RESEARCH ARTICLE

Role of Lysosomal Gene Variants in Modulating *GBA*-Associated Parkinson's Disease Risk

Letizia Straniero, PhD,[1,2] Valeria Rimoldi, PhD,[1,2] Edoardo Monfrini, MD,[3,4] Salvatore Bonvegna, MD,[5] Giada Melistaccio, MSc,[1] Julie Lake, BSc,[6] Giulia Soldà, PhD,[1,2] Massimo Aureli, PhD,[7] Shankaracharya, PhD,[8] Pamela Keagle, BSc,[8] Tatiana Foroud, PhD,[9] John E. Landers, PhD,[8] Cornelis Blauwendraat, PhD,[6] Anna Zecchinelli, MD,[5] Roberto Cilia, MD,[10] Alessio Di Fonzo, MD, PhD,[3,4] Gianni Pezzoli, MD,[5,11] Stefano Duga, PhD,[1,2] and Rosanna Asselta, PhD[1,2]*

[1]*Department of Biomedical Sciences, Humanitas University, Milan, Italy*
[2]*Humanitas Clinical and Research Center, IRCCS, Milan, Italy*
[3]*IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Neurology Unit, Milan, Italy*
[4]*Dino Ferrari Center, Neuroscience Section, Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy*
[5]*Parkinson Institute, ASST Gaetano Pini-CTO, Milan, Italy*
[6]*Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA*
[7]*Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy*
[8]*Department of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts, USA*
[9]*Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana, USA*
[10]*Fondazione IRCCS Istituto Neurologico Carlo Besta, Parkinson and Movement Disorders Unit, Milan, Italy*
[11]*Fondazione Grigioni per il Morbo di Parkinson, Milan, Italy*

ABSTRACT: Background: To date, variants in the *GBA* gene represent the most frequent large-effect genetic factor associated with Parkinson's disease (PD). However, the reason why individuals with the same *GBA* variant may or may not develop neurodegeneration and PD is still unclear.

Objectives: Therefore, we evaluated the contribution of rare variants in genes responsible for lysosomal storage disorders (LSDs) to *GBA*-PD risk, comparing the burden of deleterious variants in LSD genes in PD patients versus asymptomatic subjects, all carriers of deleterious variants in *GBA*.

Methods: We used a custom next-generation sequencing panel, including 50 LSD genes, to screen 305 patients and 207 controls (discovery cohort). Replication and meta-analysis were performed in two replication cohorts of *GBA*-variant carriers, of 250 patients and 287 controls, for whom exome or genome data were available.

Results: Statistical analysis in the discovery cohort revealed a significantly increased burden of deleterious variants in LSD genes in patients ($P = 0.0029$). Moreover, our analyses evidenced that the two strongest modifiers of *GBA* penetrance are a second variation in *GBA* (5.6% vs. 1.4%, $P = 0.023$) and variants in genes causing mucopolysaccharidoses (6.9% vs. 1%, $P = 0.0020$). These results were confirmed in the meta-analysis, where we observed pooled odds ratios of 1.42 (95% confidence interval [CI] = 1.10–1.83, $P = 0.0063$), 4.36 (95% CI = 2.02–9.45, $P = 0.00019$), and 1.83 (95% CI = 1.04–3.22, $P = 0.038$) for variants in LSD genes, *GBA*, and mucopolysaccharidosis genes, respectively.

Conclusion: The identification of genetic lesions in lysosomal genes increasing PD risk may have important implications in terms of patient stratification for future therapeutic trials. © 2022 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson Movement Disorder Society.

Key Words: Parkinson's disease; *GBA;* lysosomal genes; mutation burden

***Correspondence to:** Prof. Rosanna Asselta, Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini, 4, Pieve Emanuele, Milan, Italy; E-mail: rosanna.asselta@hunimed.eu

Previous affiliation for Dr. Roberto Cilia: Parkinson Institute, ASST Gaetano Pini-CTO, Milan, Italy.

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Although for decades Parkinson's disease (PD) was considered the prototype of a nongenetic disease, it is now recognized as a complex multifactorial neurodegenerative disorder, with a relevant fraction of genetic heritability. About 15% of patients report a family history of PD, whereas the vast majority of patients have sporadic PD, which is likely related to the combined effect of predisposing genetic variants and environmental risk factors.[1] Heterozygous variations in *GBA* (encoding beta-glucocerebrosidase, OMIM*606463), which cause the recessive lysosomal storage disorder (LSD) Gaucher disease (GD), are the most frequent large-impact genetic risk factor for PD. Many studies have shown an increased frequency of *GBA* variants in PD compared to controls.[2-4] *GBA* variants were shown to confer a five- to seven-fold increased risk of PD[4,5] and to modify PD manifestations, causing earlier age of onset, more severe cognitive dysfunction, and accelerated progression of the neurodegenerative process.[6-8]

Concerning the mechanism relating *GBA* variants to PD, it has been suggested that either a chronic loss of enzymatic activity or a possible toxic gain of function of the mutated glucocerebrosidase results in lysosomal dysfunction and endoplasmic reticulum stress could contribute to disease pathogenesis.[9] However, the observation that carriers of *GBA* variants yielding smaller enzymatic residual activity have a more severe phenotype suggests that the pathogenic mechanism is more likely attributable to loss-of-function rather than gain-of-function effects.[10,11]

Substantial evidence highlights a broader importance of lysosomal mechanisms in PD susceptibility and pathogenesis.[12-14] This idea is strengthened by multiple lines of evidence, suggesting a wider contribution of genetic variations in lysosomal genes to PD. Besides *GBA*, other genes involved in LSDs were associated with a higher risk of PD (eg, *SMPD1*, *SCARB2*, and *GALC*).[15,16] Additional lysosomal genes with an increased burden of variants in PD patients were recently reported (*ATP13A2*, *LAMP1*, *TMEM175*, and *VPS13C*).[17,18] Moreover, (1) typical motor symptoms of PD such as tremor, bradykinesia, and rigidity were described in patients affected by LSDs (GM1 and GM2 gangliosidosis, neuronal ceroid lipofuscinoses, and Fabry disease),[15] and (2) an excessive burden of LSD gene variants was found in PD patients by studying 54 LSD genes in exome data from 1156 patients versus 1679 controls.[19] Little is known about the mechanisms that underly the increased susceptibility of *GBA* mutation carriers to PD. The lifelong PD penetrance of *GBA* variants ranges between 10% and 30%[20,21] and significantly differs when comparing carriers of low- and high-risk variants.[22,23] However, the reason why patients with the same variant (even within the same family) may or may not develop neurodegeneration and PD is obscure.

To shed light on this important topic, a large genome-wide association study exploring the genetic modifiers of risk and age at onset in *GBA*-PD patients was recently conducted.[24] The two main loci identified as possible modifiers in *GBA* carriers were *SNCA* and *CTSB* (ie, variants rs356219 and rs1293298, lying in close proximity of the two genes), both implicated in the lysosomal autophagy pathway. Interestingly, though a genetic risk score based on common PD genetic factors contributed only little to predicting the *GBA*-associated risk for the disease, common variants in genes implicated in lysosomal function were found to exert the largest effect on *GBA*-PD risk.[24]

In this context, there must be other factors, including rare genetic variants, which are still largely unexplored, and/or environmental and aging-related factors, involved in modulating *GBA*-PD risk. We therefore decided to explore whether the burden of rare lysosomal gene variants contributes to the differences in *GBA* mutation penetrance, under the hypothesis that multiple LSD gene variants may contribute, in association with *GBA* dysfunction, to lysosomal impairment.

Patients and Methods

This study was approved by local ethics committees and was conducted according to the Declaration of Helsinki and the Italian legislation on sensitive personal data recording. All participants signed an informed consent.

Study Participants

Discovery cohort: we selected 305 patients diagnosed with typical PD[25] and 207 asymptomatic (as assessed after medical examination) controls who carried a *GBA* variant causative for GD and/or associated with PD (Supplementary Table 1 and Supplementary Figure 1 and 2). All subjects were Italians of southern European origin. A total of 285 patients were enrolled by the Parkinson Institute Biobank of Milan, Italy (https://www.parkinson.it/biobanca), and an additional 20 patients were recruited at the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy).

As for genotyping, 80% of PD patients and 46% of controls were identified, thanks to a recent study in which they were analyzed for the four most common PD-related *GBA* variants: c.1093G > A, p.E365K; c.1223C > T, p.T408M; c.1226A > G, p.N409S; and c.1448T > C, p.L483P; and the legacy names were p.E326K, p.T369M, p.N370S, and p.L444P, respectively. These missense substitutions altogether account for up to 80% of *GBA* deleterious variants in PD; p.N370S and p.L444P represent recognized GD pathogenic mutations, whereas p.E326K and p.T369M are PD-

associated non-GD variants.[26] All four genetic defects were genotyped using a multiplex allele-specific polymerase chain reaction (PCR) assay.[23,27] The remaining 54% of controls were from the MIGen cohort[28] and were previously analyzed by exome sequencing; the remaining 20% of patients underwent a diagnostic next-generation sequencing (NGS) gene panel. This approach allowed the identification of additional *GBA* rare variant carriers. All the identified *GBA* variants were confirmed by Sanger sequencing.

Replication cohorts: for replication studies, we had access to two replication cohorts. The first one (replication cohort 1) was composed of 140 *GBA*-PD patients and 156 controls of European origin, collected by the Parkinson's Progression Markers Initiative (PPMI), with whole genome sequencing performed at the Laboratory of Neurogenetics, National Institutes of Health, (Bethesda, MD), and available at https://www.ppmi-info.org/ and https://amp-pd.org/. Data were processed as previously described by Iwaki et al.[29] The second cohort (replication cohort 2) comprised 110 *GBA*-PD patients and 131 controls (dbGaP accession numbers: phs000668 and phs000398) of Caucasian origin bearing a PD-related *GBA* variant, recruited through the Parkinson Study Group as part of the PROGENI study, using an affected sibling recruitment design (only one sibling for each family was included in our analysis).[30]

Targeted NGS

DNA was extracted from peripheral blood using a Chemagic Star workstation (Hamilton, ON, Canada). Targeted NGS was performed on 401 DNAs (those not previously analyzed by whole-exome sequencing). The target regions were captured using a custom-designed HaloPlex Target Enrichment kit (Agilent, Santa Clara, CA), according to the manufacturer's protocol. The full coding regions and the intron–exon boundaries (25 nucleotides upstream and downstream each exon) of 50 LSD genes and 13 genes associated with PD were included for ~190 kb (Supplementary Table 2). According to Blauwendraat and colleagues,[31] only PD genes classified as "highly or very highly confident" were included. To reduce time and costs, we used a pooling strategy mixing five DNA samples for each library. The obtained libraries were sequenced (paired-end, 2x150bp) on a NextSeq500 (Illumina, San Diego, CA). The mean depth coverage across all libraries was ~1300×. NGS data were analyzed through an in-house pipeline: first, good-quality reads (ie, having a read mapping quality >20) were aligned to the reference genome (release hg19, February 2009) using the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/), variant calling was performed using Comprehensive Read Analysis for Identification of Single Nucleotide Polymorphisms from Pooled sequencing (https://bansal-lab.github.io/software/crisp.html), and finally, variants were annotated with Annovar (https://annovar.openbioinformatics.org/en/latest/).

Variant Selection

Independent of the sequencing procedure (exome or targeted NGS), variants covered by at least 20 reads and with a frequency <1% in the non-Finnish European population (GnomAD v2.1.1 https://gnomad.broadinstitute.org/) were selected and classified in one of the following functional categories: (1) loss-of-function (LoF) variants (frameshift, nonsense, and splicing variants, ie, affecting intronic positions ±1 and ±2); (2) missense variants already reported as pathogenic in LSDs/PD; and (3) all other missense variants without a clear pathogenic role. Regarding the last group, the deleteriousness of these variants was estimated using the CADD (Combined Annotation Dependent Depletion) score (https://cadd.gs.washington.edu/), and the variants with a score >25 were retained for further analyses (Supplementary Figure 2).

Variant Validation

All candidate variants identified in the discovery cohort were confirmed by Sanger sequencing; in the case of samples multiplexed for the targeted NGS, this step also allowed the identification of the individual of the pool carrying the variation. Briefly, amplicons spanning the exons of interest were designed, and direct sequencing of the obtained PCR products was carried out using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Thermo Fisher Scientific, Carlsbad, CA) and analyzed on an ABI-3500 Genetic Analyzer (Thermo Fisher Scientific).

When possible, the phase of *GBA* variants was defined in those patients who were carriers of at least two variants in the gene. Amplicons containing the two variations were amplified and cloned in the pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. The obtained plasmids were used to transform JM109 competent cells (Promega); colony PCR and subsequent Sanger sequencing allowed the characterization of each allele.

Statistical Analysis

The Sequence Kernel Association Test-Optimal (https://cran.r-project.org/web/packages/SKAT/SKAT.pdf) was implemented in R using SKAT v1.3.2.1 to determine the difference in the aggregate burden of rare LSD and PD gene variants between PD patients and controls. Concerning LSD gene variants, we performed the same analysis (adjusted for sex, age, and the 10 main principal components, when available) for each of the following categories: LoF variants, missense variants already associated with LSDs or PD, and

missense variants with CADD >25. Burden analysis for each LSD gene was also performed. For chromosome-X genes, we considered uniform and complete X-inactivation in women and a similar effect size between men and women. Therefore, women were considered to have 0, 1, or 2 copies of an allele, whereas men were considered to have 0 or 2 copies of the same allele (ie, male hemizygotes were considered equivalent to female homozygotes).

The threshold for significance was set to $P < 0.05$; we considered the multiple testing issue by correcting each P according to the number of evaluated variant categories (see table legends).

Replication Studies and Meta-Analysis

For replication studies, we extracted LSD variants from all *GBA* carriers of the two cohorts following the same filtering criteria used for the Italian set and performed burden analyses as described earlier.

In the meta-analysis, we focused on all genes/variant categories/disease classes that showed a significant association in the discovery cohort. *P*-values, pooled odds ratios (ORs), and confidence intervals (CIs) were calculated using the Mantel–Haenszel model.[32] A Breslow–Day test for heterogeneity (with Tarone's correction) was performed for testing differences in OR between sample populations.[33,34]

Results

Overall Screening Results

A cohort of 512 subjects (305 patients and 207 controls, Supplementary Table 1), all carriers of at least one *GBA* variant, were analyzed using exome sequencing (111 individuals) or a custom NGS panel (401 individuals) comprising 50 lysosomal genes causing LSDs and 13 known PD genes (Supplementary Table 2). After the filtering step (described earlier), we found 184 different variants in 201 individuals (Supplementary Table 3), all validated by Sanger sequencing: the majority of them were missense (88%), and 12% were LoF variants (8% nonsense/frameshift and 4% splicing variants). Among missense variants, those with a CADD score comprised between 25 and 30 were the most frequent (40%), followed by missense variants already associated with LSD or parkinsonism (31%) and by those with a CADD >30 (17%).

Increased Burden of LSD Variants in *GBA*-PD

Mutation burden analysis was performed separately for lysosomal and known PD genes. *GBA* was included in the list of LSD genes to search for additional variants other than the one used for patient enrollment. Only the second *GBA* variant (variant 2) was incorporated

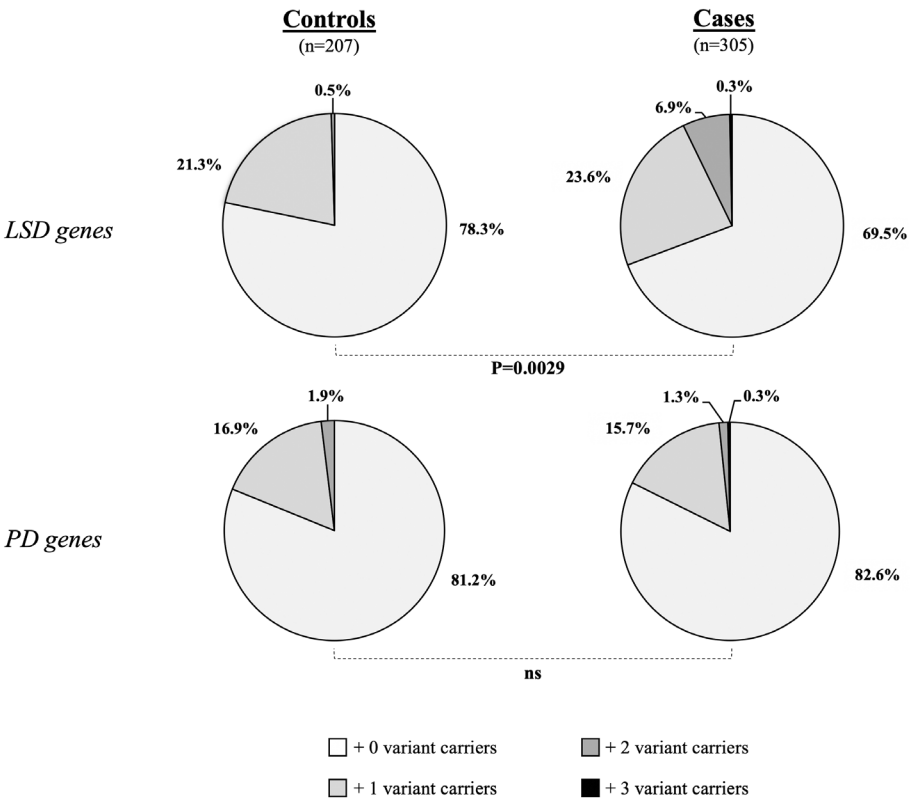
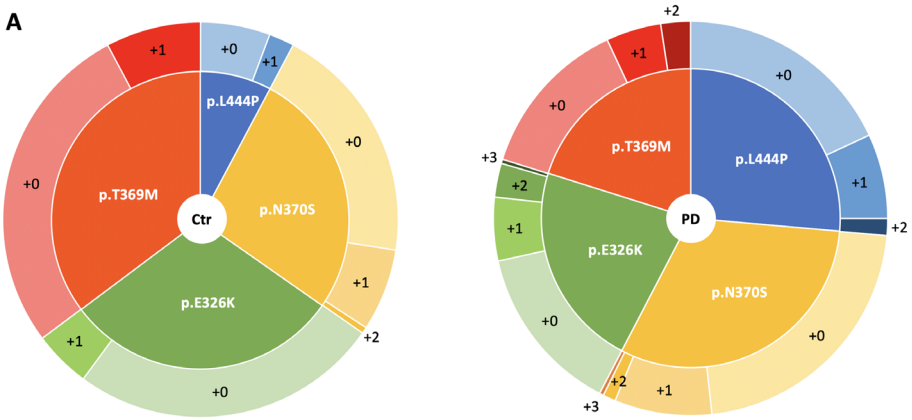


FIG. 1. Distribution of selected variants in LSD- and PD-related genes among patients and controls. SKAT-O (Sequence Kernel Association Test-Optimal) analyses were performed on variants identified in our cohort of *GBA*-mutated patients and controls after sequencing 50 LSD and 13 PD genes.

A



B

		+0	+1	+2	+3	P
p.L444P	Ctr (n=15)	73.3%	26.7%			ns
	PD (n=76)	68.4%	26.3%	5.3%		
p.N370S	Ctr (n=52)	73.1%	25.0%	1.9%		ns
	PD (n=90)	70.0%	25.6%	3.3%	1.1%	
p.E326K	Ctr (n=58)	84.5%	15.5%			0.0004
	PD (n=64)	62.5%	23.4%	12.5%	1.6%	
p.T369M	Ctr (n=68)	77.9%	22.1%			0.023
	PD (n=57)	65.5%	22.4%	12.1%		

FIG. 2. Distribution of LSD variants according to *GBA* genotype in PD patients and controls. (**A**) Graphical representation of the distribution of LSD deleterious variants among patients and controls classified depending on their *GBA* variant. (**B**) Detailed table of the obtained results. [Color figure can be viewed at wileyonlinelibrary.com]

in the analysis, considering that all the analyzed subjects are carriers of at least one *GBA* PD-associated variant (variant 1) according to our a priori enrollment criteria. In individuals carrying two *GBA* variants, variants 1 and 2 were assigned according to their severity, as specified in Supplementary Table 4.

Considering LSD genes, a significantly higher proportion of individuals carrying a deleterious variant were observed in PD patients compared to controls (30.8% and 21.7%, respectively, $P = 0.0029$) (Fig. 1). This result is remarkably strong considering that the power calculation taking into account the sample size of our discovery cohort and the overall number of variants identified in LDS genes indicated 63% of probability to find an association at $P < 0.05$. This enrichment is significant even after excluding the second *GBA* variant (27.5% in patients vs. 20.3% in controls, $P = 0.019$) (Table 1). Conversely, we found no significant difference in the number of deleterious variants in known PD genes between patients and controls (17.4% vs. 18.8%, respectively, $P = 0.28$) (Fig. 1).

To disentangle the relative contribution of LSD variants to the risk for *GBA*-PD, subjects were also subclassified according to their *GBA* variant (only the four most common PD-related variants were considered; Rec*Nci*I alleles were included in the p.L444P group), and for each group the same burden analysis was performed. Interestingly, a significant enrichment of variants in LSD genes was present only in PD patients who carried a non GD-causing *GBA* variant (ie, the p.E326K or p.T369M variants, $P = 0.0004$ and $P = 0.023$, respectively) (Fig. 2).

Focusing on the different functional categories of analyzed variants, missense substitutions causing LSD were present in 15.7% of PD patients and in 8.7% of controls ($P = 0.022$), whereas those with CADD >25 accounted for 16.4% of PD patients compared to 9.7% of controls ($P = 0.041$). As for LoF variants, no difference between patients and controls was observed (3.3% vs. 3.4%, $P = 0.85$) (Table 1).

Burden analysis was also performed categorizing LSD genes according to the disease type for which they are responsible (Supplementary Table 2).[35] The most interesting result concerns the mucopolysaccharidosis-related genes, in which a higher burden of deleterious variants was observed in patients compared to controls (6.9% vs. 1%, $P = 0.0020$) (Table 1).

Single-Gene Analysis: A Second Variant in *GBA* Is the Main Risk Modulator for *GBA*-PD

Analyzing the distribution of variants in different LSD genes in all subjects enrolled, we observed that the gene with the highest number of variants was *GBA* (n = 20), followed by *GNPTAB*, *NPC1*, and *SMPD1* (all with n = 10). Other eight genes (*CLN5*, *CTSF*, *GAA*, *GLA*, *GUSB*, *HEXB*, *MAN2B1*, and *MCOLN1*) carried more than five genetic defects, whereas the vast

TABLE 1 *Burden analyses of LSD variants*

	Frequencies		
	Controls (%) n = 207	PD (%) n = 305	P*
All LSD variants	21.7	30.8	**0.0029**
All LSD variants excluding *GBA*	20.3	27.5	**0.019**
Variant categories			
LoF	3.4	3.3	0.85
Known mutations	8.7	15.7	0.022
CADD >25	9.7	16.4	0.041
Diseases[a]			
Sphingolipidoses	8.2	12.1	0.23
Mucopolysaccharidoses	1.0	6.9	**0.0020**
Neuronal lipofuscinoses	2.9	4.3	0.57
Glycoproteinoses	1.9	4.6	0.18
Integral membrane protein disorders	5.8	4.9	0.12
Posttranslational modification defects	1.4	3.3	0.35

[a]Variants in LSD genes were classified according to the diseases for which they are responsible.[35]
Abbreviations: PD, Parkinson's disease; LoF, loss of function; CADD, Combined Annotation Dependent Depletion.
*SKAT-O (Sequence Kernel Association Test-Optimal) P-values adjusted for age/sex. Significant P-values are in bold (P-values corrected for multiple testing were for variant categories, P = 0.05/3 = 0.016, and for disease categories, P = 0.05/6 = 0.0083).

majority of genes showed four or fewer variants (Supplementary Table 5).

Single-gene association tests were performed only for those genes with the number of variations greater than or equal to five (Fig. 3). Variants in the *GBA* gene were present at a significantly higher frequency in patients (5.6%) compared to controls (1.4%, $P = 0.023$), suggesting that a second variant in *GBA* represents the strongest modulator of the disease penetrance among *GBA* mutation carriers. All 20 individuals carrying an additional variant in *GBA* were analyzed to determine the phase of the two variants. For 17 of them, a strategy combining long PCR and cloning allowed the determination of the phase: in the majority of patients (71%), an in-trans association was experimentally confirmed (Supplementary Table 4).

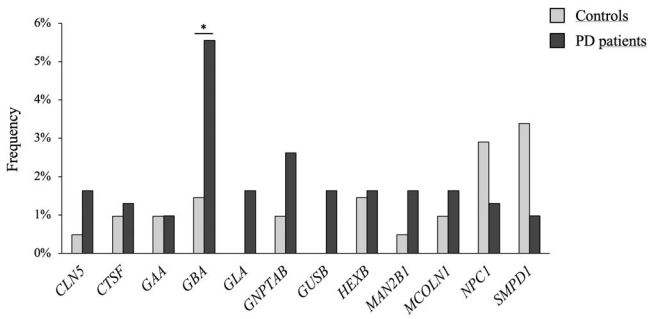


FIG. 3. Single-gene burden analyses. SKAT-O (Sequence Kernel Association Test-Optimal) single-gene association tests were performed only on genes with a number of identified variants greater than or equal to five. * = $P < 0.05$ (corrected *P*-value threshold = 0.05/12 = 0.0042).

Meta-Analysis Confirmed the Role of Lysosomal Genes as Modulators of PD Penetrance

To confirm our results, the same burden analyses for LSD variants were performed in two independent replication cohorts of similar size. These were composed of European-descent individuals, for 250 *GBA*-PD patients and 287 *GBA*-mutated asymptomatic controls, all with exome or genome data available.

In replication cohort 1, we observed suggestive evidence of an enrichment in LSD variants in PD patients (33.6% vs. 27.6% in patients and controls, respectively, $P = 0.16$), mainly driven by variations in genes responsible for sphingolipidoses and neuronal lipofuscinoses (Table 2A). In replication cohort 2, we replicated the significant burden of LSD variants in PD patients compared to asymptomatic *GBA* carriers (34.5% vs. 24.4%, $P = 0.00051$); this enrichment was also confirmed excluding all the second *GBA* variants from the analysis (Table 2A). In both replication cohorts, the single-gene analysis confirmed the major role as a modulator of PD penetrance of a second mutation in the *GBA* gene (replication cohort 1: 7.9% vs. 2.5%, $P = 0.032$, and replication cohort 2: 8.2% vs. 0.8, $P = 0.00068$) (Table 2A).

We also performed a meta-analysis on the variant categories that were significantly enriched in the discovery cohort (Table 2B; Supplementary Figure 3). As for LSD deleterious variants, we observed a pooled OR of 1.42 (95% CI = 1.10–1.83, $P = 0.0063$). The effect of variants in mucopolysaccharidosis genes seemed to be even higher, with an OR of 1.83 (95% CI = 1.04–3.22, $P = 0.038$). Finally, a second mutation in the *GBA* gene emerged as the genetic risk factor, with the highest impact on the modulation of the penetrance of *GBA* mutations (OR = 4.36, 95% CI = 2.02–9.45, $P = 0.00019$).

Discussion

A major finding of our work is that the overall burden of deleterious variants in lysosomal genes is significantly higher in *GBA*-PD patients compared to asymptomatic *GBA*-variant carriers. This signal is mostly due to the presence of a second deleterious

TABLE 2 *Replication studies and meta-analysis*

A. Replication studies

	Replication cohort 1			Replication cohort 2		
	Frequencies			Frequencies		
	Controls (%) n = 156	PD (%) n = 140	*P*[a]	Controls (%) n = 131	PD (%) n = 110	*P*[a]
All LSD variants	27.6	33.6	0.16	24.4	34.5	**0.00051**
All LSD variants excluding *GBA*	25	28.6	0.78	24.4	27.3	**0.0078**
Variant categories						
LoF	1.9	4.3	0.11	4.6	5.5	0.61
Missense	25.6	30.7	0.28	19.8	30.0	**0.00051**
Diseases						
Sphingolipidoses	7.7	14.3	0.051	8.4	17.3	**0.0033**
Mucopolysaccharidoses	8.3	5.0	0.16	3.1	7.3	*0.025*
Neuronal lipofuscinoses	5.1	2.9	0.07	5.3	1.8	0.17
Glycoproteinoses	3.2	4.3	0.82	3.1	7.3	*0.014*
Integral membrane protein disorders	3.2	6.4	0.77	3.8	0.9	0.14
Posttranslational modification defects	1.9	4.3	0.90	1.5	1.8	0.69
Genes[b]						
GBA	2.5	7.9	*0.032*	0.8	8.2	**0.00068**

B. Meta-analysis

	P[a] (discovery)	*P*[a] (replication1)	*P*[a] (replication2)	Pooled *P*	Pooled OR[c]	95% CI for pooled OR[c]	*P* for heterogeneity[d]
All LSD variants	**0.0029**	0.16	**0.00051**	**0.0063**	1.42	1.10–1.83	0.83
All LSD variants excluding *GBA*	**0.019**	0.78	**0.0078**	0.077	1.27	0.97–1.64	0.75
Mucopolysaccharidoses	**0.0020**	0.16	*0.025*	**0.038**	1.83	1.04–3.22	0.0052
GBA	**0.023**	*0.032*	**0.00068**	**0.00019**	4.36	2.02–9.45	0.56

[a]Significant adjusted SKAT-O (Sequence Kernel Association Test-Optimal) *P*-values are in bold (*P*-values corrected for multiple testing were for variant categories, $P = 0.05/3 = 0.016$; for disease classes, $P = 0.05/6 = 0.0083$; and for single-gene analysis, $P = 0.05/2 = 0.025$). *P*-values <0.05 are in italics.
[b]Genes with significant burden test in the discovery cohort.
[c]Pooled ORs/CIs were calculated using the Mantel–Haenszel model.
[d]*P*-values for heterogeneity were calculated using Breslow–Day test with Tarone's correction.

variant in *GBA* or in one of the genes related to mucopolysaccharidoses (MPS). Interestingly, no significant increased burden of variants was found for the set of the known PD genes we analyzed. This is in line with what was reported by Blauwendraat and colleagues,[24] who found that the contribution of known PD loci in modulating the risk of *GBA*-PD is not large. A partially surprising result is the finding that only missense defects (associated with LSDs or showing a CADD >25) seem to play an important role in increasing the risk of PD, whereas LoF alleles in lysosomal genes are equally distributed among patients and controls. With all the caution required considering the small number of null alleles found, this could be related to the expression of a dysfunctional lysosomal enzyme in the organelle, which could add further stress by promoting lysosomal dysfunction and/or protein aggregation.

Our burden analysis in the discovery cohort also highlighted the role of MPS genes as modulators of *GBA*-PD risk, which was confirmed in the meta-analysis (OR = 1.83, 95% CI = 1.04–3.22, $P = 0.038$). This observation is in line with several findings relating MPS

to PD: (1) the identification of *GUSB* (responsible for MPS type VII) as the candidate gene of a novel PD locus,[36] (2) the discovery of a common *NAGLU* (MPS IIIB) haplotype associated with PD (OR = 1.3),[37] and (3) the observation of α-synuclein deposits in the brain of MPS IIIA and MPS IIIB patients.[37,38]

Considering single-gene analyses, we found that a second "hit" in this gene represents the most important contribution to disease risk, reaching a remarkable OR of 4.36 in the meta-analysis (95% CI = 2.02–9.45, $P = 0.00019$). This was not completely unexpected, as it was previously proposed that non-GD-causing variants in *GBA* (ie, p.E326K, p.T369M) are frequently associated with a second genetic defect in the gene in PD patients and more important that age of onset is also lowered if you carry two GBA variants[24,39,40]; it is also true that no clear-cut results were obtained by similar investigations,[41,42] possibly because of the lack of statistical power in the examined cohorts. In particular, previous studies dealt with only a few dozen *GBA*-PD patients and, more important, with small cohorts of healthy unrelated individuals carrying *GBA* mutations. We tried to overcome this problem by performing a large genotyping effort (>11,000 unrelated samples screened, all of Italian origin) to identify more than 500 individuals who are carriers of one PD-related *GBA* variant.[23] In addition, we tried to assess, whenever possible, the phase of association of the two *GBA* variants, and in most cases we could demonstrate an in-trans association, thus supporting the concept of a double hit in a lysosomal gene in determining a higher risk for PD.

We are aware that our work has some limitations: (1) the design of our NGS panel includes only the coding regions of the selected LSD genes, so the potential contribution of the noncoding regulatory variants in the promoter and untranslated regions, as well as in the deep intronic portions, was not assessed; in this respect, the contribution of a second variant in the *GBA* gene or of variants in other lysosomal genes could have been underestimated; (2) the pooled sequencing strategy might have missed some mutated alleles, even though we confirmed 100% of identified variants by Sanger sequencing; (3) patient stratification according to *GBA* mutations might introduce a population bias as, for example, the p.N370S variant as well as some *SMPD1* variants is more frequent among individuals of Askenazi Jewish descent, whereas the p.E326K and p.T369M are more frequent among northern Europeans[43]; and (4) replication cohorts are smaller than the discovery cohort because of the difficulty in finding patient/control samples with mutations in *GBA* and exome/genome data available.

In conclusion, the present study points out the contribution of deleterious variants in LSD genes as modifiers of PD penetrance in *GBA* carriers. Early detection of individuals with higher PD risk may be beneficial for future neuroprotective treatments. ●

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Data Availability Statement

Data available on request from the authors.

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Supporting Data

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