuronal cells, we investigated

differentiated SH-SY5Y cells. This dopaminergic cell line expresses detectable levels of endogenous α-syn, which do not differ between glucose- or galactose-culturing conditions (Fig 4D,F). However, cells grown in the galactose medium exhibited significantly increased levels of p-syn (Fig. 4D,E), confirming the data obtained in over-expressing cells. Taken together, these findings indicate that the complex genetic background of idiopathic patients promotes per se α-syn stress and that suppression of mitochondrial function to mitigate synucleinopathy is ineffective in PD cells.

Discussion

The data presented in this study are completely consistent and confirm previous observations from others and our laboratories showing impairment in mitochondrial function in PD.[6,8] However, in this study we report the unexpected and surprising finding that mitochondrial function in PD patients peripheral fibroblasts can be potentiated in conditions forcing OXPHOS, that is, in a galactose medium, which were used to reveal alterations in genetically stratified PD fibroblasts also in previous studies.[30-32] This evidence provides an alternative perspective on the current view of mitochondria in PD and suggests that, rather than being irreversibly damaged, mitochondrial function is suppressed. A possible hypothesis to explain this observation is that in PD mitochondria may suffer from intrinsic anomalies resulting in harmful consequences if the organelles function at normal levels and/or that mitochondrial activity may promote other PD-related pathogenic processes. Consistent with the latter hypothesis, the galactose medium induced an increase in α-syn stress—indicated by increased p-syn levels—in the Green Fluorescent Protein-synuclein (GFP-syn) expressing fibroblasts from healthy subjects. Experiments in engineered fibroblasts and differentiated SHSY-5Y cells also revealed that α-syn stress occurs at high levels in PD cells when grown in glucose and does not increase in galactose, therefore indicating that the complex genetic background of idiopathic PD patients promotes α-syn stress per se. Additional studies will be necessary to unravel possible connections between α-syn aggregation and mitochondrial respiration and their potential role in PD pathogenesis.

A protective role for the suppression of mitochondrial function is consistent with recent hypotheses suggesting that neurodegeneration in Alzheimer's disease is caused by metabolic alterations and that dysfunctional neurons upregulate mitochondrial respiration according to an inverse-Warburg effect pathogenic paradigm.[33,34] We have recently demonstrated that mild inhibition of complex I caused by nitrite-mediated complex I S-nitrosation is protective in multiple models of PD and improves bioenergetic efficiency in the fibroblasts of PD patients, but not in matched controls.[12] A protective role for mitochondrial suppression is further substantiated by other independent studies that have shown that the reversible complex I inhibitor Mitochondrial division inhibitor 1 (Mdivi-1)[35] is protective in PD animal models[11,13] and that proteolytic degradation of complex I attenuates ROS production in damaged, depolarized mitochondria.[36] In addition, it has been shown that fibroblasts from patients harboring mutations in the PD-associated gene ATPase cation transporting 13A2 (*ATP13A2*) display higher, rather than lower, mitochondrial oxygen consumption, which is in turn associated with multiple mitochondrial anomalies.[37] Together with the evidence presented in this study, these data suggest that—at least in some subtypes of PD—mitochondrial function could be an amenable target for disease modification.

Two mitochondrial variables—reserve capacity and rotenone-sensitive (ie, complex I driven) respiration—correlate with the SENS-PD scale. These results confirm the pivotal role of complex I in PD pathobiology and are consistent with previous studies indicating that reserve capacity is very sensitive to stress and therefore particularly suited to detect systemic physiological anomalies.[38,39] The SENS-PD scale addresses clinical features that mostly do not improve on dopaminergic treatment.[16] It is likely that these predominantly nondopaminergic items more accurately reflect the severity and progression of the underlying disease pathobiology.[1]

We used CART analysis[20]—a machine-learning methodology using recursive partitioning—to classify patients on the basis of clinical and bioenergetic measures. CART (which has been already used to analyze biomedical problems[40] and has been used to stratify patients[26]) has several advantages over traditional approaches such as generalized linear models (eg, linear regression, logistic regression, among others). First, the method is nonparametric and thus does not assume any distribution model for the dependent variable. Second, it can handle a large set of explanatory variables, and it automatically selects the most important variables to be used in the final model. Compared to classical regression methods where variable selection is an open problem with no definitive answer,[41] CART analysis is data driven and identifies interactions objectively, in an unbiased manner, and does not require any input from the researcher.

Not surprisingly, the highest hierarchical discriminant of clinical phenotypes is disease duration, with longer durations associated with more severe clinical presentation. The other classifying variables selected by CART among the multitude of demographic, clinical, and bioenergetic parameters are related to mitochondrial function, and the analysis indicates that patients falling in classes with higher respiration present with increased severity of symptoms. Of note, 2 independent statistical methods—that is, multivariate analysis of variance and CART analysis—identify mitochondrial respiration as a

predictor of clinical symptoms. These data substantiate the relevance of mitochondrial biology in PD pathogenesis and further support the hypothesis that suppression of mitochondrial function during PD pathogenesis might represent a protective adaptive response.

Using the SENS-PD scale as response variable led to better separation of patients with shorter disease duration and milder symptoms, therefore confirming the concept that signs outside the dopaminergic domain—which are less sensitive to dopaminergic medications, may manifest at earlier stages, and are enriched in the SENS-PD scale—can be highly informative for PD phenotyping and to monitor the disease progression.[1] Here we propose a novel approach; its methodologies, algorithms, and the consequent results must be replicated in independent experiments. More studies and subsequent publications obtained in possibly larger cohorts also backing up the Seahorse approach with other methodologies will be necessary to conclusively confirm a possible association between peripheral mitochondrial function and clinical symptoms and the clinical and scientific relevance.

In summary, our study reveals new aspects of mitochondrial biology in PD, indicates a connection between clinical and laboratory measures, and may lay a foundation for better stratification of patients. ■

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Supporting Data

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