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Glucocerebrosidase activity in Parkinson's disease with and without *GBA* mutations

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Glucocerebrosidase (*GBA*) mutations have been associated with Parkinson's disease in numerous studies. However, it is unknown whether the increased risk of Parkinson's disease in *GBA* carriers is due to a loss of glucocerebrosidase enzymatic activity. We measured glucocerebrosidase enzymatic activity in dried blood spots in patients with Parkinson's disease ($n = 517$) and controls ($n = 252$) with and without *GBA* mutations. Participants were recruited from Columbia University, New York, and fully sequenced for *GBA* mutations and genotyped for the *LRRK2* G2019S mutation, the most common autosomal dominant mutation in the Ashkenazi Jewish population. Glucocerebrosidase enzymatic activity in dried blood spots was measured by a mass spectrometry-based assay and compared among participants categorized by *GBA* mutation status and Parkinson's disease diagnosis. Parkinson's disease patients were more likely than controls to carry the *LRRK2* G2019S mutation ($n = 39$, 7.5% versus $n = 2$, 0.8%, $P < 0.001$) and *GBA* mutations or variants (seven homozygotes and compound heterozygotes and 81 heterozygotes, 17.0% versus 17 heterozygotes, 6.7%, $P < 0.001$). *GBA* homozygotes/compound heterozygotes had lower enzymatic activity than *GBA* heterozygotes (0.85 μmol/l/h versus 7.88 μmol/l/h, $P < 0.001$), and *GBA* heterozygotes had lower enzymatic activity than *GBA* and *LRRK2* non-carriers (7.88 μmol/l/h versus 11.93 μmol/l/h, $P < 0.001$). Glucocerebrosidase activity was reduced in heterozygotes compared to non-carriers when each mutation was compared independently (N370S, $P < 0.001$; L444P, $P < 0.001$; 84GG, $P = 0.003$; R496H, $P = 0.018$) and also reduced in *GBA* variants associated with Parkinson's risk but not with Gaucher disease (E326K, $P = 0.009$; T369M, $P < 0.001$). When all patients with Parkinson's disease were considered, they had lower mean glucocerebrosidase enzymatic activity than controls (11.14 μmol/l/h versus 11.85 μmol/l/h, $P = 0.011$). Difference compared to controls persisted in patients with idiopathic Parkinson's disease (after exclusion of all *GBA* and *LRRK2* carriers; 11.53 μmol/l/h, versus 12.11 μmol/l/h, $P = 0.036$) and after adjustment for age and gender ($P = 0.012$). Interestingly, *LRRK2* G2019S carriers ($n = 36$), most of whom had Parkinson's disease, had higher enzymatic activity than non-carriers (13.69 μmol/l/h versus 11.93 μmol/l/h, $P = 0.002$). In patients with idiopathic Parkinson's, higher glucocerebrosidase enzymatic activity was associated with longer disease duration ($P = 0.002$) in adjusted models, suggesting a milder disease course. We conclude that lower glucocerebrosidase enzymatic activity is strongly associated with *GBA* mutations, and modestly with idiopathic Parkinson's disease. The association of lower glucocerebrosidase activity in both *GBA* mutation carriers and Parkinson's patients without *GBA* mutations suggests that loss of glucocerebrosidase function contributes to the pathogenesis of Parkinson's disease. High glucocerebrosidase enzymatic activity in *LRRK2* G2019S carriers may reflect a distinct pathogenic mechanism. Taken together, these data suggest that glucocerebrosidase enzymatic activity could be a modifiable therapeutic target.

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Abbreviation: UPDRS = Unified Parkinson's Disease Rating Scale

Introduction

Mutations in the glucocerebrosidase (*GBA*) gene are associated with Parkinson's disease. This association has been established in patients with mutations in two *GBA* alleles (i.e. patients with Gaucher disease; Neudorfer *et al.*, 1996; Mitsui *et al.*, 2009), and in carriers of a single *GBA* mutation (i.e. *GBA* heterozygotes; Aharon-Peretz *et al.*, 2004; Lwin *et al.*, 2004; Halperin *et al.*, 2006; Goker-Alpan *et al.*, 2008; Bras *et al.*, 2009; Kalinderi *et al.*, 2009; Mitsui *et al.*, 2009; Sidransky *et al.*, 2009; Giraldo *et al.*, 2011). *GBA* encodes the lysosomal enzyme glucocerebrosidase, a glucosylceramide hydrolase that plays an important role in sphingolipid degradation, especially in the macrophage/monocyte cell lineage. In Gaucher disease, diminished glucocerebrosidase enzymatic activity accounts for glucocerebroside accumulation in the spleen, liver and bone marrow. Gaucher disease is the most common lysosomal storage disease in the general population and the most common genetic disorder in Ashkenazi Jews, up to 6% of whom are carriers of *GBA* mutations (Horowitz *et al.*, 1999). Among Ashkenazi Jews, the vast majority of patients with Gaucher disease carry at least one N370S mutation, a missense mutation that retains 30% of glucocerebrosidase enzymatic activity and is linked to Type 1 Gaucher disease, the milder non-neuronopathic form. Here and thereafter, we use the term *GBA* mutation to describe pathogenic changes in the *GBA* gene (previously associated with Gaucher disease), a polymorphism if it is clearly not associated with Gaucher disease, or a variant if it is unclear or controversial (e.g. E326K).

The mechanism by which *GBA* mutations are linked to Parkinson's disease remains unknown. As mutations lead to a reduction in glucocerebrosidase enzymatic activity, and more 'severe' mutations (e.g. 84GG) are associated with higher risk for Parkinson's disease compared to 'milder' mutations (e.g. N370S; Gan-Or *et al.*, 2008), loss of glucocerebrosidase enzymatic activity may be a pathogenic mechanism. However, the fact that most *GBA* homozygotes and compound heterozygotes (i.e. patients with Gaucher disease) will never develop Parkinson's disease (Rosenbloom *et al.*, 2011; Alcalay *et al.*, 2014) in spite of diminished glucocerebrosidase enzymatic activity, while *GBA* heterozygotes are at a higher risk for Parkinson's disease compared to controls, suggests an additional mechanism (Mazzulli *et al.*, 2011; Sidransky and Lopez, 2012). Little is known about glucocerebrosidase enzymatic activity in carriers of specific heterozygous *GBA* mutations, and there is currently no evidence of sphingolipid accumulation in heterozygotes (as opposed to patients with Gaucher disease); however, increased production of cytosolic reactive oxygen species has been demonstrated in fibroblasts from *GBA* heterozygotes with and without Parkinson's disease (McNeill *et al.*, 2014). Glucocerebrosidase enzymatic activity measurement in dried blood spots is a validated methodology (Zhang *et al.* 2008), which is clinically approved to screen for Gaucher disease. Here, our aims were to measure glucocerebrosidase enzymatic activity in dried blood spots from Parkinson's disease patients with and without *GBA* mutations and from controls to (i) assess whether glucocerebrosidase enzymatic activity is reduced in heterozygote *GBA* carriers with Parkinson's disease compared to non-carriers; and (ii) explore the association between glucocerebrosidase enzymatic activity, Parkinson's disease risk, and Parkinson's disease severity.

Materials and methods

Participants and clinical evaluation

Participants in the study ('SPOT') included Parkinson's disease patients and non-blood related controls (mostly spouses) from the Center for Parkinson's Disease at Columbia University Medical Center in New York, NY. The cohort's demographics (but not genotype) were previously described in a report of participants' attitude towards genetic testing (Sakanaka *et al.*, 2014). In brief, a blood sample and demographics including Ashkenazi Jewish ancestry, medical history, medication, Parkinson's disease family history (Marder *et al.*, 2003), the Unified Parkinson's Disease Rating Scale (UPDRS; Fahn, 1987) in the 'ON' state and the Montreal Cognitive Assessment (Nasreddine *et al.*, 2005) were collected from consecutive Parkinson's disease cases, as defined by the United Kingdom Parkinson's Disease Brain Bank criteria (except that we did not exclude cases with a family history of Parkinson's disease) (Hughes *et al.*, 1992), and a convenience sample of consecutive non-blood related control individuals, mostly spouses.

Evaluation of Parkinson's cases and controls was identical. All study procedures were approved by the Columbia University institutional review board, and all participants signed informed consent.

Sequencing and genotyping of *GBA* mutations

DNA was extracted using a standard salting-out method. All participants were genotyped for *GBA* mutations in two ways. First, participants were screened as previously described for 10 *GBA* mutations and variants (Alcalay *et al.*, 2013, 2014), and, given the high prevalence of *LRRK2* G2019S in the Ashkenazi Jewish population (Alcalay *et al.*, 2013), all study participants were genotyped for *LRRK2* G2019S mutation (Alcalay *et al.*, 2013).

We subsequently fully sequenced the *GBA* gene in all patients with Parkinson's disease and controls, regardless of whether any of the 10 *GBA* mutations/variants were found. Supplementary Table 1 details the primers and conditions used for the amplification and sequencing of the *GBA* gene. PCRs were performed using the AmpliTaq Gold® DNA Polymerase (Applied Biosystems) according to the manufacturer's instructions. PCR products were sequenced using a 3730XL DNAnalyzer (Applied Biosystems) and the chromatograms were analysed using the Genalys 3.3b software (Takahashi *et al.*, 2003). The success rate of the sequencing was 96%, and all mutations detected by screening were replicated by sequencing. For Parkinson's disease cases with compound heterozygote *GBA* mutations/variants, family members were analysed to confirm whether the mutations were in *cis* (same allele) or *trans* (opposite allele).

Glucocerebrosidase enzymatic activity assay

Dried blood spots were obtained as previously described (Olivova *et al.*, 2008; Reuser *et al.*, 2011). In brief, blood samples were collected in a 10 cm^3 EDTA tube. Seventy-five microlitres of blood was 'spotted' on each of five circles on a filter paper (Whatman® 903 protein saver card) and dried at room temperature for at least 4 h. Absorbent filter paper was then stored in a sealed plastic bag with desiccants and a humidity indicator in a −20°C freezer and later shipped to the laboratories at room temperature. Upon receipt, the samples were stored at −80°C before analysis.

Glucocerebrosidase enzymatic activity was measured using a previously published protocol as part of a multiplex assay together with four additional lysosomal enzymes (Zhang *et al.*, 2008). In summary, glucocerebrosidase was extracted from a 3.2-mm diameter punch from a dried blood spot sample in 70 µl of 20 mM sodium phosphate buffer (pH 7.1) on a 96-well plate. Ten microlitres of dried blood spot extract was added to 15 µl of glucocerebrosidase substrate/internal standard mixtures (The Center for Disease Control and Prevention, Georgia, Atlanta), 0.67 mM of C12-glucocerebroside and 13.33 µM C14-ceramide in the citrate-phosphate (0.31/0.620 M) buffer (Sigma) with sodium taurocholate (16 g/l, Sigma). The substrate has previously been selected because it does not exist in human blood, and due to the fact it has a similar structure to the smallest natural glucocerebrosidase substrate. Sealed plates were incubated on an orbital shaker at 37°C for 20 h. Reactions were quenched with 100 µl of organic solution (ethyl acetate:methanol, 1:1) following liquid-liquid and solid phase extractions. The samples were dried under nitrogen, sealed and stored at −20°C. Prior to tandem mass spectrometry (MS/MS) analysis, plates were thawed and reconstituted with 200 µl of a solvent mixture (80:20 acetonitrile:water containing 0.2% formic acid).

All analyses were monitored on an API 4000 triple quadrupole mass spectrometer (ABSciex) by selected ion monitoring mode (Multiple Reaction Monitoring, MRM). The enzyme activity of each sample was calculated from the ion abundance ratio of product to internal standard as measured by the mass spectrometer. Background activity of a blank filter paper was subtracted from the dried blood spot activity. Activity was expressed as micromoles of product per litre of whole blood per hour (µmol/l/h). Two quality control samples with previously established activity levels for each enzyme and disease positive samples were included in each plate for quality control. All Genzyme scientists were blinded to Parkinson's disease and genetic status.

Statistical analysis

Demographics, level of glucocerebrosidase enzymatic activity and frequency of *GBA* mutations were compared between Parkinson's disease cases and controls using the Student *t*-test for continuous variables, and the chi-square and Fisher's exact tests for categorical variables.

Including Parkinson's disease cases and controls, glucocerebrosidase enzymatic activity was compared among individuals with two *GBA* mutations (Gaucher disease patients and compound heterozygotes of a mutation and a variant), *GBA* heterozygotes and non-carriers by the Student *t*-test and ANOVA. In addition, we compared heterozygote carriers of each *GBA* mutation and variant, as well as of the *LRRK2* G2019S mutation, separately to non-*GBA* non-*LRRK2* carriers.

To test whether glucocerebrosidase enzymatic activity was associated with demographic characteristics, we analysed glucocerebrosidase enzymatic activity by age, sex and Ashkenazi Jewish ancestry using the Pearson correlation coefficient and *t*-tests as appropriate. Analyses were performed on controls only and repeated including the entire cohort (including Parkinson's disease cases and *GBA* and *LRRK2* mutation carriers).

We used the Student *t*-test to compare glucocerebrosidase enzymatic activity between Parkinson's disease cases and controls, first including all participants, and second after excluding: (i) *GBA* and *LRRK2* G2019S mutation carriers; (ii) those with a family history of Parkinson's disease in a first degree relative; and (iii) patients with Parkinson's disease with disease age at onset $\leqslant$40 years to exclude potential Parkinson's disease cases with parkin (*PARK2*), *PINK1* (also known as *DJ-1*) mutations, which were not tested. To examine the association between glucocerebrosidase enzymatic activity (predictor) and Parkinson's disease status (outcome), we constructed logistic regression models. To do so, glucocerebrosidase enzymatic activity was normalized to the batch in which the samples were analysed. Each batch included at least three replicates of two quality control controls with previously established activity

ranges. The activity of each sample was divided by the mean activity of the quality control samples from the same run. Logistic models included normalized glucocerebrosidase enzymatic activity, age, and gender as predictors.

To test the association between glucocerebrosidase enzymatic activity and Parkinson's disease characteristics in non-*GBA* non-*LRRK2* G2019S carriers we divided the non-*GBA* non-*LRRK2* G2019S Parkinson's disease cohort into tertiles based on glucocerebrosidase enzymatic activity. The Student *t*-test and chi square test were used to compare demographics and disease characteristics among the tertiles. A multivariate logistic regression model was constructed to test the association between membership in the higher tertile of glucocerebrosidase enzymatic activity (outcome) and demographics and disease characteristics (predictor).

Analyses were performed using SPSS Statistics version 19.0 software.

Results

Cohort characteristics

The study included 517 Parkinson's disease patients and 252 controls. Parkinson's disease patients and controls were similar in age, education and Ashkenazi Jewish ancestry, but by design (using spouse controls) patients with Parkinson's disease were more likely to be male than controls (Table 1). Patients with Parkinson's disease had mean disease duration of 6.8 ± 6.3 years and were more likely than controls to carry the *LRRK2* G2019S mutation (7.5% versus 0.8%, $P < 0.001$) and *GBA* mutations or variants (17.0% versus 6.7%, $P < 0.001$). Among *GBA* variant/mutation carriers, 53.4% (47 of 88) of Parkinson's disease participants and 35.2% (6 of 17) of control participants (carriers without Parkinson's) reported at least one Ashkenazi Jewish grandparent.

GBA mutations and glucocerebrosidase enzymatic activity

Seven patients with Parkinson's disease were found to have two *GBA* mutations or variants. Four of these seven were N370S homozygotes and one was a compound heterozygote for the N370S and A456P/L444P mutations, consistent with Gaucher disease. Two carried a T369M variant in addition to either N370S or 84GG. In both cases, analysis of family members confirmed that the T369M variant was in *trans* to the other mutation. Two N370S homozygotes and the 84GG/T369M compound heterozygote Parkinson's disease patients were first identified through this study and were referred for clinical testing and evaluation. The frequency of *GBA* and *LRRK2* G2019S mutations by Parkinson's disease status is presented in Table 2.

As expected, glucocerebrosidase enzymatic activity in *GBA* homozygotes/compound heterozygotes was significantly lower and did not overlap with activity in *GBA* heterozygotes or non-carriers (Fig. 1). Mean glucocerebrosidase enzymatic activity in heterozygotes [including patients with Parkinson's ($n = 81$) and heterozygotes without Parkinson's disease ($n = 17$), $n = 98$] was lower than in non-*GBA* non-*LRRK2* carriers (7.88 µmol/l/h versus 11.93 µmol/l/h, $P < 0.001$) and higher than in *GBA* homozygotes/compound heterozygotes (7.88 µmol/l/h versus 0.85 µmol/l/h, $P < 0.001$). When each *GBA* mutation or variant group was compared independently to non-*GBA* non-*LRRK2* carriers, glucocerebrosidase enzymatic activity was lower in each of the mutations or variants which were present in at least four participants (N370S, L444P, 84GG, R496H, E326K, T369M; Table 3 and Supplementary Table 3), and was higher when compared independently with glucocerebrosidase enzymatic activity in *GBA* homozygotes/compound heterozygotes. Glucocerebrosidase enzymatic activity was higher in the 36 *LRRK2* G2019S carriers who did not carry *GBA* mutations than in non-*GBA* non-*LRRK2* carriers.

Glucocerebrosidase enzymatic activity by age, gender, ancestry and Parkinson's disease status

We examined whether age and gender are associated with glucocerebrosidase enzymatic activity among controls with no *GBA* and no *LRRK2* G2019S mutations. There was no correlation between glucocerebrosidase enzymatic activity and age (Pearson correlation < 0.001, $P = 0.998$) and there was no difference in enzymatic activity by gender (males 12.03 µmol/l/h, females 12.08 µmol/l/h, $P = 0.914$). There was no association between age, gender and glucocerebrosidase enzymatic activity when analyses were repeated including all participants (i.e. including Parkinson's cases and mutation carriers). We further compared glucocerebrosidase enzymatic activity between those with four Ashkenazi Jewish grandparents ($n = 222$) and those without Ashkenazi Jewish grandparents ($n = 381$), excluding those with mixed ancestry, *GBA* or *LRRK2* mutations. There was no difference in glucocerebrosidase activity (11.99 µmol/l/h versus 11.73 µmol/l/h, $P = 0.343$) between Ashkenazi Jews and non-Jews.

Mean glucocerebrosidase enzymatic activity was modestly lower in Parkinson's disease patients ($n = 517$) than in controls ($n = 252$) (11.14 µmol/l/h versus 11.85 µmol/l/h, $P = 0.011$), but ranges overlap (Parkinson's disease glucocerebrosidase enzymatic range: 0.005–30.30 µmol/l/h; control range: 4.45–25.95µmol/l/h). When analyses were repeated excluding *GBA* and *LRRK2* G2019S mutation carriers, all those with a family history of Parkinson's disease, and Parkinson's disease patients with disease age at onset ⩽40, glucocerebrosidase enzymatic activity was lower in Parkinson's participants ($n = 301$) than in controls ($n = 219$) (11.53 µmol/l/h, range: 5.20–24.27 µmol/l/h versus 12.11 µmol/l/h, range: 5.07–25.95 µmol/l/h,

Table 1 Demographics, *GBA* mutation status and glucocerebrosidase enzymatic activity in Parkinson's disease cases and controls

		Parkinson's cases ($n = 517$)	Controls ($n = 252$)	*P*-value
Mean age in years, (SD)		66.0 (10.5)	65.3 (9.6)	0.324
Males, % (*n*)		63.4 (328)	33.3 (84)	<0.001
Subjects with at least one Ashkenazi Jewish grandparent, % (*n*)		44.7 (231)	38.5 (97)	0.327
Subjects with family history of Parkinson's disease in first-degree relative, % (*n*)[a]		18.2 (92)	4.8 (12)	<0.001
LRRK2 G2019S carriers, % (*n*)		7.5 (39)	0.8 (2)	<0.001
GBA mutation/variant status, % (*n*)	2 mutations/variants	1.4 (7)[b]	0.0 (0)	<0.001
	1 mutation/variant	15.7 (81)	6.7 (17)	
	0 mutations/variants	83.0 (429)	93.3 (235)	
Mean glucocerebrosidase enzymatic activity in μmol/l/h, (SD)		11.14 (3.77)	11.85 (3.40)	0.011
Education in years, (SD)		16.6 (2.9)	16.7 (2.7)	0.746
UPDRS part III, (SD)		18.0 (10.6)	1.0 (1.9)	<0.001
Montreal Cognitive Assessment, (SD)		25.2 (3.7)	27.0 (2.2)	<0.001
Mean Parkinson's age-at-onset, (SD)		59.2 (11.6)		
Levodopa equivalent daily dose in mg, (SD)		539 (461)		

[a]Family history information was not available on 12 Parkinson's disease cases and four controls.
[b]Two of the Parkinson's disease cases with two mutations/variants carried the T369M variant in addition to the N370S or 84GG mutations.

Table 2 Frequency of *GBA* and *LRRK2* G2019S mutations and variants by Parkinson's disease status

	Parkinson's cases ($n = 517$)	Controls ($n = 252$)	*P*-value
GBA homozygotes and compound heterozygotes	1.4% (7)	0.0% (0)	<0.001
All *GBA* heterozygotes	15.7% (81)	6.7% (17)	<0.001
N370S	7.0% (36)[a]	1.6% (4)	0.004
L444P	1.4% (7)	0.4% (1)	0.284
84GG	0.8% (4)	0.0% (0)	0.309
R496H	0.8% (4)	0.0% (0)	0.309
IVS2 + 1	0.4% (2)	0.0% (0)	<1
K-27R	0.4% (2)	0.0% (0)	<1
E326K variant	2.5% (13)	1.2% (3)	0.289
T369M variant	1.0% (5)	1.6% (4)	0.486
Other *GBA* variants or mutations[b]	1.6% (8)	2.0% (5)	n/a
LRRK2 G2019S	7.5% (39)[a]	0.8% (2)	<0.001
Non-*GBA* non-*LRRK2* carriers	75.4% (395)	92.4%(233)	<0.001

[a]Four *LRRK2* G2019S carriers also carried the *GBA* N370S mutation and one carried the *GBA* R44C variant.
[b]Thirteen *GBA* variants and mutations were present in only one study participant. Eight in Parkinson's disease cases (L461P, V294M, A456P, G241R, rearrangement on exon 8, Q-8H, R44C, and N392S) and five in controls (S110A, T410M, F-36V, P387P, and E349K). See Supplementary Table 2.

$P = 0.036$). Glucocerebrosidase enzymatic activity differences between Parkinson's cases and controls did not reach statistical significance when we excluded all *GBA* carriers but not G2019S carriers. Normalized glucocerebrosidase enzymatic activity (predictor) was associated with Parkinson's status (outcome) in logistic regression models adjusted for age and gender [odds ratio (OR) = 1.9, 95% confidence interval (CI) = 1.2–3.0, $P = 0.003$]. The association was maintained (OR = 2.2, 95% CI = 1.2–4.0, $P = 0.012$) when mutation carriers were excluded [excluding *GBA* and *LRRK2* G2019S mutation carriers, those with first degree family history of Parkinson's, as well as Parkinson's disease participants with age at onset $\leqslant 40$ ($n = 36$)].

Glucocerebrosidase enzymatic activity and Parkinson's phenotype in idiopathic Parkinson's disease

To test the association between glucocerebrosidase enzymatic activity and Parkinson's disease phenotype, we divided the Parkinson's disease cases who were non-*GBA* non-*LRRK2* G2019S carriers into tertiles based on glucocerebrosidase enzymatic activity. Comparison of the demographics and disease characteristics among the tertiles is presented in Table 4. Higher glucocerebrosidase enzymatic activity was associated with male gender, younger age at onset, longer disease duration, higher daily levodopa

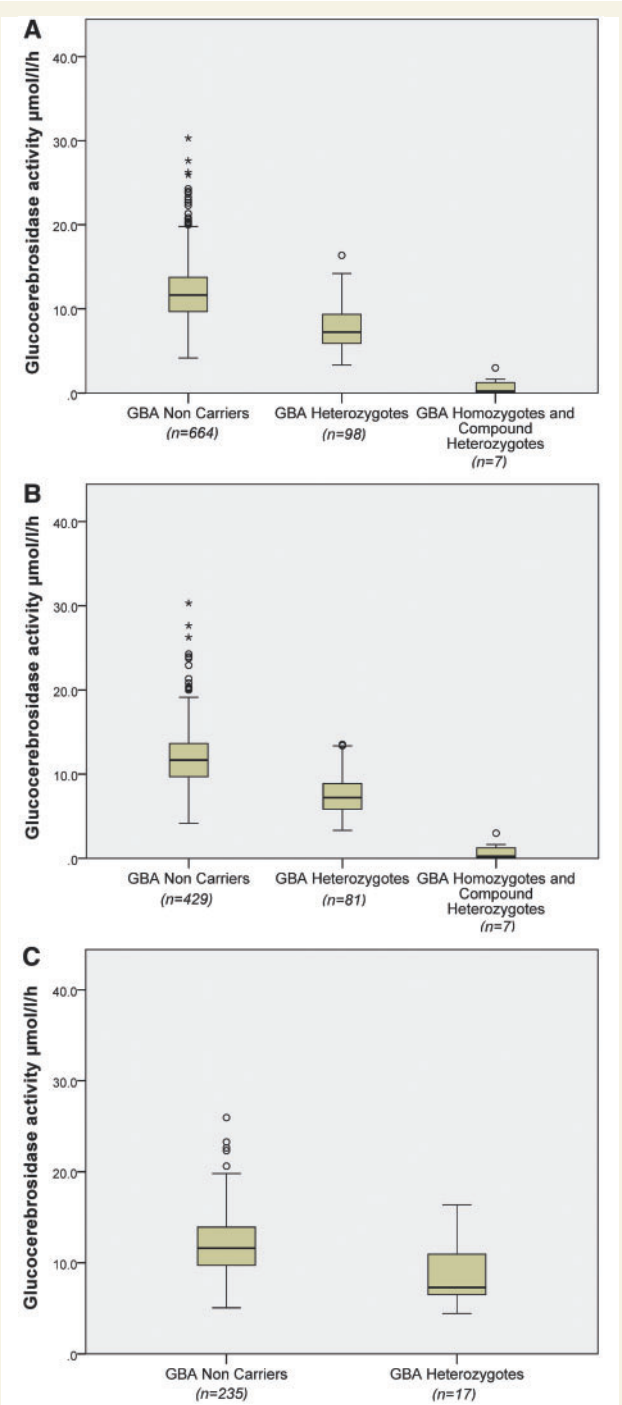

Figure 1 Glucocerebrosidase enzymatic activity in Parkinson's participants and controls stratified according to *GBA* status: non-*GBA* mutation carriers, heterozygote carriers (of variants and or mutations) and carriers of two mutations (of variants or mutations). (**A**) entire cohort ($n = 769$); (**B**) Parkinson's participants only ($n = 517$); (**C**) controls only ($n = 252$).

dose and worse performance on the UPDRS and Montreal Cognitive Assessment.

Given the different characteristics among the tertiles we constructed a multivariate logistic regression model in which membership in the highest glucocerebrosidase enzymatic activity tertile (versus the lower and middle tertiles) was the outcome, and sex, age, disease duration, family history of Parkinson's disease, education, levodopa equivalent daily dose, UPDRS-III and Montreal Cognitive Assessment were the predictors. In the model, younger age at exam (OR = 0.98, 95% CI = 0.95–1.00, $P = 0.048$), longer Parkinson's disease duration (OR = 1.07, 95% CI = 1.03–1.11, $P = 0.002$), and lower Montreal Cognitive Assessment scores (OR = 0.92, 95% CI = 0.86–0.99, $P = 0.026$) were significantly associated with the highest glucocerebrosidase enzymatic activity tertile.

Discussion

Better understanding of the link between *GBA* mutations and Parkinson's disease may shed light on the pathophysiology of Parkinson's disease and lead to novel therapeutic approaches for treating the disease. This is the first study to measure glucocerebrosidase enzymatic activity in dried blood spots in Parkinson's disease cases with and without *GBA* mutations and in controls. Our findings show that *GBA* heterozygotes with Parkinson's disease have lower glucocerebrosidase enzymatic activity than controls, and that low enzymatic activity is modestly associated with Parkinson's disease even among those without *GBA* or *LRRK2* mutations. These findings suggest that low glucocerebrosidase enzymatic activity may be a risk factor for Parkinson's disease.

Glucocerebrosidase enzymatic activity by mutation status

Glucocerebrosidase enzymatic activity is routinely tested during the workup for Gaucher disease, but little is known about enzymatic activity in healthy individuals or in heterozygotes; it is unknown how glucocerebrosidase enzymatic activity is regulated, or how gender and age affect enzymatic activity. The reports on glucocerebrosidase enzymatic activity in *GBA* heterozygotes predate genetic testing, and describe reduced activity in family members who are obligate carriers (Raghavan *et al.*, 1980; Svennerholm *et al.*, 1980; Grabowski *et al.*, 1982). Consistent with these reports, we observed lower mean glucocerebrosidase enzymatic activity in heterozygotes when compared to non-carriers, with overlapping ranges of activity. Here, we report that glucocerebrosidase enzymatic activity in dried blood spots measured by tandem mass spectrometry can accurately distinguish between patients with Gaucher disease and unaffected individuals (*GBA* heterozygotes or non-carriers). We also show that the mean enzymatic activity among heterozygotes is significantly lower than that of both non-carriers with Parkinson's disease and controls. Surprisingly, when each mutation was tested individually, carriers of mutations that are considered 'milder', i.e. associated with non-neuronopathic Gaucher disease (including N370S and R496H), had similar if not lower enzymatic

Table 3 Glucocerebrosidase enzymatic activity by *GBA* and *LRRK2* G2019S mutation status[a]

Mutation status	*n*	Mean glucocerebrosidase enzymatic activity in μmol/l/h (SD)	Range	*P*-value (compared to non-carriers)
GBA homozygotes and compound heterozygotes	7	0.85 (1.11)	0.005–3.00	<0.001
N370S	40	6.42 (1.72)	3.23–11.72	<0.001
L444P	8	7.66 (2.28)	4.88–11.26	<0.001
84GG	4	7.13 (1.29)	5.46–8.47	0.003
R496H	4	8.10 (2.52)	4.83–10.92	0.018
IVS2 + 1	2	7.97 (1.00)	7.26–8.67	0.082
E326K	16	9.81 (2.87)	5.2–16.37	0.009
T369M	9	7.64 (1.71)	6.12–11.10	<0.001
LRRK2 G2019S[b]	36	13.69 (4.84)	8.70–30.30	0.002
Non-*GBA* and non-*LRRK2* carriers	628	11.93 (3.21)	4.17–25.95	

[a]Including both Parkinson's disease and control participants.
[b]Four *LRRK2* G2019S carriers that carried the N370S mutation and one that carried the R44C variant were removed from the analysis. Their glucocerebrosidase enzymatic activity was similar to other *GBA* mutation carriers (mean 7.3 ± 2.7 μmol/l/h).

Table 4 Demographics and Parkinson's disease characteristics by glucocerebrosidase enzymatic activity tertiles among Parkinson's patients who are non-carriers of *GBA* or *LRRK2* G2019S mutations (*n* = 395)

	Lower glucocerebrosidase enzymatic tertile (*n* = 131)	Middle glucocerebrosidase enzymatic tertile (*n* = 132)	Higher glucocerebrosidase enzymatic tertile (*n* = 132)	*P*-value[a]
Mean glucocerebrosidase enzymatic activity in μmol/l/h, (SD) [range]	8.59 (1.19) [4.17–10.39]	11.55 (0.68) [10.40–12.83]	15.26 (2.39) [12.84–24.27]	<0.001
Males, % (*n*)	55.7 (73)	67.4 (89)	69.7 (92)	0.040
First-degree family history, % (*n*)	14.7 (19)	14.7 (19)	20.6 (27)	0.340
Mean age in years, (SD)	66.7 (9.6)	66.0 (10.6)	65.0 (12.2)	0.429
Mean age-at-Parkinson's disease onset, years (SD)	60.8 (10.1)	60.6 (11.4)	56.0 (13.5)	0.001
Disease duration, years (SD)	5.8 (5.6)	5.5 (4.7)	9.0 (8.1)	<0.001
Education in years, (SD)	16.7 (2.9)	16.6 (3.1)	16.4 (2.4)	0.723
Levodopa equivalent daily dose in mg, (SD)	466 (410)	482 (407)	651 (549)	0.002
UPDRS-III, (SD)	16.2 (9.8)	18.1 (10.0)	19.3 (10.9)	0.046
Montreal Cognitive Assessment, (SD)	25.9 (3.0)	25.6 (3.8)	24.5 (4.1)	0.004

[a]*P*-value obtained by the chi square test comparing the three tertiles.

activity than heterozygote carriers of 'severe' mutations (including L444P, 84GG and IVS-2 + 1). In addition, we show that two *GBA* variants, E326K and T369M, whose contribution to Gaucher disease in the homozygote state is controversial (Horowitz *et al.*, 2011), are associated with lower enzymatic activity than non-carriers. The *GBA* E326K and T369M variants have been associated with Parkinson's disease in some but not all studies (Clark *et al.*, 2007, 2009; Nichols *et al.*, 2009; Duran *et al.*, 2013). The observation in this study may help explain the reported association between these mutations and Parkinson's disease (Clark *et al.*, 2007; Duran *et al.*, 2013), if this link is mediated through reduced glucocerebrosidase enzymatic activity. It is important to note that most heterozygotes in this study (82.7%, Table 1) are Parkinson's disease cases, and glucocerebrosidase enzymatic activity in heterozygotes without Parkinson's disease was available only in 17 cases, most of whom carried the E326K and T369M variants or variants of unknown significance (Supplementary Table 2).

Glucocerebrosidase enzymatic activity and Parkinson's disease

When the entire cohort was included, mildly lower glucocerebrosidase enzymatic activity was observed in Parkinson's disease after adjustment for age and gender. The association persisted after excluding *GBA* mutation carriers, suggesting that reduced glucocerebrosidase enzymatic activity may be independently associated with Parkinson's disease. However, the mechanism of the association was not studied here. We did not test whether glucocerebrosidase expression was reduced in Parkinson's cases compared to controls. Other factors (e.g. genes that modify glucocerebrosidase enzymatic activity such as

SCARB2; Velayati *et al.*, 2011), may also increase the risk for Parkinson's disease. In addition, the role of the glucocerebrosidase activators—e.g. Saposin C (Salvioli *et al.*, 2000)—in Parkinson's risk is unknown. Measuring Saposin C levels in lysosomes of patients with Parkinson's disease and controls may further clarify the role of Saposin C in Parkinson's risk. Of special interest are those without *GBA* mutations who have glucocerebrosidase enzymatic activity lower than heterozygotes. Exploring the cause of lower enzymatic activity in this group (Supplementary material) may shed light on glucocerebrosidase modifiers. However, reduced glucocerebrosidase enzymatic activity alone is not a sufficient cause of Parkinson's disease, given that most patients with Gaucher disease, who by definition have diminished enzymatic activity, will never develop Parkinson's disease (Rosenbloom *et al.*, 2011; Alcalay *et al.*, 2014). Moreover, reduced glucocerebrosidase enzymatic activity is not a necessary cause of Parkinson's disease, as there is a significant overlap in glucocerebrosidase enzymatic activity between Parkinson's disease cases and controls. Therefore, glucocerebrosidase enzymatic activity alone cannot be used as a biomarker or a screening tool to identify Parkinson's disease.

The pathophysiological mechanism of the link between *GBA* mutations, reduced glucocerebrosidase activity and Parkinson's disease remains unclear. Several studies have found an increase in alpha synuclein levels and/or aggregation, both *in vitro* and *in vivo*, in the setting of mutant *GBA* expression or glucocerebrosidase loss of function (Cullen *et al.*, 2011; Mazzulli *et al.*, 2011; Sardi *et al.*, 2011; Woodard *et al.*, 2014). However, the relative contribution of loss of glucocerebrosidase activity versus toxic gain of function remains controversial. Interestingly, one study found that the glucocerebrosidase substrate glucosylceramide can bind to alpha-synuclein oligomers (Mazzulli *et al.*, 2011). Here, we did not measure glucocerebrosidase substrates in our samples, and future studies are needed to evaluate if the reduced glucocerebrosidase activity is also associated with higher levels of substrate accumulation.

Low glucocerebrosidase enzymatic activity has been previously reported in Parkinson's disease and Lewy body dementia in CSF (Balducci *et al.*, 2007; Parnetti *et al.*, 2009, 2014) and in two brain autopsy studies (Gegg *et al.*, 2012; Murphy *et al.*, 2014), findings which are consistent with our observation. Gegg *et al.* measured glucocerebrosidase enzymatic activity in brains of *GBA* heterozygotes ($n = 14$), sporadic Parkinson's disease ($n = 14$) and controls ($n = 10$). Compared to controls, they found that *GBA* heterozygotes had decreased glucocerebrosidase enzymatic activity in all brain regions except for the frontal cortex, and sporadic Parkinson's disease brains also showed lower glucocerebrosidase enzymatic activity in the substantia nigra and cerebellum. Murphy *et al.* (2014) examined neuropathological tissue of sporadic Parkinson's brains ($n = 19$) and age matched controls ($n = 10$) all without *GBA* mutations. Both glucocerebrosidase protein and enzymatic activity were reduced in the anterior cingulate gyrus of Parkinson's cases when compared to controls. The importance of our finding that glucocerebrosidase enzymatic activity is lower in dried blood spots of Parkinson's disease cases versus controls is that blood samples can be obtained prospectively and longitudinally (as opposed to the retrospective nature of autopsy samples), and in a simpler and less invasive procedure than lumbar puncture (Olivova *et al.*, 2008; Reuser *et al.*, 2011). The marked differences in mean glucocerebrosidase enzymatic activity among patients with Gaucher disease, heterozygotes and non-*GBA* carriers further validates the reliability of glucocerebrosidase enzymatic activity measurement in dried blood spots. The correlation between glucocerebrosidase enzymatic activity in dried blood spots and glucocerebrosidase enzymatic activity in spinal fluid, and more importantly in brain parenchyma, remains unknown and should be explored in follow-up studies.

We also tested the association between glucocerebrosidase enzymatic activity and Parkinson's disease characteristics and severity markers. Based on the more severe Parkinson's disease phenotype of *GBA* carriers compared to non-carriers (Neumann *et al.*, 2009; Alcalay *et al.*, 2012), we hypothesized that among non-carriers, high glucocerebrosidase enzymatic activity would be associated with milder disease severity. Higher glucocerebrosidase enzymatic activity was associated with male gender, younger age at onset, longer disease duration, higher daily levodopa dose and worse performance on the UPDRS and Montreal Cognitive Assessment, which are markers of more advanced Parkinson's disease. In regression models, only age, disease duration and Montreal Cognitive Assessment performance were associated with high glucocerebrosidase enzymatic activity. Worse Montreal Cognitive Assessment score may represent worse cognitive functioning. However, longer Parkinson's disease duration in a cross-sectional study may be viewed as a marker of a more benign form of Parkinson's disease, where those with the potentially protective effect of high glucocerebrosidase enzymatic activity were more likely to survive and participate in the study. A similar phenomenon was observed in our report on parkin-Parkinson's disease (Alcalay *et al.*, 2012). Therefore, our finding of longer disease duration among Parkinson's disease cases in the higher tertile of glucocerebrosidase enzymatic activity may be interpreted as a marker of a milder disease course. However, we cannot conclude whether higher glucocerebrosidase enzymatic activity is associated with a milder course (earlier age-at-onset, but longer disease duration and slower progression). Longitudinal follow up including comprehensive evaluation is required to conclude whether glucocerebrosidase enzymatic activity may be a marker of disease severity in idiopathic Parkinson's disease.

Glucocerebrosidase enzymatic activity in *LRRK2* G2019S carriers

Interestingly, we found that *LRRK2* G2019S carriers have higher glucocerebrosidase enzymatic activity than

non-carriers (Table 3), even higher than controls without Parkinson's disease. This finding will require replication. The mechanism by which glucocerebrosidase enzymatic activity is increased in dried blood spots of *LRRK2* carriers is unknown. One possible explanation is that mutant LRRK2 may cause lysosomal compartment expansion (Orenstein *et al.*, 2013), which in turn is associated with increased glucocerebrosidase enzymatic activity. Alternatively, it is possible that mutated LRRK2 affects retromer function and thus alters sorting and turnover of glucocerebrosidase (MacLeod *et al.*, 2013).

The strengths of our study include the large number of participants, all of whom were carefully evaluated by a single movement disorders specialist for research purposes, and the simplicity of obtaining the biological product, the dried blood spot. However, we acknowledge that the Parkinson's disease and control groups were not matched for gender (which is not associated with glucocerebrosidase activity) and we also have a relatively smaller number of male controls ($n = 84$). The differential glucocerebrosidase enzymatic activity in heterozygotes and non-carriers (as performed in a laboratory that was blinded to genotype) further validates the glucocerebrosidase enzymatic activity measurement.

The major limitation of our study is its cross-sectional nature. We cannot conclude if high enzymatic activity is associated with a milder disease (Alcalay *et al.*, 2012). The lack of longitudinal data limits our ability to assess potential changes in glucocerebrosidase enzymatic activity because of Parkinson's disease treatment (levodopa) or as Parkinson's disease progresses. We cannot conclude if low glucocerebrosidase enzymatic activity is an independent risk factor for Parkinson's disease unless controls or *GBA* carriers with low activity are followed. Comparison of glucocerebrosidase enzymatic activity between older *GBA* heterozygotes with and without Parkinson's disease would help determine if lower glucocerebrosidase enzymatic activity is associated with Parkinson's disease in this population and may be used as a biomarker for increased Parkinson's disease risk.

Longitudinal follow-up would allow us to conclude whether higher glucocerebrosidase enzymatic activity is associated with a slower rate of Parkinson's disease progression and whether progression differs by type of *GBA* mutation.

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Conflict of interest

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Supplementary material

Supplementary material is available at *Brain* online.

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