RESEARCH ARTICLE

Harmonizing Genetic Testing for Parkinson's Disease: Results of the PARKNET Multicentric Study

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ABSTRACT: Background and Objective: Early-onset Parkinson's disease (EOPD) commonly recognizes a genetic basis; thus, patients with EOPD are often addressed to diagnostic testing based on next-generation sequencing (NGS) of PD-associated multigene panels. However, NGS interpretation can be challenging in a diagnostic setting, and few studies have addressed this issue so far.

Methods: We retrospectively collected data from 648 patients with PD with age at onset younger than 55 years who underwent NGS of a minimal shared panel of 15 PD-

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[Correction added on 12 October 2023, after first online publication: The ninth author's degree is updated in this version.].

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related genes, as well as PD-multiplex ligation-dependent probe amplification in eight Italian diagnostic laboratories. Data included a minimal clinical dataset, the complete list of variants included in the diagnostic report, and final interpretation (positive/negative/inconclusive). Patients were further stratified based on age at onset ≤40 years (very EOPD, n = 157). All variants were reclassified according to the latest American College of Medical Genetics and Genomics criteria. For classification purposes, PD-associated *GBA1* variants were considered diagnostic.

Results: In 186 of 648 (29%) patients, the diagnostic report listed at least one variant, and the outcome was considered diagnostic (positive) in 105 (16%). After reanalysis, diagnosis changed in 18 of 186 (10%) patients, with 5 shifting from inconclusive to positive and 13 former positive being reclassified as inconclusive. A definite diagnosis was eventually reached in 97 (15%) patients, of whom the majority carried *GBA1* variants or, less frequently, biallelic *PRKN* variants. In 89 (14%) cases, the genetic report was inconclusive.

Conclusions: This study attempts to harmonize reporting of PD genetic testing across several diagnostic labs and highlights current difficulties in interpreting genetic variants emerging from NGS-multigene panels, with relevant implications for counseling. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; EOPD; next-generation sequencing; gene panel; variant classification

Introduction

Patients with early-onset Parkinson's disease (EOPD) are mostly defined as subjects with disease onset before 50 years.[1] It has been clearly established that EOPD often recognizes a genetic basis.[2] Although *PRKN* is the most commonly mutated gene in recessively inherited EOPD with onset before 40 years, several other genes have also been implicated.[3] In particular, heterozygous pathogenic variants in *LRRK2* and *GBA1*, both dominantly inherited with low to very low penetrance, have been frequently detected in patients with EOPD, with ethnic-specific variability.[4] In the past, these genes used to be Sanger sequenced in selected patients (eg, based on positive family history, very early onset, atypical presentations). However, most PD genes have numerous exons and lack mutational hot spots, making Sanger sequencing impractical in the diagnostic setting.

The advent of next-generation sequencing (NGS) has revolutionized the diagnosis of genetically heterogeneous diseases such as EOPD, allowing to sequence all disease-related genes simultaneously and rapidly in large cohorts of patients, and has now fully substituted Sanger sequencing in most diagnostic labs. In contrast, NGS unraveled a much higher genetic variability than expected, resulting in the identification of several variants of difficult interpretation, which often represent a challenge in the diagnostic setting.

Although labs are bound to adopt specific criteria to classify variants according to their presumed pathogenicity, guidelines on which variants should be included in the diagnostic report and how certain variants should be interpreted are still missing, leading to wide variability and inconsistencies in genetic testing reports.

Within the PARKNET project, this study aimed to harmonize the interpretation of PD genetic testing across the network, as well as to provide a comprehensive assessment of the genetic basis of EOPD in Italy.

Patients and Methods

Patient Recruitment

The National Virtual Parkinson Institute, a consortium of Italian Scientific Institutes for Medical Research and Care (Istituti di Ricovero e Cura a Carattere Scientifico [IRCCS]) focused on PD research and management funded by the Ministry of Health, has undertaken PARKNET, an ambitious project to share knowledge and expertise, as well as clinical and genetic data of patients with PD among participating centers. Data-sharing agreements were signed by each institute.

In the frame of PARKNET, we retrospectively collected data from 648 patients with PD with age at onset (AO) younger than 55 years referred for PD genetic testing in the years 2017–2022 at one of the following institutions: Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan, Foundation IRCCS Istituto Neurologico Carlo Besta of Milan, IRCCS Humanitas Research Hospital of Rozzano, IRCCS Ospedale Policlinico San Martino of Genoa, IRCCS Istituto delle Scienze Neurologiche of Bologna, Foundation Mondino IRCCS of Pavia, IRCCS Istituto Neurologico Mediterraneo of Isernia, and Foundation Santa Lucia IRCCS of Rome (Fig. S1). Diagnosis of PD was always made by specialists in movement disorders using the Movement Disorder Society clinical diagnostic criteria.[5] The decision to set the cutoff of AO to 55 years was stimulated by the observation that several patients with onset between 50 and 55 years had also been referred to diagnostic genetic testing, even if not properly defined as EOPD. Shared data included a minimal clinical dataset (AO, family history for PD, consanguinity), as well as the complete

list of variants included in the diagnostic report and the final interpretation of results: either positive (one or more variants listed in the report, allowing to reach a definite genetic diagnosis), inconclusive (one or more variants listed in the report, but not sufficient to reach a genetic diagnosis), or negative (no variants listed in the report). Written informed consent for genetic analyses for diagnostic and research purposes was obtained from all patients. Stratification of collected data was performed according to AO, differentiating a subgroup of patients with very EOPD (vEOPD; AO ≤ 40 years), and to family history for PD (Fig. S4).

Genetic Analysis

The study design is schematically shown in Figure S2.

Patients were included if both a diagnostic gene panel and a dosage analysis for *SNCA*, *PRKN*, *PINK1*, and *PARK7* had been performed. Panels varied among institutions, but all shared a minimal set of 15 genes associated with autosomal dominant (*SNCA*, *LRRK2*, *VPS35*, *GBA1*), X-linked (*RAB39B*), and autosomal recessive PD (*PRKN*, *PINK1*, *PARK7*, *ATP13A2*, *PLA2G6*, *DNAJC6*, *SYNJ1*, *FBXO7*, *VPS13C*, *PTRHD1*) (Table S1). NGS enrichment was based on either HaloPlex (Agilent), SureSelect XT (Agilent), Nextera (Illumina), or TruSeq Custom Amplicon (Illumina). All labs guaranteed a mean 30-fold coverage of at least 90% nucleotides across all genes. Multiplex ligation-dependent probe amplification (MLPA) was performed to determine exon copy-number variants (CNVs) using P051-D2 and P052-D2 kits (MRC Holland). Sanger sequencing was used to confirm all reported variants. Each center was requested to indicate all single-nucleotide variants (SNVs) and CNVs included in the genetic report, and whether it was defined positive or inconclusive. Data related to each variant were collected from online databases (gnomAD v2.1.1,[6] dbSNP, ClinVar).

Harmonization of the Interpretation Pipeline

The harmonization process consisted of applying the same internationally adopted criteria to classify each reported variant. Each SNV was (re)classified as benign (<−6 points), likely benign (from −6 to −1 points), variants of unknown significance (VUSs; from 0 to 5 points), likely pathogenic (from 6 to 9 points), or pathogenic (≥10 points), according to the latest American College of Medical Genetics and Genomics (ACMG) criteria, because not all laboratories had formerly used this classification.[7,8] ACMG classification was first obtained using Varsome Premium[9] and then manually curated to take into account additional relevant factors, such as data from the recent literature or cosegregation with another deleterious variant (in case of recessive genes). *GBA1* variants previously classified as "complex," "severe," "mild," and "risk" were all classified as pathogenic.[10] To calculate the diagnostic yield, we included only variants classified as pathogenic and likely pathogenic, hereby collectively referred to as "deleterious variants." Notably, according to recent evidences, the PVS1 criterion was not flagged for loss-of-function *LRRK2* variants.[11] All deleterious *GBA1* variants were considered diagnostic, although being aware of their very low penetrance. Regarding autosomal recessive genes, only biallelic deleterious variants were considered diagnostic.[12,13]

Statistical Analysis

χ^2 test was used to assess statistically significant differences within PD and vEOPD groups. A P value <0.05 was set to consider a difference between groups statistically significant.

Results

Harmonization and Diagnostic Yield

We reviewed the diagnostic reports of 648 patients with PD with AO ≤ 55 (mean AO, 42.6 ± 9.7 years; male: n = 369, 57%) (Table 1).

In 186 of 648 patients (29%), at least one SNV/CNV was listed in the report. The identified variants were considered diagnostic in 105 patients (16%), whereas in 81 cases (13%) the report listed one or more variants, but the outcome was considered inconclusive.

After harmonization, the interpretation of genetic testing changed in 18 of 186 patients (10%). In five, the outcome was modified from inconclusive to diagnostic after reclassifying the reported *GBA1* variants as pathogenic. Conversely, 13 reports previously considered as diagnostic were declassified as inconclusive, including four cases with single heterozygous pathogenic variants in a recessive gene and nine cases where the reported variants were reclassified as VUS or likely benign (Table S3).

The new diagnostic yield was 15% (97/648); in an additional 89 patients (14%), the variants listed in the report were considered inconclusive (Tables 1 and S4). Deleterious variants in the *GBA1* gene (n = 58, 8.0%; mean AO: 43.6 ± 9.3 years) and biallelic deleterious SNVs/CNVs of *PRKN* (n = 18, 2.6%; mean AO: 33.3 ± 11.2 years) were the most common diagnoses, followed by heterozygous deleterious variants in *LRRK2* (n = 9, including p.G2019S n = 6, 0.9%, mean AO: 42.3 ± 12.8 years; p.R1441C n = 3, 0.5%, mean AO: 40.7 ± 20.6 years), biallelic deleterious variants in *PINK1* (n = 7, 1.1%; mean AO: 32.6 ± 12.5 years), and heterozygous deleterious variants in *SNCA* (n = 3, 0.5%, mean AO: 44.6 ± 12.6 years). Single patients carried deleterious variants in *RAB39B*, *PARK7*, *VPS13C*, and *PLA2G6*, respectively.

TABLE 1 *Demographic data and genetic results*

	PD (AO ≤ 55 y)	vEOPD (AO ≤ 40 y)
Demographic data		
Total patients, n	648	157
Mean AO ± SD	42.6 ± 9.7 y	32.3 ± 9.7 y
Male sex, n (%)	369 (57)	105 (67)
Positive family history for PD, n (%)	172 (26.5)	59 (37.6)
No variant alleles listed in genetic report, n (%)	462 (71.3)	86 (54.8)
At least one variant listed in genetic report, n (%)	186 (28.7)	71 (45.2)
Genetic results (postharmonization)		
Positive diagnoses (P/LP variants), n (%)	97 (15.0)	38 (24.2)
• *GBA1*[a]	58 (9.0)	14 (8.9)
• *PRKN* biallelic[a]	18 (2.8)	12 (7.6)
• *LRRK2*[a]	9 (1.4)	4 (2.5)
• *PINK1* biallelic	7 (1.1)	6 (3.8)
• *SNCA*	3 (0.5)	1 (0.6)
• *PARK7* biallelic	1 (0.2)	1 (0.6)
• *RAB39B*	1 (0.2)	1 (0.6)
• *VPS13C* biallelic	1 (0.2)	–
• *PLA2G6* biallelic[b]	1 (0.2)	–
Inconclusive diagnoses, n (%)	89 (13.7)	33 (21.0)
• Monoallelic P/LP variants in recessive genes	13 (2.0)	6 (2.5)
• Other variants (VUS, LB, or B)	76 (11.7)	27 (17.2)

[a]One patient tested positive for both *GBA1* and *PRKN*.
[b]One patient tested positive for both *LRRK2* and *PLA2G6*.
Abbreviations: PD, Parkinson's disease; AO, age at onset; vEOPD, very early-onset Parkinson's disease; SD, standard deviation; VUS, variant of unknown significance; LB, likely benign; B, benign.

Notably, two patients received a double genetic diagnosis: one carried a *GBA1* heterozygous variant and a *PRKN* homozygous variant, and another carried the *LRRK2* p.G2019S variant in combination with a homozygous likely pathogenic missense variant in *PLA2G6*. In 16 patients who received a definite diagnosis, the diagnostic report also listed one or more additional variants in a distinct gene, which were classified as VUSs or likely benign/benign in all cases but one (a heterozygous likely pathogenic variant in *DNAJC6*).

Among the 89 patients with inconclusive reports, 13 carried a single heterozygous deleterious variant in a recessive gene (*PRKN* n = 7, *PINK1* n = 2, *PLA2G6* n = 3, *PARK7* n = 1), whereas 5 additional cases were biallelic carriers of two variants in a recessive gene (*PINK1* n = 2, *PLA2G6* n = 1, *FBXO7* n = 1, *VPS13C* n = 1), both classified either as VUS or benign/likely benign, thus not allowing to reach a definite diagnosis. The remaining patients carried single or multiple nondeleterious variants in one or more genes.

Focusing on the vEOPD subgroup (n = 157; male n = 105, 67%; mean AO: 32 ± 9.7 years), 71 of 157 (45%) had one or more variants reported, which were initially considered diagnostic in 43 (27%) and inconclusive in 28 (18%). Postharmonization, a definite diagnosis could be confirmed in 38 patients (25%). Deleterious *GBA1* variants (n = 14, 9%; mean AO: 32.3 ± 8.7) and biallelic *PRKN* SNVs/CNVs (n = 12, 8%; mean AO: 30 ± 8.9) were the most common diagnoses, followed by biallelic *PINK1* variants (n = 6, 4%; mean AO: 28.8 ± 8.4 years). Other genetic causes were rarer.

Variants

Overall, 263 variant alleles in 14 genes were listed in the examined genetic reports (Tables 2 and S5). Among these alleles, 148 were classified as deleterious, 50 as VUSs, and 65 as benign or likely benign. Of the deleterious variants, 134 were diagnostic, whereas 14 represented single heterozygous variants in recessive genes.

GBA1 was the most commonly mutated gene, with 70 variant alleles reported, of which 65 (93%) were classified as deleterious. The founder p.N409S (N370S) and p.L483P (L444P) variants were the commonest, representing nearly half of the reported variants (n = 30, 43%), either alone or in combination with other *GBA1* variants [p.R87W (R48W), p.R159Q (R120Q), p.E365K (E326K)]. In the *PRKN* gene, 55 variant alleles were reported. The majority of *PRKN* variants were pathogenic or likely pathogenic (n = 43, 78%), with the majority being represented by CNVs, in particular deletions or duplications of exon 3. Of the 28 *PINK1* variant alleles, more than half were deleterious (n = 16, 57%, of which 13 were truncating). Conversely, of 34 *LRRK2* variant alleles, only 9 (26%) could be classified as deleterious, including 6 p.G2019S and 3 p.R1441C.

Discussion

In this study, we reviewed the genetic reports of 648 patients with PD with AO ≤55 years provided by eight Italian genetic laboratories in the frame of the PARKNET project, with the main goal to harmonize the interpretation of diagnostic PD genetic tests across all participating centers. In addition, we used this large wealth of data to assess the diagnostic yield and

TABLE 2 *Genetic findings: summary of the most involved genes*

	Total	Positive P/LP variants, biallelic in AR genes	Inconclusive		
			Monoallelic P/LP variants in AR genes	VUSs	LB/B variants
All genes	**263**	**134 (51%)**	**14 (5.3%)**[a]	**50 (19%)**	**65 (24.7%)**
GBA1	70	**65 (92.9%)**	–	5 (7.1%)	0
LRRK2	34	9 (26.5%)	–	10 (29.4%)	**15 (44.1%)**
SNCA	3	**3 (100%)**	–	0	0
VPS35	4	0	–	3 (75%)	1 (25%)
PRKN	55	**36 (65.4%)**	7 (12.7%)	7 (12.7%)	5 (9.1%)
PINK1	28	**14 (50.0%)**	2 (7.1%)	8 (28.6%)	4 (14.3%)
PARK7	12	2 (16.7%)	1 (8.3%)	1 (8.3%)	**8 (66.7%)**
ATP13A2	2	0	0	0	**2 (100%)**
PLA2G6	17	2 (11.8%)	3 (17.6%)	**6 (35.3%)**	**6 (35.3%)**
DNAJC6	7	0	1[a] (14.3%)	2 (28.6%)	**4 (57.1%)**
SYNJ1	6	0	0	3 (50%)	3 (50%)
FBXO7	6	0	0	2 (33.3%)	**4 (66.7%)**
VPS13C	18	2 (11.1%)	0	3 (16.7%)	**13 (72.2%)**
PTRHD1	0	0	0	0	0
RAB39B	1	1 (100%)	–	0	0

Note: Number and frequency of variant alleles divided by gene and American College of Medical Genetics and Genomics classification (out of a total of 263 alleles reported in 186 subjects with Parkinson's disease). For the purpose of this table, homozygous variants are counted twice. Some variants (within the same gene or distinct genes) co-occurred in the same individual. The two *GBA1* complex alleles are counted as single variants. Percentages are calculated on the total number of variants for each gene. The most prevalent category for each gene is highlighted in bold.

[a]One heterozygous LP variant in *DNAJC6* occurred in a patient carrying a *GBA1* deleterious variant.

Abbreviations: AR, autosomal recessive; VUS, variant of unknown significance.

determine the contribution of each gene to the diagnosis of EOPD in Italy.

To harmonize interpretation of genetic results, we applied the latest ACMG guidelines to (re)classify each variant that had been listed in the diagnostic reports; in line with these widely accepted guidelines, only patients carrying pathogenic or likely pathogenic variants in a PD-related gene (biallelic in case of recessive genes) were considered "positive," for example, received a definite diagnosis of a genetic disease. Notably, not all laboratories were CLIA accredited, and some had not adopted ACMG criteria to classify variants. Interestingly, the reanalysis led to changing the outcome of the diagnostic report in 18 patients (10%): 5 could eventually receive a definite diagnosis after upgrading of the detected *GBA1* variants to pathogenic, whereas in 13 patients, the former genetic diagnosis could not be confirmed, either because the identified variant was (re) classified as VUSs or likely benign or due to the lack of a second deleterious variant in a recessive gene. This outcome change has major implications for genetic counseling of patients and families, considering at one end the diagnostic delay and, at the other end, the former delivery of a genetic diagnosis that could not be confirmed based on further assessments.

Despite the rising awareness toward PD genetic testing and the increasing requests of diagnostic tests for patients with EOPD, no specific guidelines exist, leading to inconsistencies across distinct laboratories in the choice of variants to be listed in the diagnostic report and, most importantly, in their final interpretation. The case of the patient carrying the homozygous variant p.A516V in *PLA2G6* is emblematic of the still-open challenge in the interpretation of variant pathogenicity and highlights the limitations of current ACMG criteria. Indeed, according to such criteria, this variant is classified as a VUS, applying PM1 (mutational hot spot) and PM2 (absence in controls) at the supporting level. Upon discovering a positive family history and reexamining clinical and imaging data, we were able to manually flag two additional criteria at the supporting level, namely, PP1 (cosegregation into multiple family members) and PP4 (highly specific phenotype), yet, to strictly adhere to current guidelines,[8] we did not flag PP5 (reliable source reports the variant as pathogenic), although the same variant had formerly been reported

in three unrelated patients with features in the spectrum of PLA2G6-associated neurodegeneration.[14-17] Thus, despite that this homozygous variant clearly appears as causative of the early-onset dystonia-parkinsonism phenotype shown by the patient, ACMG criteria still do not reach the sufficient strength to unequivocally classify the variant as pathogenic/likely pathogenic. This case well illustrates the current controversies and methodological challenges in reaching a genetic diagnosis that should be properly recognized toward continuous improvement of diagnostic criteria. In this light, ClinGen has recently established an international PD gene and variant curation expert panel to develop consensus over genes with low confidence that have been cited in the literature and/or included on commercially available genetic testing panels, as well as over the pathogenic relevance of specific gene variants (https://clinicalgenome.org/). Yet, this effort is still ongoing and, even upon completion, it will require some time to enter routine clinical practice. Our work highlights the importance of developing international consensus guidelines for PD genetic testing, providing guidance to the diagnostic laboratories as regard genes to be included in NGS panels, as well as consolidated criteria for variant classification, interpretation, and reporting.

In this cohort of 648 PD probands, a genetic diagnosis could be reached in 15% of patients and increased to 24% in the subgroup of vEOPD. These figures are in line with previous NGS studies in French, Spanish, Chinese, and Taiwanese populations,[18-21] suggesting that the diagnostic yield is similar across different populations worldwide and not strictly correlated with population-specific genetic variants. However, the frequency of founder mutations is different across populations: for instance, *LRRK2* p.G2019S and *GBA1* p.N409S (N370S) are more common in European patients, whereas *LRRK2* p.G2385R and *GBA1* p.L483P (L444P) occur more frequently in Asians.[22,23]

Notably, *GBA1* variants emerged as the commonest determinants in EOPD, being detected in 9% of the whole cohort and accounting for more than half of all genetic diagnoses. There is now consensus evidence that GBA1 heterozygous variants cause autosomal dominant PD with highly reduced penetrance (https://www.clinicalgenome.org/affiliation/40079/), and therefore such variants should be included in the diagnostic reports and included in the assessment of the diagnostic yield of PD genetic testing. Although the two founder variants, p.N409S (N370S) and p.L483P (L444P), were the most frequent ones, they collectively accounted for less than half of all *GBA1* diagnoses, confirming our previous findings that sequencing of the entire *GBA1* gene is necessary to avoid false negative results.[10]

The *PRKN* gene was the second most frequently mutated gene in this study: frequencies are consistent to those of different populations,[18,21] with the exception of Ashkenazi Jews, who seem to be more rarely mutated.[13] Up to 40% of variant alleles were represented by CNVs, highlighting the importance of complementing the diagnostic testing with molecular strategies able to identify *PRKN* genomic rearrangements.

LRRK2 p.G2019S and p.R1441C were collectively found in ~1.4% of patients with PD with AO ≤55 years, in line with previous reports in the Italian population.[24-26] This low frequency of *LRRK2* diagnoses compared with other studies may reflect the fact that *LRRK2*-related PD may manifest at older ages.[24,26] Moreover, it is expected that several patients with EOPD (especially those with positive family history) have already been screened for *LRRK2* p.G2019S and p.R1441C in the past years, and thus may have been missed by this study.

PINK1 biallelic and *SNCA* heterozygous pathogenic variants occurred in 1.1% and 0.5% of patients, respectively, whereas few other genes (*PARK7*, *VPS13C*, *PLA2G6* or *RAB39B*) were mutated in single patients, usually presenting more complex phenotypes. It must be noted that the cohort screened in this study was characterized by a pure parkinsonian syndrome, which likely explains the underrepresentation of genes associated with more complex phenotypes (ie, *SYNJ1*, *ATP13A2*, *FBXO7*, *PTRHD1*). Subgroup stratification showed enrichment of pathogenic variants in patients with vEOPD, leading to a higher diagnostic yield ($p = 0.00002$). In particular, patients with AO ≤40 years presented a higher chance to have biallelic *PRKN* or *PINK1* variants compared with the entire cohort ($P = 0.00007$).

Upon reclassification of variants, 89 genetic tests (14%) yielded inconclusive results, yet important differences should be highlighted within this group. Indeed, 13 patients carried a single heterozygous pathogenic or likely pathogenic variant in a recessive gene, suggesting the existence of a second cryptic variant escaping NGS and MLPA testing, and thus prompting further studies to explore this possibility. In two additional cases, two VUSs in *trans* were detected in a recessive gene (either *PINK1* or *PLA2G6*). Although this variant's classification impeded to reach a definite diagnosis at present, functional experiments may be warranted to prove pathogenicity of these VUSs, which would potentially allow to reclassify them as deleterious.

Conversely, 74 reports listed heterozygous variants in one or more genes, which were classified as VUSs, likely benign, or benign. Similar variants were also listed in the reports of 16 patients who already had a definite diagnosis related to the presence of deleterious variants in another PD gene. It is questionable whether these variants should have been included at all in the diagnostic reports because, according to ACMG guidelines,

VUSs should be reported only where further testing or investigations have the potential to change their classification to likely pathogenic, which is improbable for benign and likely benign variants, and also for some low-score VUSs. In fact, these variants are almost invariably unlikely to be disease causing and could be potentially confusing if included in a report. Notably, most variant alleles in the *SNCA*, *GBA1*, and *PRKN* genes were deleterious (many of them having already been reported in patients with PD); conversely, nearly half of *PINK1* and the majority of *LRRK2*, *PARK7*, *PLA2G6*, and *VPS13C* variant alleles were classified as nondeleterious, suggesting caution when interpreting variants encountered in these genes. It is also worth noticing that variant classification is still a highly dynamic process, with frequent fine-tuning of the adopted criteria that might result in classification changes, especially for VUSs. In this light, laboratories should implement a regular reassessment of NGS data, especially regarding variants whose classification is not straightforward.

Finally, it is interesting to note that, despite NGS and MLPA screening, the majority of patients with PD remained without a genetic diagnosis. Many Whole Exome Sequencing projects have been recently conducted in large cohorts of patients but have resulted in very few novel PD-causative genes.[27] These observations suggest either the possibility of an incomplete detection of variants in already known PD genes using conventional molecular approaches, or the existence of other still-unknown genetic factors, possibly acting in a multifactorial model to influence PD susceptibility even in patients with early onset of the disease.[28,29] It is expected that large ongoing projects on PD genetics, such as the worldwide GP2 effort (Global Parkinson's Genetic Project; https://gp2.org), will eventually address this major unsolved issue.

We acknowledge some limitations of this study. First, this is a retrospective multicentric study, and despite that we included only patients who underwent both NGS of a minimal shared gene set and MLPA analysis, testing methodologies, as well as criteria for reporting variants, varied among labs, possibly affecting sensitivity and specificity of testing. Moreover, all panels were designed to target only exonic regions and intron-exon boundaries, missing possible pathogenic variants and rearrangements within noncoding regions. Also, *GBA1* complex rearrangements could have been missed or underestimated using this NGS approach because of the very high sequence homology of *GBA1* with its pseudogene, leading to misalignment of reads. Indeed, only two complex alleles were reported in the entire cohort. Furthermore, as mentioned earlier, *LRRK2* carrier frequency could also have been underestimated because of previous screenings of patients with a positive family history. Finally, the authors acknowledge the potential inclusion of genetic data formerly reported in other studies, because the occasional patients may have independently undergone sampling for diagnostic and research purposes tests in different centers, and with diverse pseudonymization.

In summary, in this article, we report the results of a project aimed at harmonizing the diagnostic testing and reporting strategies across different institutions in Italy. We confirm that *GBA1* and *PRKN* are the commonest mutated genes in PD with early AO, and that a very small set of genes (including *GBA1*, *LRRK2*, *PRKN*, *PINK1*, and *SNCA*) accounts for nearly all mutated PD patients lacking atypical features. Assessment of variants according to the latest ACMG criteria led to change the diagnostic status in 18 of 186 patients, with relevant consequences for counseling of patients and their families. This highlights the importance of developing an international consensus strategy for PD genetic testing, variant interpretation, and reporting in the diagnostic setting. ■

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Data Availability Statement

The complete list of variants is available in Table S2. The data that supports the findings of this study are available in the supporting information of this article.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.