

Association of caffeine and related analytes with resistance to Parkinson disease among *LRRK2* mutation carriers

A metabolomic study

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Abstract

Objective

To identify markers of resistance to developing Parkinson disease (PD) among *LRRK2* mutation carriers (*LRRK2+*), we carried out metabolomic profiling in individuals with PD and unaffected controls (UC), with and without the *LRRK2* mutation.

Methods

Plasma from 368 patients with PD and UC in the *LRRK2* Cohort Consortium (LCC), comprising 118 *LRRK2+*/PD+, 115 *LRRK2+*/UC, 70 *LRRK2-*/PD+, and 65 *LRRK2-*/UC, and CSF available from 68 of them, were analyzed by liquid chromatography with mass spectrometry. For 282 analytes quantified in plasma and CSF, we assessed differences among the 4 groups and interactions between *LRRK2* and PD status, using analysis of covariance models adjusted by age, study site cohort, and sex, with *p* value corrections for multiple comparisons.

Results

Plasma caffeine concentration was lower in patients with PD vs UC ($p < 0.001$), more so among *LRRK2+* carriers (by 76%) than among *LRRK2-* participants (by 31%), with significant interaction between *LRRK2* and PD status ($p = 0.005$). Similar results were found for caffeine metabolites (paraxanthine, theophylline, 1-methylxanthine) and a nonxanthine marker of coffee consumption (trigonelline) in plasma, and in the subset of corresponding CSF samples. Dietary caffeine was also lower in *LRRK2+*/PD+ compared to *LRRK2+*/UC with significant interaction effect with the *LRRK2+* mutation ($p < 0.001$).

Conclusions

Metabolomic analyses of the LCC samples identified caffeine, its demethylation metabolites, and trigonelline as prominent markers of resistance to PD linked to pathogenic *LRRK2* mutations, more so than to idiopathic PD. Because these analytes are known both as correlates of coffee consumption and as neuroprotectants in animal PD models, the findings may reflect their avoidance by those predisposed to develop PD or their protective effects among *LRRK2* mutation carriers.

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Glossary

A2AR = adenosine 2A receptor; **ACN** = acetonitrile; **ANCOVA** = analysis of covariance; **BH** = Benjamini-Hochberg; **IPA** = isopropanol; **LC/MS** = liquid chromatography with mass spectrometry; **LCC** = LRRK2 Cohort Consortium; **LRRK2** = leucine-rich repeat kinase 2; **mH&Y** = modified Hoehn & Yahr scale; **MJFF** = Michael J. Fox Foundation for Parkinson's Research; **MoCA** = Montreal Cognitive Assessment; **OR** = odds ratio; **PD** = Parkinson disease; **UC** = unaffected controls.

Leucine-rich repeat kinase 2 (LRRK2) gene mutation is considered a major causative influence on Parkinson disease (PD), and demonstrates variable age-dependent, incomplete penetrance.^{1,2} This incomplete penetrance suggests that other genetic or environmental factors modulate the gene's expression or its effects on PD pathophysiology. The identification of such modulators could pave the way for future preventative and disease-modifying therapies. Our recent work identified higher levels of urate in LRRK2 mutation carriers without PD, suggesting that plasma urate could be a marker of resistance against developing PD in mutation carriers.³ Alcalay et al.⁴ studied urinary bis(monoacylglycerol) phosphate isoforms and found slightly higher levels of 2,20-di-18:1-bis(monoacylglycerol)phosphate in LRRK2+/PD+ compared to LRRK2+/unaffected controls (UC).

In this study, we carried out metabolomic profiling in participants enrolled in the LRRK2 Cohort Consortium (LCC). To our knowledge, there has only been one plasma metabolomic study of LRRK2 mutation carriers, and its investigation of purine metabolites was limited to 12 LRRK2+/PD+ participants and 21 LRRK2+/UC.⁵ Our study sought to identify markers of resistance to developing PD in LRRK2 mutation carriers, and to characterize a metabolomic signature of pathogenic LRRK2 mutations. Among our prespecified hypotheses was that caffeine and its related analytes are reduced in patients with PD compared to UC, with reduction similar in those with and without LRRK2 mutations. Our expectation was based on the link between caffeine intake to reduced risk of PD⁶⁻⁸ as well as on the demonstration of their lower serum levels in idiopathic PD.^{9,10}

Methods

Participants

Plasma and CSF were obtained from participants enrolled in the LCC. The LCC was established in 2009 and has been coordinated and funded by the Michael J. Fox Foundation for Parkinson's Research (MJFF). The LCC includes participants diagnosed with idiopathic PD (LRRK2-/PD+), pathogenic LRRK2 gene mutation carriers with PD (LRRK2+/PD+), LRRK2 gene mutation carriers without evidence of PD (LRRK2+/UC), and unaffected noncarrier controls (LRRK2-/UC). The LCC comprises 3 distinct studies, all of which were drawn upon in selecting samples for our research: 23andMe blood collection study, the LRRK2 longitudinal study, and the LRRK2 cross-sectional study. Participants in the LRRK2 longitudinal study and LRRK2 cross-sectional study were enrolled in family-, community-, or clinic-based studies from North

America, Europe, Asia, and North Africa. The LRRK2 cross-sectional study was subdivided into the cross-sectional North American site cohort and the cross-sectional Europe, Asia, and North African site cohort, with their biological samples originally stored in separate biorepositories (Coriell and BioRep, respectively) before all LCC biosamples were transferred for collective management at Indiana University. Therefore, in our study we adjusted analyses for these 4 distinctly processed LCC study site cohorts: the 23andMe blood collection cohort, the LRRK2 longitudinal cohort, the LRRK2 cross-sectional North American cohort, and the LRRK2 cross-sectional Europe, Asia, and North African cohort. Further information on the LCC was published previously¹¹ and is available at michaeljfox.org/data-sets and michaeljfox.org/lccinvestigators.

From these 4 LCC study site cohorts, plasma specimens were selected to best match age and sex across the genotype and disease status groups, similar to our prior study.³ Sixteen percent of requested plasma specimens had corresponding CSF specimens available. MJFF provided codes linking each LCC biospecimen to the associated participant's genotype, disease status, age, sex, and clinical data including caffeine intake only after our finalized analyte values were submitted to the MJFF LCC depository.

Samples

The LRRK2 longitudinal study and both of the LRRK2 cross-sectional studies had standardized protocols for plasma collection, whereas the 23andMe blood collection study did not (michaeljfox.org/data-sets and 23andme.com/pd/). CSF was collected only in the LRRK2 cross-sectional study (table e1, data available from Dryad, doi:10.5061/dryad.nzs7h44pj). As per protocol, samples were to be collected between 8 and 10 AM with participants strongly advised to be in a fasting state, with a minimum of 8 hours since last meal or food intake. Training videos were provided for collecting, storing, and shipping samples. Further information is provided at files. michaeljfox.org/LRRK2_Cohort_Consortium_Biologics_Manual%20_US.pdf. Dietary caffeine questionnaires were completed by participants in the LRRK2 longitudinal study and the LRRK2 cross-sectional study. Using data from the Food and Drug Administration, we converted the cups of caffeinated coffee, black tea, green tea, and soda into milligrams of caffeine per day (fda.gov/consumers/consumer-updates/spilling-beans-how-much-caffeine-too-much).

Standard protocol approvals, registrations, and patient consents

We did not require ethical approval for this study as it involved anonymized, minimal risk LCC data.

Sample preparation

Plasma and CSF samples received from the LCC were stored at -80°C and were thawed for assay on wet ice. Plasma samples ($10\ \mu\text{L}$) were spun down and then transferred to polypropylene 96-well V-bottom half deep-well plates. Next, methanol ($200\ \mu\text{L}$) containing internal standards was added into each well using the Velocity 11 Bravo Liquid Handling Platform (Agilent Technologies, Santa Clara, CA). Plates were shaken for 5 minutes at room temperature and placed at -20°C for 1 hour to allow further precipitation of proteins. Plates were then centrifuged at $4,000g$, 4°C for 20 minutes. The supernatant was divided into 2 aliquots: a $100\ \mu\text{L}$ aliquot was transferred to 96-well plates with glass inserts for direct liquid chromatography with mass spectrometry (LC/MS) analyses of lipids and metabolites (tables e2–e9, data available from Dryad, doi:10.5061/dryad.nzs7h44pj); a second $50\ \mu\text{L}$ aliquot was transferred to a separate 96-well plate with glass inserts, dried down under gentle nitrogen stream, and resuspended in $100\ \mu\text{L}$ of 92.5/5/2.5 acetonitrile (ACN)/isopropanol (IPA)/water with 5 mM ammonium formate and 0.5% formic acid for LC/MS analysis of GlcCer and GalCer species (tables e2–e9, data available from Dryad, doi:10.5061/dryad.nzs7h44pj).

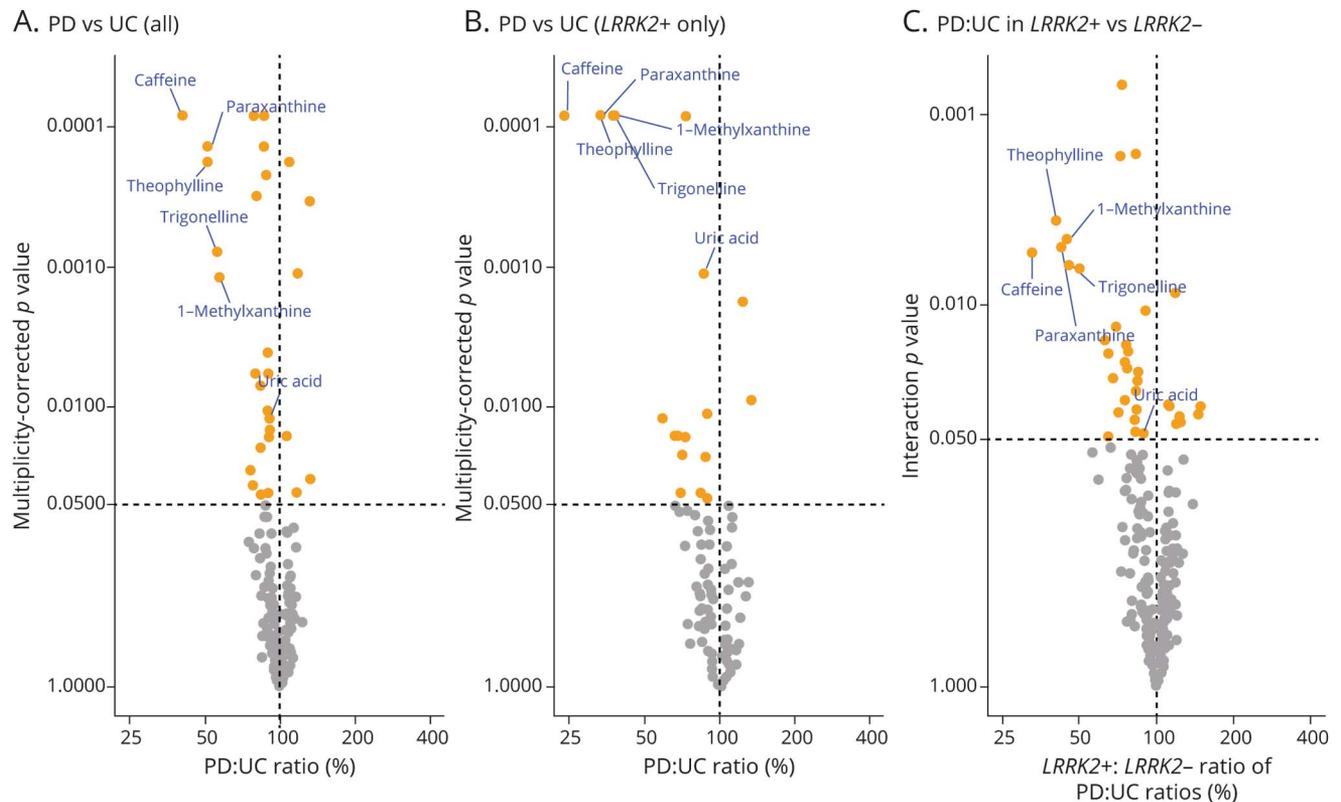
CSF samples ($20\ \mu\text{L}$) were spun down, transferred into 96-well plates with glass inserts, then methanol ($100\ \mu\text{L}$)

containing internal standards was added into each well using the Velocity 11 Bravo Liquid Handling Platform (Agilent Technologies, Santa Clara, CA). Plates were sealed, shaken for 5 minutes at room temperature, and then centrifuged at $1,000g$ at 4°C for 2 minutes. Next, the samples were divided into 2 aliquots: a $60\ \mu\text{L}$ aliquot was transferred to 96-well plates with glass inserts for direct LC/MS analyses of lipids and metabolites; a $30\text{-}\mu\text{L}$ aliquot was transferred to a separate 96-well plate with glass inserts, dried down under gentle nitrogen stream, and resuspended in $30\ \mu\text{L}$ of 92.5/5/2.5 ACN/IPA/water with 5 mM ammonium formate and 0.5% formic acid for LC/MS analysis of GlcCer and GalCer species (tables e2–e9, data available from Dryad, doi:10.5061/dryad.nzs7h44pj).

Data reporting

For each analyte, both un-normalized peak area (units = area) and peak area normalized to an internal standard (units = area ratio) were calculated and reported. For urate, concentration was calculated (units = mg/dL) as a product of the corresponding area ratio and the amount of internal standard used. Peak areas with signal-to-noise <3 were not reported. The primary analysis outcome is the internal standard normalized area ratio (“area ratio” or relative abundance).

Figure 1 Volcano plots of plasma metabolomics results



Volcano plots of plasma metabolomics results comparing Parkinson disease (PD):unaffected controls (UC) (ratio for all LRRK2 Cohort Consortium participants analyzed (A), PD:UC ratio for LRRK2+ participants (B), and PD:UC ratio in LRRK2+ vs LRRK2- participants (C), p Values were related to the PD:UC ratio (A and B) or LRRK2+:LRRK2- ratio (C) for each measured analyte adjusted for age, sex, study site cohort, and (in A only) LRRK2 status.

Table 1 Baseline demographics and features by *LRRK2* and Parkinson disease (PD) status of participants contributing analyzed plasma samples

Demographic features	<i>LRRK2</i> –/UC (n = 65)	<i>LRRK2</i> –/PD+ (n = 70)	<i>LRRK2</i> +/UC (n = 115)	<i>LRRK2</i> +/PD+ (n = 118)	p Value ^a
Age, y, mean (SD)	63.0 (9.1)	64.4 (8.5)	61.0 (8.4)	63.9 (8.6)	0.03
Male, n (%)	28 (43)	38 (54)	52 (45)	55 (47%)	0.56
Study site cohort					
<i>LRRK2</i> longitudinal (n = 87)	19	27	14	27	
<i>LRRK2</i> cross-sectional North America (n = 68)	21	20	11	16	<0.001
<i>LRRK2</i> cross-sectional Europe, Asia, North Africa (n = 126)	25	23	30	48	
23andMe (n = 87)	0	0	60	27	
Hours fasted, ^b mean (SD) [n with this info]	9.5 (5.4) [n = 63]	8.4 (5.5) [n = 63]	10.1 (5.0) [n = 50]	9.7 (5.2) [n = 85]	0.36
Caucasian race, n (%) [n with this info]	62 (95) [n = 65]	70 (100) [n = 70]	55 (100) [n = 55]	88 (97) [n = 91]	0.16
MoCA, mean (SD) [n with this info]	26.4 (2.5) [n = 64]	26.2 (4.4) [n = 66]	26.5 (2.7) [n = 51]	24.5 (4.6) [n = 76]	0.001
mH&Y, median (IQR) [n with this info]	0 (0) [n = 65]	2 (0.5) [n = 69]	0 (0) [n = 54]	2 (0.5) [n = 88]	<0.001
Dietary caffeine intake, mg/d, mean (SD) [n with this info]	146.6 (101.2) [n = 48]	216.2 (193.9) [n = 51]	241.6 (148.1) [n = 43]	157.4 (124.6) [n = 70]	0.002

Abbreviations: IQR = interquartile range; mH&Y = modified Hoehn & Yahr scale; MoCA = Montreal Cognitive Assessment; UC = unaffected controls.

^a Analysis of variance for comparison across groups of age, hours, MoCA, and dietary caffeine intake. Kruskal-Wallis for comparison across groups of mH&Y. χ^2 for comparison across groups of male sex, study site cohort, and Caucasian race.

^b A minimum of 8 hours fasting prior to blood draw between 8 and 10 AM was advised for all participants (except those in the 23andMe cohort).

A total of 282 analytes were quantified in the plasma and CSF. We excluded 7 analytes from our PD and UC comparisons, and from our volcano plots (figure 1, A–C), as they correlated closely with antiparkinsonian medication use (i.e., levodopa, its metabolites dopamine, 3-methoxytyrosine, dopamine 3-*o*-sulfate, dopamine 4-*sulfate*, 3-hydroxytyrosol, and the lysine metabolite 3-hydroxy-N6,N6,N6-trimethyllysine). Plasma urate concentrations measured previously by high-performance liquid chromatography in the same samples³ correlated well with those measured by LC/MS in the current study ($r = 0.95$).

Results from 4 of 380 available LCC plasma specimens received for this study did not pass quality control checks and were excluded from further analysis prior to unblinding. Following formal unblinding (but while unaware of individual participants' analyte values) we identified 9 UC classified participants as having features indicative of a PD or related diagnosis (e.g., clinician rating of PD probability of 50%–100%, modified Hoehn & Yahr scale [mH&Y] scale score ≥ 1 , or Unified Parkinson's Disease Rating Scale part 3 score ≥ 18), suggesting initial misclassification of their enrollment status. After review of these cases with their site clinicians and study cohort staff (and while unaware of any analyte values participants), with concurrence of MJFF staff, we corrected the classification status of 1 participant and excluded from analysis the remaining 8 participants. Of note, these LCC participant classification

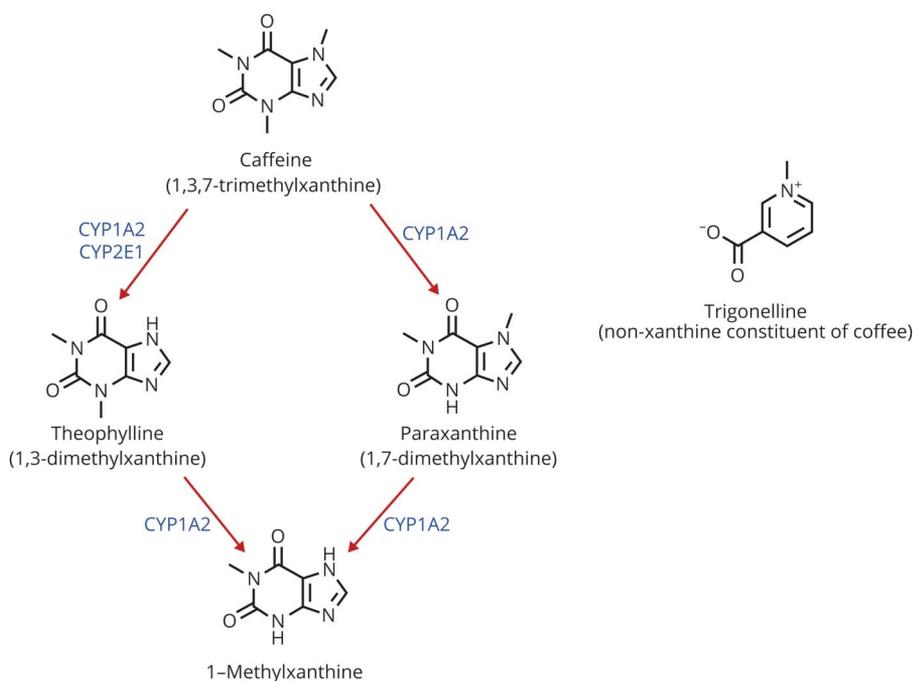
corrections were made since our previously published analysis of the dataset.³

Statistical analysis

Sample size (n = 380, with n = 120 for each of *LRRK2*+/PD+ and *LRRK2*+/UC, the 2 critical comparison groups) was determined based on estimation of sample size required to achieve adequate power for replication of a urate difference between these groups in the original test sample analysis (n = 64 for each of *LRRK2*+/PD+ and *LRRK2*+/UC groups; $p = 0.047$).

A statistical analysis plan was specified prior to unblinding of investigators to *LRRK2* genotype, PD status, and all other clinical parameters linked to plasma and CSF samples. For plasma and CSF metabolites, we reported the geometric mean of relative abundance. For all plasma analytes, estimates of mean differences between PD and UC participants and between *LRRK2*+ and *LRRK2*– participants were generated with a robust analysis of covariance (ANCOVA) model with log₂ (area ratio) as the dependent variable, and adjusting by PD status cohort (PD vs UC), *LRRK2* gene status, sex, age (modeled as cubic spline, with a knot at 60 years), and substudy (the 4 study site cohorts). Interaction terms of PD status cohort \times *LRRK2* status \times sex and PD status cohort \times substudy were included. The ANCOVA model was the basis for pairwise tests for the contrasts of interest (PD vs UC, overall or within *LRRK2* \pm subgroups), associated p values, and adjusted between-groups effect sizes. To partially control

Figure 2 Chemical structures and metabolic pathways of caffeine and related analytes (paraxanthine, theophylline, 1-methylxanthine, and trigonelline)



the false discovery rate, we employed a Benjamini-Hochberg (BH) correction to the p values for the PD vs UC (overall, and within each of the *LRRK2*⁺ and *LRRK2*⁻ groups) comparisons. Statistical significance was judged at a 2-sided 0.05 significance level.

For the smaller subset of participants with CSF samples, between-group estimates were generated from a joint CSF/plasma linear mixed effects model, considering the CSF/plasma measurements as repeated, within-participant measures for each analyte, adjusting for the same set of covariates as above. A similar, albeit simplified set of interaction terms was included in the model (cohort \times *LRRK2* status \times sample type, sex \times sample type, and age \times sample type).

Data availability

Raw data from this study are available from online LCC data request at michaeljfox.org/data-sets. Supplemental data (tables e1–e9, doi:10.5061/dryad.nzs7h44pj) are stored on Dryad.

Results

Table 1 presents baseline characteristics of the 368 LCC participants whose plasma was analyzed for this study, stratified by PD status (1:1 for diagnosed PD:UC) and *LRRK2* gene status (2:1 for *LRRK2*⁺:*LRRK2*⁻). Differences among the 4 groups' features highlight the value in adjusting for age, sex, and study site cohort. As expected, Montreal Cognitive Assessment (MoCA) scores were lower in the PD groups compared to UC ($p < 0.05$). Data on time fasted prior to

blood draw and on race, MoCA, and mH&Y assessments were available for most participants except for the 24% from the 23andMe blood collection study (which provided plasma only from *LRRK2*⁺ individuals). Regarding specific pathogenic *LRRK2* variants present, 91% ($n = 107$) of the *LRRK2*⁺/PD⁺ and 92% ($n = 106$) *LRRK2*⁺/UC groups carried the G2019S *LRRK2* mutation. Other *LRRK2* variants in the *LRRK2*⁺/PD⁺ group were R1441G ($n = 4$), R1441C ($n = 1$), N1437H ($n = 2$), L1795F ($n = 2$), C228S ($n = 1$), and unknown ($n = 1$). Other *LRRK2* variants in the *LRRK2*⁺/UC group were R1441G ($n = 4$), R1441C ($n = 1$), N1437H ($n = 1$), L1114L ($n = 1$), and L1795F ($n = 2$).

Comparing the concentrations of 282 LC/MS-quantified plasma metabolites between people with and without PD irrespective of *LRRK2* gene status revealed a cluster of 5 analytes showing the greatest differences, excluding metabolites linked to antiparkinsonian medication. All 5 are caffeine-related analytes (figure 2) and are lower in the participants with PD than UC, as depicted in figure 1A in a volcano plot of metabolomics data with effect size (ratio for PD vs UC values, adjusted for age, sex, study site cohort, and *LRRK2* status) plotted against BH-adjusted p values. Compared to UC, those with PD had lower plasma levels not only of caffeine (by 71%) but also of its partially demethylated xanthine-based metabolites paraxanthine (1,7-dimethylxanthine), theophylline (1,3-dimethylxanthine), 1-methylxanthine (by 57%, 56%, and 49%, respectively), and trigonelline, a non-xanthine constituent of coffee (by 52%), with associated BH-adjusted p values all ≤ 0.001 (table 2). Consistent with their metabolic and

Table 2 Caffeine, its metabolites, and trigonelline in plasma of LRRK2 Cohort Consortium participants by LRRK2 and Parkinson disease (PD) status with adjusted geometric mean concentrations (95% confidence interval)

Plasma analyte	LRRK2-/UC (n = 65)	LRRK2-/PD+ (n = 70)	LRRK2+/UC (n = 115)	LRRK2+/PD+ (n = 118)	BH-adjusted <i>p</i> value, for PD vs UC	Interaction <i>p</i> value, PD:UC for LRRK2+ vs -
Caffeine	9.7 (6.1–15.5)	6.7 (4.2–10.9)	12.2 (7.9–18.7)	2.9 (2.0–4.2)	<0.001	0.005
Paraxanthine	2.9 (2.1–4.1)	2.3 (1.6–3.3)	3.7 (2.7–5.1)	1.2 (0.9–1.6)	<0.001	0.005
Theophylline	1.7 (1.2–2.4)	1.3 (0.9–1.9)	2.1 (1.5–3.0)	0.7 (0.5–0.9)	<0.001	0.004
1-Methylxanthine	0.0007 (0.0005–0.001)	0.0006 (0.0004–0.0008)	0.001 (0.0008–0.001)	0.0004 (0.0003–0.0005)	0.001	0.005
Trigonelline	1.3 (0.9–1.8)	1.1 (0.8–1.5)	1.9 (1.4–2.6)	0.7 (0.5–0.9)	<0.001	0.006

Abbreviations: BH = Benjamini-Hochberg; UC = unaffected controls.

dietary relationships, plasma caffeine correlated well with its demethylation products (paraxanthine, theophylline, and 1-methylxanthine) and its coffee co-constituent, trigonelline, with Spearman coefficients (*r*) of approximately 0.8, 0.8, 0.5, and 0.5, respectively, in participants with PD.

Stratifying by genotype reveals that the extent to which these caffeine-related analytes are lower in PD is substantially greater among pathogenic LRRK2 mutation carriers (figure 1, B and C, and table 2). In LRRK2+/PD+ plasma, caffeine itself was 76% lower than in plasma of LRRK2+/UC participants, whereas in idiopathic PD plasma caffeine was 31% lower than in LRRK2-/UC participants (*p* for LRRK2 × PD status interaction = 0.005). Similarly, levels in PD were even lower among LRRK2+ vs LRRK2- participants for paraxanthine (66% vs 21%), theophylline (67% vs 21%), 1-methylxanthine (62% vs 14%), and trigonelline (63% vs 15%), with *p* for interaction <0.01 for each. Of note, fasting times prior to blood draw did not differ appreciably across groups (table 1), suggesting that the lower levels of analytes from caffeinated beverages observed in the plasma of people with PD are not due to their having fasted longer.

Dietary caffeine consumption questionnaire data were available for 212 of the participants with plasma caffeine metabolites (table 1). In this subset, participants with PD with the LRRK2 mutation consumed significantly (41%) less caffeine (in mg/d) when compared to UC with the LRRK2 mutation (*p* < 0.003), and there was a significant interaction effect with the LRRK2 mutation (*p* < 0.001). Dietary caffeine consumption positively correlated with plasma caffeine metabolite concentration (*r* = 0.3). When caffeine metabolite concentration was adjusted for dietary caffeine intake, the significant difference between PD and controls (*p* = 0.01) and between LRRK2+/PD+ and LRRK2+/UC (*p* = 0.01) persisted, although the interaction effect for LRRK2 mutation was not significant (*p* = 0.38).

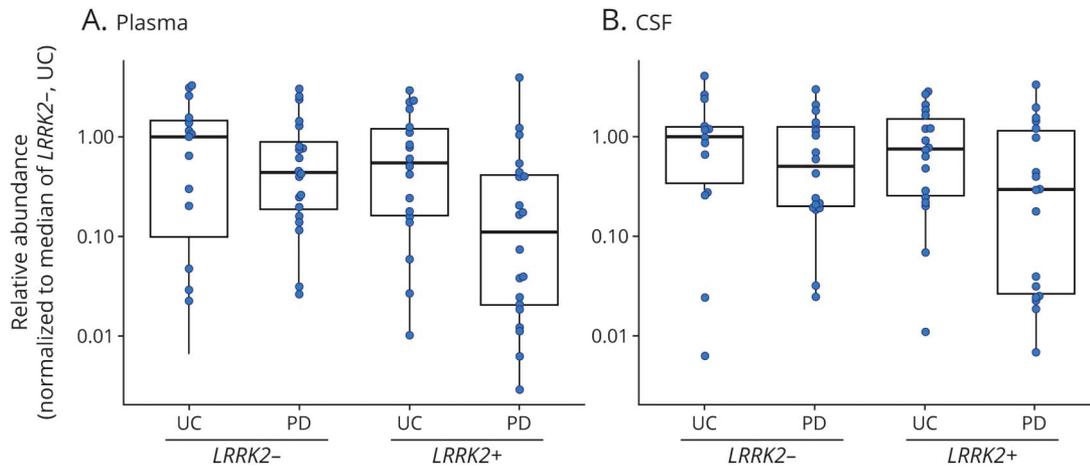
Similar to plasma caffeine, plasma levels of the endogenous purine urate were found to be a marker of PD resistance among LRRK2 mutation carriers in our earlier, nonmetabolomic

analysis in the nearly identical LCC sample set.³ Although urate also inversely associates with PD among LRRK2+ more than LRRK2- participants, the associations are weaker than for the caffeine-related analytes³ and the evidence for an interaction between urate, LRRK2 genotype, and PD status reached only marginal statistical significance in our more recent analysis (*p* = 0.047) (figure 1, A–C).

Matching CSF samples were available for 68 participants with analyzed plasma: LRRK2-/UC (n = 14), LRRK2-/PD+ (n = 18), LRRK2+/UC (n = 18), and LRRK2+/PD+ (n = 18), all of whom were from the LRRK2 cross-sectional study (table e-1, data available from Dryad, doi:10.5061/dryad.nzs7h44pj). In figure 3, we present boxplot illustrations of the overlapping plasma and CSF samples of the 4 groups by LRRK2 gene status and PD status. Among the LRRK2 mutation carriers in this subset, both CSF and plasma caffeine levels were significantly lower in participants with PD than in UC (after adjustment for age, sex, and study site cohort): by 74% in CSF (*p* < 0.02) and by 76% in plasma (*p* = 0.01), consistent with a strong positive correlation between the entire sample of plasma and CSF caffeine concentrations (*r* = 0.90). By contrast, among LRRK2- participants in this subset, CSF caffeine was 23% lower and plasma caffeine was 24% higher in participants with PD compared to controls (*p* = 0.65 and 0.71, respectively).

Similar associations were observed for CSF levels of caffeine's dimethyl metabolites paraxanthine and theophylline, and of trigonelline, whose plasma and CSF levels also closely correlated (*r* = 0.94, 0.94, and 0.90, respectively). 1-methylxanthine was not measured in the CSF. Among the LRRK2 mutation carriers in this subset, both CSF and plasma levels of paraxanthine, theophylline, and trigonelline were lower in participants with PD than in UC after adjustment: by 73%, 74%, and 70% for CSF (*p* = 0.003, 0.003, and <0.001) and by 74%, 76%, and 69% for plasma (*p* = 0.002, <0.002, and 0.001), respectively. By contrast, among LRRK2- participants in this subset, levels of paraxanthine, theophylline, and trigonelline were largely indistinguishable after adjustment between those with and without PD, being higher in CSF by 2%, 1%, and 9%

Figure 3 Concentrations of caffeine in matched plasma and CSF samples of LRRK2 Cohort Consortium (LCC) participants



Concentrations of caffeine in matched plasma (A) and CSF (B) samples of LCC participants by *LRRK2* and Parkinson disease (PD) status, adjusted for age, sex, and study site cohort. UC = unaffected controls.

in those with vs without PD ($p = 0.97, 0.99, \text{ and } 0.82$), and in plasma by 20%, 19%, and 37% in those with vs without PD ($p = 0.70, 0.72 \text{ and } 0.40$). Fasting times prior to lumbar puncture, like those for blood collection, did not differ across the 4 groups at approximately 12 hours ($p = 0.29$).

Exploratory stratification showed that the low caffeine association with PD among *LRRK2* mutation carriers was present irrespective of sex (62% lower with PD among men and 85% lower with PD among women; $p = 0.004$ and <0.001 , respectively) or age (65% lower with PD among those younger than 60 years at baseline, and 83% lower among those 60 years or older at baseline; $p < 0.02$ and <0.001 , respectively). Similarly, lower caffeine with PD among *LRRK2* mutation carriers was just as strong when PD cases were restricted to the 22% who appear not to have been taking levodopa (based on its plasma metabolite 3-methoxytyrosine having relative abundance levels less than 0.1), being 78% lower with PD ($p < 0.001$), or to those at an early clinical stage (mH&Y 2.5 or lower), being 76% lower with PD ($p = 0.002$), suggesting that the lower caffeine levels with PD among *LRRK2* mutation carriers is not likely due to an antiparkinsonian medication effect or more advanced disease. In contrast to *LRRK2* mutation carriers, those without mutations (*LRRK2*⁻) showed a non-significant, weaker link between low caffeine and PD, with caffeine 39% lower in men ($p = 0.25$) and 21% in women ($p = 0.56$).

Discussion

In this metabolomics study of *LRRK2* mutation carriers, we identified the dietary purine caffeine and its metabolites as the most affected pathway in plasma with significantly lower levels of caffeine, its demethylation products, and trigonelline, a nonpurine marker of coffee consumption in participants with PD compared to UC, and to a significantly greater extent among pathogenic *LRRK2* mutation carriers than among those without a mutation.

Our observation of lower plasma caffeine concentrations in participants with PD confirms recent metabolic⁹ and metabolomic¹⁰ findings, consistent with well-established epidemiologic evidence for increased risk of developing idiopathic PD in individuals who consume fewer caffeinated beverages, based on dietary recall.¹² Interestingly, Fujimaki et al.⁹ did not find significantly lower caffeine intake in participants with PD despite lower serum levels of caffeine and its metabolites in their cohort, raising the possibility that the lower levels could result from reduced bio-availability (e.g., due to reduced gastrointestinal absorption). By contrast, in the larger LCC cohort assessed here, a lower intake among PD vs control participants, and a significantly lower intake in participants with *LRRK2*^{+/PD} compared to *LRRK2*^{+/UC}, suggest that lower concentrations of caffeine and its metabolites circulating in people with PD reflects, at least in part, their lesser consumption of this dietary purine. Of note, the finding of comparably reduced plasma levels of trigonelline, a nonpurine constituent and plasma biomarker of coffee¹³ (which comprised 84% of the total daily caffeine consumption), further supports a dietary basis for the lower caffeine concentrations in PD vs controls. However, our results are also consistent with a role of differential absorption, metabolism, or clearance of caffeine in PD given the persistent, significantly lower level of caffeine and its related analytes in our participants with PD with or without a pathogenic *LRRK2* mutation, compared to their control counterparts, following adjustment for dietary caffeine intake.

The unexpected finding that caffeine's and related analytes' associations with resistance to PD are substantially greater among *LRRK2* mutation carriers than noncarriers appears robust and intriguing. Levels of these analytes were lower in both the plasma and CSF of participants with PD vs UC among *LRRK2* mutation carriers, whereas among noncarriers these analytes were not significantly reduced in patients with PD vs control participants. Direct evidence for interaction between PD and *LRRK2* status ($p < 0.01$ for each of the 5

caffeine-related analytes) suggests a true gene–environment interaction, rather than sample size bias resulting from the (2:1) predominance of *LRRK2*⁺ over *LRRK2*[−] participants in this study's LCC sampling.

Corroborating these metabolomic data, we obtained complementary epidemiologic evidence that participants with PD consumed significantly less caffeine compared to UC only among *LRRK2* mutation carriers. Interestingly, our findings of *LRRK2*–caffeine intake interaction substantiate earlier findings of Kumar et al.¹⁴ They studied the interplay between caffeine consumption and a different *LRRK2* mutation—the R1628P risk variant in a Chinese population. They similarly reported a significant *LRRK2*–caffeine interaction, with a lower odds ratio (OR) of developing PD among R1628P *LRRK2* mutation carriers who consumed caffeine (OR 3.1) than among mutation carriers who were nonconsumers (OR 15.4) when compared to caffeine consumers without the mutation,¹⁴ suggesting that the biology underlying the *LRRK2*–caffeine interaction entails the common ability of COR domain–based R1628P and kinase domain–based G2019S mutations to increase kinase activity.^{15–17}

Similar but indirect evidence for caffeine's greater association with resistance to genetic forms of PD was recently provided by Angelopoulou et al.,¹⁸ who explored environmental factors in early-onset (before 50 years of age) compared to later-onset PD in a Greek cohort. They observed that coffee drinking was associated with a lower risk of early but not later-onset PD. They also detected a linear dose association between coffee exposure and the risk of developing familial as well as early-onset PD.¹⁸ As early-onset PD and familial PD are more likely to be genetic than later-onset PD, these data together with those of Kumar et al.¹⁴ and ours suggest that caffeine could be broadly associated with PD gene penetrance.

Of note, no statistically significant analyte–*LRRK2* mutation interaction has been reported previously for PD risk at the metabolome level. Urate—an endogenous antioxidant and end product of purine metabolism—was found in our preceding study of the nearly identical LCC cohort to be associated with PD among *LRRK2* mutation carriers to a greater extent than in noncarriers.³ However, in contrast to caffeine's link to PD resistance, that of urate was relatively modest and only slightly greater among *LRRK2* mutation carriers than noncarriers (figure 1, A–C).

Our findings for CSF levels of caffeine and related analytes were similar to those for their plasma counterparts, despite the lower number of CSF (n = 68) than plasma (n = 368) samples analyzed. The similarities are in keeping with the close correlation between plasma and CSF concentrations for these analytes. These findings support the use of peripheral (e.g., plasma) samples in assessing the caffeine metabolic pathway in relation to PD risk.

Caffeine is the most widely consumed psychoactive substance and a nonselective antagonist of the adenosine 2A receptor (A2AR). It also possesses neuroprotective properties in animal models of PD.

Both its psychostimulant actions and protective effects on dopaminergic neurons likely rely on A2ARs,^{19–21} with A2AR blockade by caffeine reducing excitotoxic and inflammatory processes.^{22–24} The association of caffeine with resistance to PD could reflect the ability of PD determinants to reduce the likelihood of caffeine intake, or conversely it could reflect a causal relationship driven by a protective effect of caffeine or a related analyte.^{23,25,26} Interestingly, *LRRK2* biology may potentiate either of these caffeine–PD relationships given its involvement in striatal neuroplasticity and nigrostriatal neurodegeneration, both of which can be attenuated by caffeine via its actions on adenosine A2ARs.^{27,28} For example, the pathogenic G2019S *LRRK2* mutation has been found to alter synaptic plasticity in the A2AR–laden striatum while bolstering resistance to social stress in young animals.²⁹ Alternatively, the well-established neuroprotective properties of caffeine or dimethyl metabolites, paraxanthine and theophylline, which are themselves A2AR antagonists^{30,31} in PD models, could be potentiated in the setting of pathogenic *LRRK2* mutations. For example, the increased kinase activity of pathogenic G2019S *LRRK2* may potentiate the dopaminergic degeneration induced by α -synuclein,³² and α -synuclein–induced dopaminergic neuron injury can be attenuated by caffeine³³ and depend upon the A2AR.³⁴ Thus, A2AR antagonists like caffeine and its metabolites may be particularly effective in attenuating *LRRK2* kinase–potentiated α -synuclein pathobiology, raising the possibility that the therapeutic potential of caffeine or other A2AR antagonists may be greater for slowing or preventing *LRRK2*–PD than for idiopathic disease. Similarly, although the nonpurine trigonelline may simply be a marker of coffee and caffeine consumption, it has been shown to have its own protective effects in a PD model.³⁵

There are several strengths to our study. First, our metabolomic analysis of *LRRK2*–PD was conducted on the largest cohort to date. Second, the cohort included matched *LRRK2* gene status as well as PD controls, allowing the opportunity to gauge interactions across genotype and disease state. Third, the depth of the LCC biorepository allowed us to assess CSF as well as plasma biomarkers of *LRRK2*–PD. Finally, we were able to correlate dietary intake with metabolomics, which is relatively novel in allowing us to explore the basis for lower caffeine-related analytes in *LRRK2*–PD.

Several limitations of our study should be noted, including potential selection bias as participants were recruited through the multiple individual study site cohorts that comprise the LCC. Although we endeavored to match or adjust for relevant covariates to reduce their influence when differing across groups, unmeasured confounders could have affected our results. Second, misclassification of PD or control participants could not be fully excluded in part based on the lack of a biomarker for definitive diagnosis of PD, although extensive phenotype data were available for most LCC participants, allowing us to cross-check and confirm classifications, and such errors would have biased us toward null results. Lastly, the results are cross-sectional, precluding direct assessment of the predictive potential of analytes on PD risk and progression.

The identification of caffeine and adenosine antagonists as potential markers of PD resistance among *LRRK2* mutation carriers supports their potential for development as biomarkers contributing to phenoconversion risk assessment among carriers, and to progression rates among participants with *LRRK2+* PD. In addition, identification of caffeine-related analytes as resistance markers raises the possibility of their development as candidate therapeutics given the relatively low risk of repurposing these dietary or pharmacologic agents—caffeine, theophylline, and trigonelline—for slowing progression in those with manifest *LRRK2+* PD, or among at-risk mutation carriers to reduce the penetrance of the disease. Next steps may include replication of these metabolomic results in an independent cohort of *LRRK2* mutation carriers and assessing their specificity in other genetic (e.g., *GBA* mutation-driven) forms of PD.

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Disclosure

G.F. Crotty reports no disclosures. R. Maciuca is a salaried employee of Denali Therapeutics Inc. E.A. Macklin reports no disclosures. J. Wang is a salaried employee of Denali Therapeutics Inc. M. Montalban is a salaried employee of Denali Therapeutics Inc. S. Davis is a salaried employee of Denali Therapeutics Inc. J.I. Alkabsh is a salaried employee of Denali Therapeutics Inc. R. Bakshi, X. Chen, and A. Alberto report no disclosures. G. Astarita is a salaried employee of Denali Therapeutics Inc. S. Huntwork-Rodriguez is a salaried employee of Denali Therapeutics Inc. M.A. Schwarzschild reports no disclosures. Go to Neurology.org/N for full disclosures.

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Xiqun Chen, MD, PhD	Department of Neurology, Massachusetts General Hospital; Harvard Medical School, Boston	Design and conceptualization of the study, major role in the acquisition of data, analysis and interpretation of the data, revising the manuscript for intellectual content

Appendix (continued)

Name	Location	Contribution
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Sarah Huntwork-Rodriguez, PhD	Denali Therapeutics Inc., San Francisco, CA	Design and conceptualization of the study, major role in the acquisition of data, analysis and interpretation of the data, revising the manuscript for intellectual content
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