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### Abstract

Pathogenic variants in the glucocerebrosidase gene (GBA) encoding the enzyme deficient in Gaucher's disease (GD) are associated with Parkinson's disease (PD). To investigate the sequence variants, their association with PD and the related phenotypes in a large cohort of European, mostly French, patients and controls, we sequenced all exons of GBA in 786 PD patients from 525 unrelated multiplex families, 605 patients with apparently sporadic PD and 391 ethnically matched controls. GBA mutations were significantly more frequent (odds ratio=6.98, 95% confidence interval 2.54-19.21; P=0.00002) in the PD patients (76/1130=6.7%) than in controls (4/391=1.0%) and in patients with family histories of PD (8.4%) than in isolated cases (5.3%). Twenty-eight different mutations were identified in patient and control groups, including seven novel variants. N370S and L444P accounted for 70% of all mutant alleles in the patient group. PD patients with GBA mutations more frequently had bradykinesia as the presenting symptom and levodopa-induced dyskinesias. The phenotype was similar in patients with one, two or complex GBA mutations, [...]

### Reference

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# Large-scale screening of the Gaucher's disease-related glucocerebrosidase gene in Europeans with Parkinson's disease

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**Pathogenic variants in the *glucocerebrosidase* gene (*GBA*) encoding the enzyme deficient in Gaucher's disease (GD) are associated with Parkinson's disease (PD). To investigate the sequence variants, their association with PD and the related phenotypes in a large cohort of European, mostly French, patients and controls, we sequenced all exons of *GBA* in 786 PD patients from 525 unrelated multiplex families, 605 patients with apparently sporadic PD and 391 ethnically matched controls. *GBA* mutations were significantly more frequent (odds ratio = 6.98, 95% confidence interval 2.54–19.21;  $P = 0.00002$ ) in the PD patients (76/1130 = 6.7%) than in controls (4/391 = 1.0%) and in patients with family histories of PD (8.4%) than in isolated cases (5.3%). Twenty-eight different mutations were identified in patient and control groups, including seven novel variants. N370S and L444P accounted for 70% of all mutant alleles in the patient group. PD patients with *GBA* mutations more frequently had bradykinesia as the presenting symptom and levodopa-induced dyskinesias. The phenotype was similar in patients with one, two or complex *GBA* mutations, although the two patients with c.1263del+RecTL and N370S/RecΔ55 mutations had signs of GD. Segregation analyses in 21 multiplex families showed that 17% of the affected relatives did not carry *GBA* mutations found in the given family, indicating heterogeneity of the aetiology, but 46% of the unaffected relatives were *GBA* mutation carriers. These genotype and clinical analyses on the largest homogeneous sample of European patients studied to date confirmed that *GBA* mutations are the most common genetic risk factor for PD, particularly in familial forms.**

## INTRODUCTION

Monogenic Parkinson's disease (PD), caused by mutations in *leucine-rich repeat kinase 2* (*LRRK2*), *α-synuclein* (*SNCA*), *parkin*, *PTEN-induced kinase 1* (*PINK1*) and *DJ-1*, is rare

(1). Most PD result from complex interactions between genes and/or environmental factors. Genetic variations may modify penetrance, age at onset, severity and progression. Polymorphic variants in *SNCA*, *MAPT* (encoding the microtubule-associated protein tau), *LRRK2* and

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*glucocerebrosidase (GBA)* are emerging as relevant susceptibility factors for PD in several populations (1).

*GBA* mutations cause Gaucher's disease (GD), a recessive glycolipid storage disorder caused by  $\beta$ -glucocerebrosidase deficiency. Three types of GD are distinguished according to the associated neurological symptoms (2). Although there was no significant overlap between GD and PD phenotypes, the indication for a relationship between the two conditions came with the observation of the occurrence of parkinsonism and Lewy body pathologies in patients with GD and their relatives and the identification of *GBA* mutations in patients with PD (3). Subsequent genotyping studies have shown an increased frequency of *GBA* mutations in cohorts of PD patients with different geographical or ethnical origins and particularly in those with early onset (4–10). *GBA* mutations are more common in the Ashkenazi Jewish population (~20% of patients with typical parkinsonism and 4% of controls), the N370S mutation (~70% of mutant alleles) being the most frequent (8,11); they are less frequent and more diverse in other ethnic groups (11). However, carrier frequency in non-Ashkenazi Jewish populations was 3%, in a study by 16 international centres, but increased to 7% in subpopulations in which the gene was fully sequenced, showing that limited screening may miss more than half of the mutant alleles (11). To accurately ascertain the frequency and the spectrum of *GBA* mutations in Europe, we screened the entire *GBA* gene in the largest series of PD patients and relatives analysed so far and in ethnicity-matched controls. Clinical features of PD patients with and without *GBA* mutations were compared.

## RESULTS

### Mutations in *GBA* and frequency of mutation carriers

*GBA* variants were identified in 44 families (8.4%) and 32 isolated cases (5.3%) (Table 1), but only in four controls (1%). The frequency of *GBA* mutations was higher ( $P = 0.04$ ) in familial PD cases [odds ratio (OR) = 8.85, 95% confidence interval (CI) 3.15–24.85;  $P = 0.00001$ ] than in isolated cases (OR = 5.40, 95% CI 1.89–15.39;  $P = 0.0004$ ) when compared with controls. Taken together, the frequency of *GBA* mutations in the 1130 unrelated PD families and isolated cases (76/1130, 6.7%) differed significantly from that in the controls (4/391, 1.0%; OR = 6.98, 95% CI 2.54–19.21;  $P = 0.00002$ ).

Available affected relatives ( $n = 260$ ) were also screened. In 21 of the 44 families carrying *GBA* variants, at least one additional first- or second-degree affected relative carried a mutation ( $n = 29$ ); patients in five of these families were discordant for the *GBA* variant (24%) (Fig. 1). Thus, 100 individuals from 76 families carried at least one mutated allele: 21 missense variants, six of which were novel, one nonsense mutation (S173X) and five multiple amino acid substitutions designated as 'complex' or 'recombinant' (Rec) alleles that presumably arose by cross-over of the functional *GBA* gene with a nearby pseudogene *GBAP* (12) (Table 1).

The common N370S mutation was found in 28 patients from 21 multiplex families, 14 isolated cases (39 heterozygotes and three homozygotes) and two controls (heterozygotes). The risk of N370S mutation carriers for PD (35/

1130, 3.1%) compared with European controls (2/391, 0.5%) was 6.22 (CI 1.49–25.98;  $P = 0.0008$ ). L444P, the second most frequent *GBA* mutation, was found in 13 patients from eight multiplex families (12 heterozygotes and one homozygote) and four heterozygous isolated cases (12/1130, 1.1%), but not in controls ( $P = 0.09$ ). Two related patients had two mutations, N370S and L444P, that were usually reported in trans in GD patients. We thus considered these patients as potential compound heterozygotes. One patient was shown to be compound heterozygous for N370S/Rec $\Delta$ 55 (Fig. 1), a 55 bp deletion in exon 9 resulting from recombination between *GBA* and *GBAP* (c.1263–1317 del) (13). Four unrelated patients carried L444P on three different recombinant alleles: Rec*NciI* (L444P, A456P, V460V) (0.18%), RecA456P (L444P, A456P) (0.09%) and the rare complex mutations, c.1263del+RecTL (c.1263–1317del, D409H, L444P, A456P, V460V) (0.09%). Together, N370S and L444P accounted for 70% (53/76) of mutant alleles in this series of European PD patients (Fig. 2).

The other variants were found only once or twice (Table 1). The nonsense mutation S173X predicted to result in a truncated protein was found in one patient and her child (Fig. 1). One patient carried the complex allele D140H + E326K shown to produce a Type 1 GD phenotype (14). The remaining 20 amino acid substitutions, including six new non-synonymous variants (G80R, G113A, I119L, S125N, P246L and P452L) (Table 1), were each found in a single family. Of note, E388K, previously identified in two Portuguese controls (15), was found in one PD patient and one control. Two novel silent variants were also detected, A190A and A446A, the latter in combination with the newly identified G113A substitution. These novel sequence changes were not found on the 782 control chromosomes. Most amino acids affected by these variants, except for G113A, are highly conserved among mammals down to *Monodelphis domestica* (I119L, P246L and E388K) or *Tetraodon nigroviridis* (G80R and S125N) (Supplementary Material, Fig. S1). Interestingly, P452L was highly conserved across all species down to *Caenorhabditis elegans*, suggesting that the P452L variant might be pathogenic. S125N, the only new variant that could be analysed for co-segregation, co-segregated with the disease (Fig. 1). Segregation analyses in 21 multiplex families showed that 17% (5/29) of the affected relatives did not carry the *GBA* mutation in their family, indicating a heterogeneous aetiology. However, 46% (23/50) of the unaffected relatives of *GBA* carrier patients were also *GBA* mutation carriers (mean age at examination  $52.5 \pm 14.7$  years, range 29–81), indicating that not all *GBA* mutation carriers develop PD. Interestingly, a 63-year-old asymptomatic woman was homozygous for the severe Y304C mutation (FPD485) (Fig. 1). In addition to the N370S mutation, the novel non-synonymous K79M variant and the synonymous Y313Y variant were found exclusively in one control subject each. None of the *GBA* carriers had the *LRRK2* G2019S mutation.

Two variants, E326K and T369M, previously described as non-pathogenic polymorphisms in patients with GD (11), were significantly associated with PD. The E326K substitution, suggested to be a neutral single nucleotide

**Table 1.** *GBA* mutations identified in this study

<i>GBA</i> mutations Allele names	cDNA	Exon	Patients with PD ( <i>n</i> = 1391) <i>n</i> (unrelated patients, <i>n</i> = 1130)		Control subjects ( <i>n</i> = 391) <i>n</i> Carrier frequency (%)	
				Carrier frequency (%) <sup>a</sup>		Carrier frequency (%)
<b>Heterozygous variants</b>						
K(-27)R/+	c.38A > G	2	1 (1)	0.09	0	0
<b>K79M</b> /+	<b>c.353A &gt; T</b>	4	0	0	1	0.26
<b>G80R</b> /+	<b>c.355G &gt; A</b>	4	1 (1)	0.09	0	0
<b>I119L</b> /+	<b>c.472A &gt; C</b>	5	1 (1)	0.09	0	0
R120W/+	c.475C > T	5	2 (1)	0.09	0	0
<b>S125N</b> /+	<b>c.491G &gt; A</b>	5	2 (1)	0.09	0	0
R131C/+	c.508C > T	5	1 (1)	0.09	0	0
S173SfsX50/+	c.635C > G	6	2 (1)	0.09	0	0
G202R/+	c.721G > A	6	2 (2)	0.18	0	0
<b>P246L</b> /+	<b>c.851C &gt; T</b>	7	1 (1)	0.09	0	0
Y304C/+	c.1028A > G	8	2 (1)	0.09	0	0
T323I/+	c.1085C > T	8	2 (1)	0.09	0	0
R329C/+	c.1102C > T	8	2 (2)	0.18	0	0
S364N/+	c.1208G > A	8	2 (1)	0.09	0	0
N370S/+	c.1226A > G	9	39 (33)	2.92	2	0.51
G377S/+	c.1246G > A	9	1 (1)	0.09	0	0
E388K/+	c.1279G > A	9	1 (1)	0.09	1	0.26
D409H/+	c.1342G > C	9	1 (1)	0.09	0	0
L444P/+	c.1448T > C	10	16 (11)	0.97	0	0
<b>P452L</b> /+	<b>c.1472C &gt; T</b>	10	1 (1)	0.09	0	0
R463C/+	c.1504C > T	10	2 (1)	0.09	0	0
R463H/+	c.1505G > A	10	4 (1)	0.09	0	0
<b>G113A/A446A</b>	<b>c.455G &gt; A</b>	5	1 (1)	0.09	0	0
	<b>c.1455A &gt; G</b>	10				
<b>Complex or recombinant alleles</b>						
RecΔ5	c.1263–1317del55	9	1 <sup>b</sup> (0)	0	0	0
Rec <i>Nci</i> I (L444P + A456P + V460V)	c.1448T > C	10	2 (2)	0.18	0	0
	c.1483G > C	10				
	c.1497G > C	10				
RecA456P (L444P + A456P)	c.1448T > C	10	1 (1)	0.09	0	0
	c.1483G > C	10				
c.1263del + RecTL (c.1263–1317del + D409H + L444P + A456P + V460V)	c.1263–1317del55	9	1 (1)	0.09	0	0
	c.1342G > C	9				
	c.1448T > C	10				
	c.1483G > C	10				
	c.1497G > C	10				
D140H + E326K/E326K	c.535G > C	5	1 (1)	0.09		
	c.1093G > A	8				
<b>Homozygotes and compound heterozygotes</b>						
N370S/N370S	c.1226A > G	9	3 (2)	0.18	0	0
L444P/L444P	c.1448T > C	10	1 (1)	0.09	0	0
N370S/RecΔ5	c.1226A > G	9	1 <sup>b</sup> (1)	0.09	0	0
	c.1263–1317del55	9				
N370S/L444P	c.1226A > G/	9	2 (1)	0.09	0	0
	c.1448T > C	10				
Total			100 (76)	6.7	4	1.0
<b>Silent variants</b>						
<b>A190A</b> /+	<b>c.687G &gt; A</b>	6	1 (1)	0.09	0	0
<b>Y313Y</b> /+	<b>c.1056C &gt; T</b>	8	0	0	1	0.26

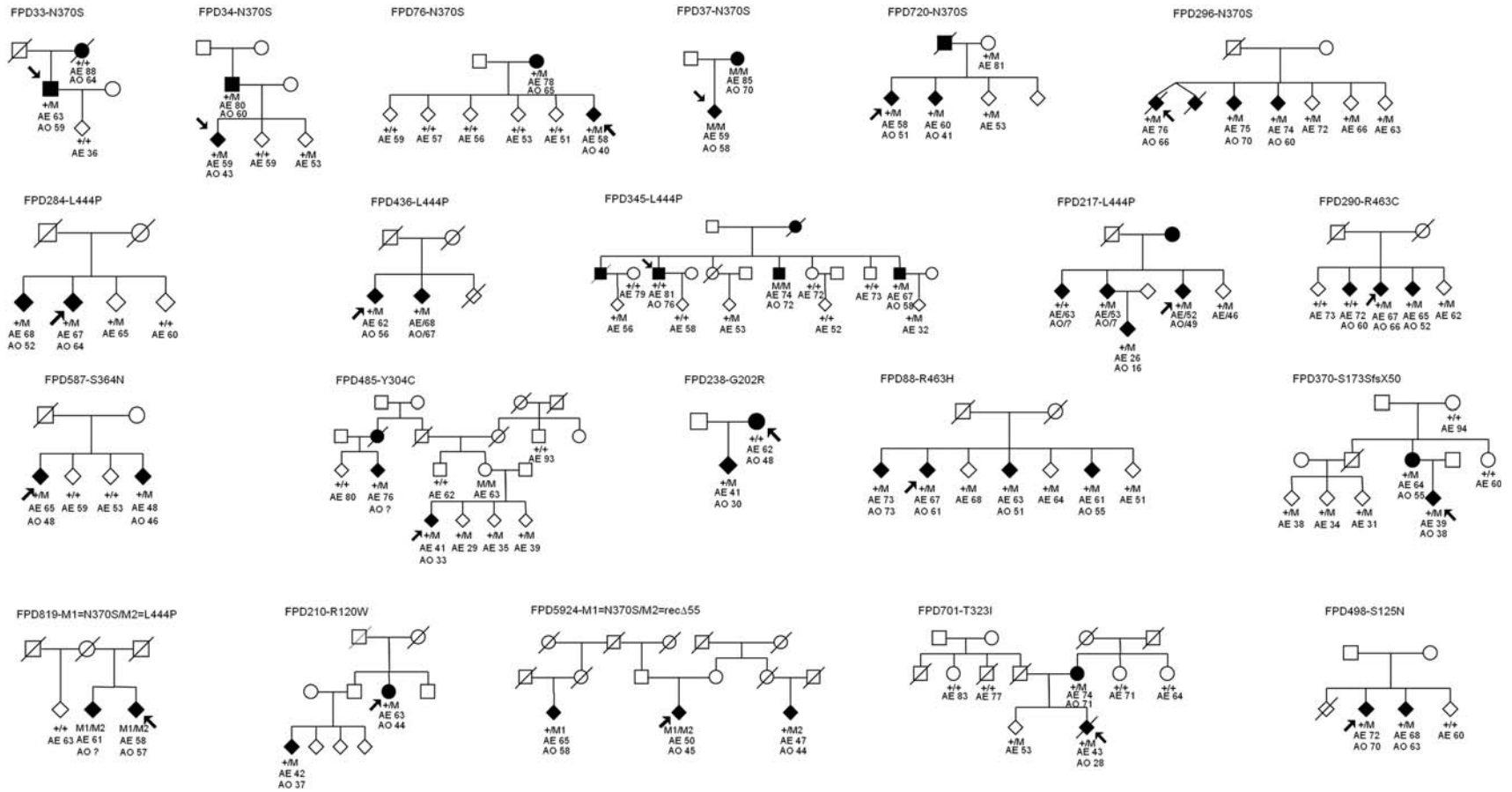
Mutation nomenclature follows HGVS recommendations: *GBA* cDNA nucleotides ('c.') are numbered with the adenine of the first ATG translation initiation codon as nucleotide + 1 (GenBank reference sequence NM\_000157.2). All allele names used in this report refer to the processed protein, excluding the 39-residue signal peptide. Novel nucleotide changes detected in this study are shown in bold.

<sup>a</sup>*GBA* mutation carrier frequency was calculated from 1130 cases counting one carrier per family.

<sup>b</sup>These two patients belong to the same family.

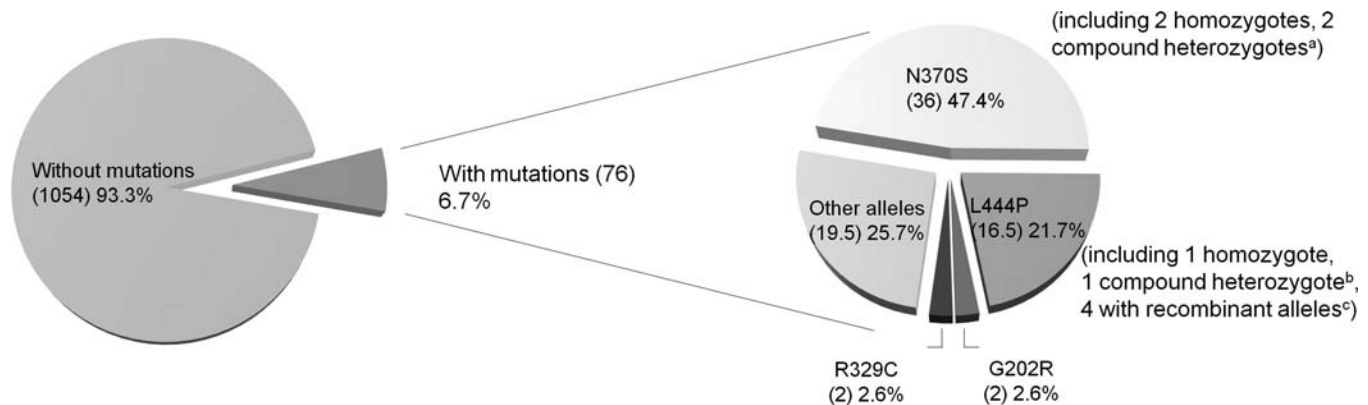
polymorphism (SNP) by functional studies (14), was identified in 49 unrelated index cases (41 heterozygotes, two compound heterozygotes with N370S, six homozygotes including one in combination with D140H; allelic frequency: 2.4%) and in eight control subjects, all in the heterozygous state (allelic

frequency: 1.0%), reflecting a trend towards an association with the disease (OR = 2.41, 95% CI 1.14–5.08; *P* = 0.02). The neutral T369M polymorphism was found in 17 unrelated PD patients (14 heterozygotes including one in combination with the complex c.1263del+RecTL mutations and three



**Figure 1.** Pedigree structures of families with *GBA* variants in which segregation analyses were performed. Affected individuals with PD are represented by black symbols and unaffected by open symbols. The arrows point to the probands. To protect confidentiality, genders of some individuals are not shown. AE, age at examination; AO, age at onset; +, wild-type; M, heterozygote mutated.





**Figure 2.** Distribution of *GBA* variants among European, mostly French, unrelated PD families. Patients with compound heterozygous mutations count for half a carrier. <sup>a</sup>N370S/L444P, N370S/RecΔ5; <sup>b</sup>N370S/L444P; <sup>c</sup>RecNciI, RecA456P, c.1263–1317del + RecTL. ‘Other alleles’ included K(–27)R, G80R, G113A, I119L, R120W, S125N, R131C, D140H + E326K/E326K, S173X, P246L, Y304C, T323I, S364N, G377S, E388K, D409H, P452L, R463C, R463H and RecΔ5.

homozygotes; allelic frequency: 0.88%) and in one control subject in the heterozygous state (allelic frequency: 0.13%). The allelic frequency of the T369M substitution was marginally higher in the PD sample than in controls (OR = 6.97, 95% CI 0.93–52.02;  $P = 0.03$ ).

#### Age at onset in carriers of *GBA* mutations with PD

The mean age at onset of PD in *GBA* mutation carriers was  $51.0 \pm 12.7$ , comparable to that of non-carriers ( $50.0 \pm 13.7$ ) (Table 2). The frequency of *GBA* mutations was similar in patients with early (6.2%, 42/681) and late onset (8.5%, 54/635) PD (OR = 0.71, 95% CI 0.47–1.08;  $P = 0.10$ ).

When stratified according to their deduced or observed effects on phenotype in patients with GD (mild *GBA* mutations are associated with non-neuronopathic Type 1 GD and severe or null mutations with severe neuronopathic Type 2 or Type 3 disease) (2,8,16), 46/100 PD patients had mild *GBA* mutations (R329C, N370S, D140H and G377S) and 43 null or severe mutations (R120W, R131C, S173X, G202R, Y304C, S364N, D409H, L444P, R463C, R463H, RecΔ55, RecNciI, RecA456P and c.1263del+RecTL) (2,17,18). The 11 PD carriers with new *GBA* variants (G80R, G113A, I119L, S125N, P246L and P452L) and previously reported variants of unknown pathogenicity [K(–27)R, T323I and E388K] were not included in the analyses. PD onset tended to be earlier in carriers of severe *GBA* mutations ( $48.3 \pm 13.5$  years) than in those with mild mutations ( $53.2 \pm 10.4$  years;  $P = 0.06$ ), particularly among isolated cases ( $39.3 \pm 12.0$  years in carriers of severe mutations compared with  $50.8 \pm 9.8$  years in those with mild mutations;  $P = 0.008$ ). When *GBA* mutations were stratified according to their type, an earlier age at onset was observed in patients with null or complex alleles ( $41.2 \pm 10.1$  years,  $n = 9$ ) than in those with missense mutations ( $52.1 \pm 11.9$  years,  $n = 76$ ;  $P = 0.01$ ). Although two controls carried the mild N370S mutation (0.05%), none carried severe mutations.

#### Clinical characteristics of mutation carriers (Table 2)

Carriers and non-carriers had similar sex distributions, ages at onset and examination, disease durations, motor

manifestations on examination and mini mental state examination (MMSE) score. Interestingly, mutation carriers more frequently had bradykinesia at onset (73%) than non-carriers (60%;  $P = 0.014$ ). They also had levodopa-induced dyskinesias more frequently (62%) than non-carriers (50%;  $P = 0.037$ ), independently of gender, dose of levodopa, disease and treatment durations (data not shown).

#### PD patients with two or complex *GBA* mutations

Eight PD patients carried two or complex *GBA* mutations, including one with the complex mutations, c.1263del+RecTL. Full clinical data were not available for one of the two patients with the N370S/L444P mutations. All but one patient had typical PD with asymmetric akinesia, rigidity and/or rest tremor, levodopa responsiveness, levodopa-induced dyskinesias and motor fluctuations. The average age at PD onset was  $53.6 \pm 17.2$  years (range 21–72), like patients with single mutations ( $50.8 \pm 12.4$  years, range 16–73). Five had no atypical symptoms, in particular no signs of GD. Because of the combination of levodopa responsiveness with disabling levodopa-induced dyskinesias, one patient with homozygous N370S mutation and onset at 52 was treated by chronic bilateral stimulation of the subthalamic nucleus (STN), which improved the asymmetric akinesia, rigidity and rest tremor as well as the motor fluctuations and dyskinesias; the patient is still improved at age 78. One PD patient was secondarily diagnosed with GD and another had clinical features suggestive of the GD phenotype, despite no formal diagnosis of GD. Due to complex neurological and extra-neurological features such as osteopenia, a large metabolic screening, including  $\beta$ -glucocerebrosidase levels in serum, was performed in the first patient who was compound heterozygous for N370S/RecΔ55, leading to the diagnosis of GD. At the age of 44, he had symmetrical levodopa-responsive parkinsonism with cervical dystonia, dysarthria and cognitive impairment (MMSE 22/30). Oculomotor movements were normal. Levodopa-induced dyskinesias and motor fluctuations appeared progressively. When examined a second time, he had no haematological or liver abnormalities, but osteopenia and decreased serum levels of  $\beta$ -glucocerebrosidase [ $0.9 \text{ nmol/mg protein/h}$  (normal range 6.5–10.5)] were

**Table 2.** Comparison of the demographic and clinical characteristics of 100 carriers and 1291 non-carriers of *GBA* mutations with PD

	<i>GBA</i> carriers <i>n</i> = 100	<i>n</i> <sup>a</sup>	<i>GBA</i> non-carriers <i>n</i> = 1291	<i>n</i> <sup>a</sup>	<i>P</i> -value
Sex ratio (men: women)	53:47	100	754:537	1291	0.29
Family history of PD (%)	69	100	56	1291	0.009
Age at PD onset					
Total (years)	51.0 ± 12.7 (16–73)	96	50.0 ± 13.7 (10–86)	1220	0.42
Early onset PD (≤50 years)	39.2 ± 7.9 (16–50)	42	39.4 ± 8.4 (10–50)	639	0.93
Late onset PD (>50 years)	60.1 ± 6.9 (51–73)	54	61.7 ± 7.3 (51–86)	581	0.10
Age at examination (years)	59.5 ± 12.4 (25–85)	98	58.8 ± 13.9 (14–90)	1284	0.69
Disease duration (years)	8.45 ± 5.83 (0–30)	96	8.74 ± 7.46 (0–63)	1219	0.61
Signs at onset (%)					
Micrographia	25	88	34	1062	0.082
Bradykinesia	73	88	60	1069	0.014
Rest tremor	51	88	59	1076	0.16
Clinical signs at examination (%)					
Rigidity	92	92	95	1134	0.215
Bradykinesia	95	93	97	1139	0.197
Rest tremor	68	90	74	1129	0.19
UDPRS III ‘off’	35 ± 19 (5–74)	36	33 ± 18 (1–123)	484	0.64
UDPRS III ‘on’	19 ± 11 (2–42)	56	19 ± 13 (0–79)	792	0.84
Hoehn and Yahr ‘on’	1.9 ± 0.8 (0–4)	52	2.1 ± 0.9 (0–5)	743	0.42
Mini mental state (/30)	27.6 ± 4.5 (5–30)	59	28 ± 3.5 (0–30)	749	0.90
Treatment and its effect					
Daily dose of levodopa (mg)	452 ± 281 (0–1550)	79	553 ± 382 (0–3000)	927	0.05
Duration of treatment (months)	87 ± 65 (3–288)	67	87 ± 74 (0.5–720)	797	0.69
Estimated improvement (%)	66 ± 22 (1–100)	61	60 ± 25 (1–100)	737	0.21
Fluctuations	64	76	59	902	0.32
Dyskinesia	62	77	50	911	0.037
Dystonia	28	75	31	894	0.63

<sup>a</sup>*n*, number of available patients.

observed. Imiglucerase treatment did not provide obvious functional benefit.

The second patient, carrying c.1263del+RecTL, had levodopa-responsive asymmetric parkinsonism and rest tremor at the age of 21, in association with segmental dystonia, mild supranuclear gaze palsy, mild pyramidal tract dysfunction and diffuse dorso-lombar pain. Brain magnetic resonance imaging was normal, but the DAT (Dopamine Transporter) scan showed severe bilateral dopaminergic denervation. Copper and ceruloplasmin serum levels were normal. Within 2 years, her status deteriorated, with postural instability, axial hypotonia and depression, and she finally became wheelchair bound at age 23. Because of levodopa-induced dyskinesias and motor fluctuations, bilateral STN chronic stimulation was begun when she was 24. Two years after the beginning of the bilateral STN chronic stimulation, the patient still had some clinical benefits, but the axial unified Parkinson’s disease rating scale (UPDRS) motor score deteriorated due to postural instability.

## DISCUSSION

This study of *GBA* mutations in a total of 1782 individuals from multiplex PD families and isolated cases and controls is one of the largest sequencing studies of *GBA* mutations reported. It allowed us to accurately estimate the frequency of mutations and to identify multiple rare variants, seven of which were novel mutations. These results validate the association between *GBA* mutations and PD reported in a recent, large multicentre analysis (11) and, in addition, provided

data from combined mutational, segregation and clinical analyses in a large and homogeneous European cohort. This enabled us to identify a sufficient number of carriers to assess the influence of the nature of the mutations on the phenotype of patients (8,16). Furthermore, this ethnically homogeneous and clinically well-defined subset of European PD patients allowed us to compare the phenotypes of patients with and without *GBA* mutations, recruited according to the same criteria. The large number of patients with familial (*n* = 525) and isolated (*n* = 605) PD yielded an accurate estimate of the frequency of *GBA* mutations in PD patients. Finally, since all available affected (*n* = 260) and unaffected (*n* = 50) relatives were also analysed, we were better able than previously (6,9,19) to evaluate the concordance of *GBA* mutation carriers within PD families.

In all, *GBA* variants were detected in 100 patients from 76 different PD families and isolated cases for an overall carrier frequency of 6.7%, which was significantly greater than in controls (1.0%, OR = 6.98). However, the frequency was significantly higher in multiplex families than in isolated cases. Although our results replicate the most robust and consistent associations of *GBA* mutations with PD in previous studies (3,4,6,8–11,15,19–25), current genome-wide association studies (26–28), including one of our own in a subset of the present cohort (M. Saad, S. Lesage, A. Saint-Pierre, J.C. Corvol, D. Zelenika, J.C. Lambert, M. Vidailhet, G.D. Mellick, E. Lohmann, F. Durif, P. Pollak, P. Damier, F. Tison, P.A. Silburn, C. Tzourio, S. Forlani, M.A. Lorient, M. Giroud, C. Helmer, F. Portet, P. Amouyel, M. Lathrop, A. Elbaz, A. Durr, M. Martinez and A. Brice, manuscript in preparation),

have not confirmed this association. The present results, however, have greatly expanded on those obtained in the recent meta-analysis, in which 297 isolated PD patients and 251 control subjects of the present cohort were genotyped for only three common *GBA* mutations (4% in patients and 0.4% in controls) (11), and demonstrate the need to sequence all exons and intronic regions of the *GBA* gene, particularly in non-Ashkenazi Jewish populations, to accurately estimate the frequency of mutations and to identify the multiple rare variants in patient and control groups. We would have missed 30% of the mutant alleles in the present series of PD patients and 50% in the controls if only the two most common mutations, N370S and L444P, were analysed. The most common mutation in the present study, N370S, found on 46% of the mutant alleles, was six times more frequent in the PD group than in controls. It was also the most frequent in Ashkenazi Jewish (>70%) and Portuguese PD patients (36%) (15). Of note, N370S was rarely found in Asian patients and controls (7,19,21,22,29), but L444P was found regardless of ethnic background, and is the most frequent mutation in the non-Ashkenazi Jewish PD population (11).

*GBA* variants were identified in 44 of the 525 multiplex families, 21 of which were assessed for co-segregation. The rate of discordance within families (24%) was lower than that in previous studies (6,9). Among discordant families, Nichols *et al.* (9) observed an earlier age at PD onset in patients with pathogenic *GBA* variants than those without. Carriers of pathogenic mutations in our five discordant families also had a lower mean age at onset ( $50.3 \pm 11.5$  years) than those without ( $62 \pm 18.7$  years). However, 46% of the unaffected family members also carried the *GBA* mutations, including an asymptomatic 63-year-old woman with the homozygous Y304C mutation. Their ages at examination, ranging from 29 to 81 years, indicate age-dependent and reduced penetrance. Fourteen out of the 23 unaffected relatives were older than the mean age at onset of PD patients with *GBA* mutations at the time they were examined.

PD onset tended to be earlier in carriers of severe *GBA* mutations, particularly in those with null or recombinant alleles. Interestingly, one carrier of the severe L444P *GBA* mutation and another with c.1263del+RecTL had juvenile PD (onset at 20 and 21 years, respectively). Of note, severe *GBA* mutations were not found in controls. Interestingly, we found eight PD patients with two or complex *GBA* mutations, including two patients with c.1263del+RecTL and N370S/Rec $\Delta$ 55, who had typical PD with early age at onset (21 and 45 years, respectively) and clinical features suggestive of GD.

Clinically, patients with *GBA* mutations were more likely to have family histories of PD and bradykinesia as presenting symptoms of PD; dyskinesias also tended to be more frequent, as previously shown (6), independently of gender, dose of levodopa, disease and treatment durations. However, in contrast with previous reports of an earlier age at onset, a higher male to female ratio and more cognitive changes among *GBA* mutation carriers (3,4,6,8,9,11,19–21, 23–25,30), we found no significant differences between mutation carriers and non-carriers. In addition, the phenotype of PD patients with single *GBA* mutations, and most carriers of two mutations, was similar to that of PD patients with no mutations. Thus, PD patients carrying two or complex *GBA*

mutations may be suitable for chronic bilateral STN stimulation, as illustrated by the two cases we report. However, further studies are needed to confirm these results.

In conclusion, this large study reinforces the role of *GBA* as a major risk factor for PD. The next challenge will be to elucidate how *GBA* mutations lead to PD. Increasing evidence suggests that mutant  $\beta$ -glucocerebrosidase protein is related to the abnormal handling and clearance of  $\alpha$ -synuclein that result in Lewy body pathologies. If  $\beta$ -glucocerebrosidase misfolding and impaired trafficking contribute to the development of PD in *GBA* mutation carriers, then chaperone-mediated therapy may be a therapeutic or preventive option to treat these patients (31).

## MATERIALS AND METHODS

### Subjects

Patients were recruited through the French network for the study of Parkinson's Disease Genetics (PDG). Local Ethics Committees approved the study, and written informed consent was obtained from all participants.

The study included 1391 PD patients, 786 patients from 525 unrelated multiplex families and 605 isolated PD cases, mostly from France (89%) (Table 3). Patients underwent standard neurological examinations, including the UPDRS, the Hoehn and Yahr scale, response to levodopa and the MMSE to evaluate cognitive function. Most (>80%) fulfilled the criteria for definite PD (32). Familial parkinsonism was defined by a positive family history compatible with PD in at least one first- or second-degree relative. For co-segregation analyses, available DNA from unaffected family members ( $n = 50$ ) was also tested for the corresponding *GBA* mutations. The controls, 391 ethnicity-matched subjects without family histories of PD, mainly spouses of patients, included 210 females and 181 males examined at age  $59.3 \pm 11.0$  (range 31–85).

Patients with *Parkin*-, *PINK1*- and *DJ-1*-associated recessive parkinsonism, with an early age at onset (<45 years), were excluded. All subjects were screened for the *LRRK2* G2019S mutation, by direct sequencing of exon 41 (32) or by Taqman Assay-by-Design SNP method (33) using SDS v2.0.1 on ABI 7500 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and any mutations identified were verified by direct sequencing.

### Molecular analyses

Peripheral blood was collected from each patient, and DNA was extracted from leucocytes according to standard procedures. Exons and flanking intronic regions of *GBA* were sequenced in all patients and controls. To avoid amplifying and sequencing the neighbouring pseudogene, *GBA* was amplified in three large fragments (a 2972 bp fragment encompassing exons 1–5; a 2049 bp fragment encompassing exons 5–7 and a 1682 bp fragment encompassing exons 8–11), using previously described primers and a unique 64°C to 54°C touch-down PCR program (Supplementary Material, Table S1). PCR products were sequenced with internal primers, adjacent to coding exons and exon–intron boundaries, using the Big Dye Terminator Cycle Sequencing



**Table 3.** Demographic and clinical characteristics of PD patients and control subjects

	Patients with PD			Control Subjects
	Total	With FH <sup>a</sup>	Sporadic	
Number of subjects	1391	786	605	391
Gender (M/F)	807/584	438/348	369/236	181/210
Age at onset (years)	50.1 ± 13.6	53.0 ± 13.8	46.4 ± 12.3	
Range	10–86	10–86	12–78	
Age at examination (years)	58.9 ± 13.8	61.2 ± 13.8	55.8 ± 13.2	59.3 ± 11.0
Range	14–90	16–90	14–85	31–85
Disease duration (years)	8.73 ± 7.34	8.09 ± 7.56	9.52 ± 6.98	
Range	0–63	0–63	0–37	

FH, family histories of PD.

<sup>a</sup>These patients derived from 525 unrelated multiplex families.

Ready Reaction kit (Applied Biosystems), as prescribed. Sequencing products were purified using the Big Dye X Terminator Purification kit (Applied Biosystems), then electrophoresed on an ABI 3730 automated sequencer and analysed with DNA Sequencing Analysis (version 5.1) and Seqscape (version 2.1.1) software (Applied Biosystems).

Mutation nomenclature follows Human Genome Variation Society (HGVS) recommendations: *GBA* cDNA nucleotides ('c.') are numbered with the adenine of the first ATG translation initiation codon as nucleotide + 1 (GenBank reference sequence NM\_000157.2). All allele names used in this report refer to the processed protein, excluding the 39-residue signal peptide. The ClustalW program on the European Bioinformatics Institute server (<http://www.ebi.ac.uk/clustalw/>) was used to align the human *GBA* protein and its closest homologues: [sequence IDs: *Homo sapiens* (NM\_000157.2), *Pan troglodytes* (ENSPTRT00000002602), *Macaca mulatta* (ENSPPYT00000000903), *Pongo pygmaeus* (ENSMMUT0000011892), *Mus musculus* (ENSMUST00000077367), *Canis familiaris* (ENSCAFT00000026904), *Felis catus* (ENSFCAT00000000900), *Bos taurus* (ENSBTAT00000019765), *M. domestica* (ENSMODT00000021686), *T. nigroviridis* (GSTENT00033926001), *Drosophila melanogaster* (FBtr0113405) and *C. elegans* (C33C12.8)].

### Statistical analyses

Mutation frequencies were calculated counting one carrier per family. Demographic and clinical characteristics of mutation carriers and non-carriers were compared with the  $\chi^2$  or Fisher's exact tests for categorical variables and the Mann–Whitney U-test for continuous variables (significant at  $P < 0.05$ ). ORs were calculated with a 95% CI for association between *GBA* mutations and PD.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement.* None declared.

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### APPENDIX

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