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MUTATION IN BRIEF

PINK1 Heterozygous Rare Variants: Prevalence, Significance and Phenotypic Spectrum

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Short Title: Heterozygous rare variants in the *PINK1* gene

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Heterozygous rare variants in the *PINK1* gene, as well as in other genes causing autosomal recessive parkinsonism, have been reported both in patients and healthy controls. Their pathogenic significance is uncertain, but they have been suggested to represent risk factors to develop Parkinson disease (PD). The few large studies that assessed the frequency of *PINK1* heterozygotes in cases and controls yielded controversial results, and the phenotypic spectrum is largely unknown. We retrospectively analyzed the occurrence of *PINK1* heterozygous rare variants in over 1100 sporadic and familial patients of all onset ages and in 400 controls. Twenty patients and 6 controls were heterozygous, with frequencies (1.8% vs. 1.5%) not significantly different in the two groups. Clinical features of heterozygotes were indistinguishable to those of wild-type patients, with mean disease onset 10 years later than in carriers of two mutations but worse disease progression. A meta-analysis indicated that, in *PINK1* heterozygotes, the PD risk is only slightly increased with a non significant odds ratio of 1.62. These findings suggest that *PINK1* heterozygous rare variants play only a minor susceptibility role in the context of a multifactorial model of PD. Hence, their

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significance should be kept distinct from that of homozygous/compound heterozygous mutations, that cause parkinsonism inherited in a mendelian fashion. © 2008 Wiley-Liss, Inc.

KEY WORDS: Parkinson disease, *PINK1*, autosomal recessive parkinsonism, heterozygous rare variants

INTRODUCTION

Parkinson disease (PD, MIM# 168600) is a common neurodegenerative disorder characterized by dopaminergic neuronal loss in the substantia nigra and other brain areas. Despite the vast majority of cases are sporadic, at least six genes have been identified so far that are responsible of autosomal dominant or recessive forms of parkinsonism (Tan and Skipper, 2007).

Homozygous or compound heterozygous mutations in autosomal recessive genes (*Parkin* [*PARK2*; MIM# 602544], *PINK1* [MIM# 608309] and *DJ-1* [*PARK7*; MIM# 602533]) cause parkinsonism that usually differs from idiopathic PD for the earlier onset (<45-50 years), the better response to Levodopa and the slower disease progression (Kubo et al., 2006). However, in a substantial proportion of mutated cases only a single heterozygous mutation (or rare variant) can be detected despite extensive analysis. These variants are occasionally found also in healthy controls and their significance and pathogenicity are largely debated. While the existence of a second, unidentified pathogenic variant in the same or in a distinct gene cannot be excluded, an alternative hypothesis suggests that heterozygous rare variants could influence the risk to develop PD or the age at onset, in the frame of a more complex genetic and environmental background. This hypothesis is supported by recent neuroimaging and neurophysiology studies that identified subclinical signs of dopaminergic dysfunction also in healthy *Parkin* or *PINK1* heterozygotes (reviewed in Klein et al., 2007).

PINK1 represents the second most frequent cause of early onset parkinsonism (EOP) after *Parkin*, with homozygous and compound heterozygous mutations accounting for about 4-5% of autosomal recessive and 1-2% of sporadic cases. Single heterozygous rare variants are rarely encountered in recessive families while they are more frequently detected in sporadic cases (Valente et al., 2004b; Bonifati et al., 2005; Klein et al., 2005; Li et al., 2005; Abou-Sleiman et al., 2006; Fung et al., 2006; Ibanez et al., 2006; Tan et al., 2006). Their prevalence differs considerably among published studies. Moreover, clinical data are available only for a limited number of heterozygotes and no phenotypic comparisons have been performed between heterozygotes and carriers of two mutations or wild-type cases.

To address these issues we assessed the frequency and phenotypes of *PINK1* heterozygotes among our cohorts of patients and controls tested for *PINK1* since the gene identification in 2004 (Valente et al., 2004a), and compared them with published data.

PATIENTS AND METHODS

Patients

We report here retrospective data on 1126 probands ascertained by nine Italian movement disorders centers (Italian PD Study Group) and tested for *PINK1* mutations since 2004. This cohort includes both familial and sporadic patients with a definite clinical diagnosis of PD and variable age at onset from the second to the ninth decade. All probands were negative for the common G2019S mutation in the *LRRK2* gene; moreover, mutations and exonic rearrangements in the *Parkin* and *DJ-1* genes were excluded in over 90% of early onset cases. A positive family history was considered when at least one first- or second-degree relative was affected by PD (diagnosed by a movement disorder specialist). Transmission was considered consistent with autosomal dominant inheritance in probands with at least one affected parent or offspring; and consistent with autosomal recessive inheritance in probands with affected siblings or in sporadic cases with consanguineous parents. The remaining familial cases with uncertain transmission (i.e. with an affected uncle), were defined as "unclear inheritance". Detailed clinical data of heterozygotes were collected from clinical charts for comparison with two groups: 1) patients carrying two PINK1 mutations (n=10), including the two probands and four affected relatives from the original PARK6 Italian families (Valente et al., 2004a) and other four subsequently identified cases (Valente et al., 2004b; Marongiu et al., 2007; unpublished data); 2) a subgroup of 320 wild-type patients for whom full medical records were available, that was representative of the whole mutation negative group in terms of age at onset (50.4 ± 11.5 vs. 50.1 ± 11.3 years) and disease duration (11.6 ± 6.7 vs. 11.3 ± 6.5 years).

Four hundred unaffected controls, unrelated to the probands and mostly recruited among spouses and care-givers, were also tested for *PINK1* mutations (mean age: 62.4 ± 9.5, range 45 - 91 years). All controls denied any

family history for movement disorders and 200 of them were directly examined by a movement disorder specialist. None of them was found to carry two mutations in the *PINK1* gene.

All patients and controls had given written informed consent prior to testing, and the study protocol had been approved by the local Ethical Committee of each participating centre.

Genetic analysis

One-hundred early onset cases and 200 controls have been previously reported (Valente et al., 2004b). In the remaining 1026 patients and 200 controls, the screening of each *PINK1* exon was performed by a two-step strategy including agarose gel electrophoresis (to detect homozygous exon deletions), and Denaturing High Performance Liquid Chromatography (DHPLC) analysis (to detect point mutations and small deletions or insertions). Samples showing an abnormal elution profile at DHPLC analysis were newly PCR-amplified and underwent direct sequencing of the entire *PINK1* coding region. The DHPLC protocol, based on a Wave DNA Fragment Analysis System (Transgenomic, Crewe, United Kingdom), was first validated and shown to be highly sensitive by testing a panel of "positive control" samples carrying known mutations or polymorphisms in each *PINK1* exon, all of which generated clearly abnormal elution profiles. Samples found to carry heterozygous variants also underwent real time quantitative PCR of each exon, to search for possible heterozygous exon rearrangements. Sequencing and real time PCR have been performed as reported (Valente et al., 2004b). Primers, PCR and DHPLC conditions are available on request.

Variants occurring with allelic frequency higher than 1% in unaffected controls were considered polymorphisms. Rare variants were included in this study only when they were predicted to alter the protein primary structure, and the same criterion was used when deducting frequencies from published reports. DNA mutation numbering was based on cDNA sequence, +1 being the first nucleotide of the ATG translation initiation codon in the reference sequence. Multiple sequence alignments of the human PINK1 protein and its homologues were generated using the ClustalW program (http://www.ebi.ac.uk/clustalw/). Prediction of the possible impact of missense variants on the PINK1 protein was obtained with PolyPhen (http://genetics.bwh.harvard.edu/pph/). Accession numbers are as follows: human *PINK1* mRNA sequence: NM_032409.1; PINK1 protein sequence: *Homo sapiens* ENSP00000364204; *Pan troglodytes* ENSPTRP00000000500; *Macaca mulatta* ENSMMUP00000000169; *Rattus norvegicus* ENSRNOP00000020820; *Mus musculus* ENSMUSP00000030536; *Danio rerio* ENSDARP00000014981; *Anopheles gambiae* AGAP004315-PA; *Drosophila melanogaster* CG4523-PA; *Caenorhabditis elegans* EEED8.9.

Statistical analysis

Frequencies of categorical data were compared using the chi-square test (with Yates' correction when appropriate) or the Fisher's exact test. Odds ratios (ORs) and 95% Confidence Intervals (CI) were calculated to estimate relative risks. Quantitative variables were described using mean value, standard deviation and range. Statistical analysis was performed using one-way ANOVA and post hoc multiple comparisons, with Bonferroni correction or Tamhane's T2 test in case of unequal variances ($p<0.10$ at Levene's test).

Published *PINK1* or *Parkin* screens were included in the meta-analysis only if both patients' and controls' samples had undergone the same protocol of mutation analysis. Thus, all studies in which control subjects had been tested only for mutations detected in patients were purposely excluded.

RESULTS

Twenty-two distinct heterozygous rare variants were found in 20 of 1126 probands and six of 400 controls, none of whom carried *PINK1* exon rearrangements. Direct sequencing and dosage analysis of all *Parkin* and *DJ-1* exons did not reveal additional rare variants. General demographic and clinical data for patients are summarized in Table 1. The mean age of the six heterozygous controls was 60.3 ± 8.0 (range 50-73 years).

PINK1 heterozygous rare variants

We identified only two clearly pathogenic mutations (c.1366C>T, p.Q456X and g.15445_15467del23), that have been shown to result in nonsense-mediated mRNA decay or the production of a truncated protein (Grunewald et al., 2007; Marongiu et al., 2007). In our series, these are the only mutations that have been reported also in homozygous or compound heterozygous patients (Bonifati et al., 2005; Hedrich et al., 2006; Ibanez et al., 2006; Zadikoff et al., 2006; Marongiu et al., 2007). All other variants were missense changes that were located across the

entire gene (Figure 1, panel A). Fourteen were identified only in our cohort while the remaining six have been reported in the heterozygous state in patients and/or controls in other studies (Table 2). Alignment of human PINK1 with its orthologues showed that missense variants affected residues that were variably conserved among species. In particular, 11 changes replaced residues conserved in all vertebrates, six variants affected residues that were conserved only in mammals while for three variants the conservation among orthologues was poor (Figure 1, panels B to D). Bioinformatic analysis using PolyPhen software indicated that only eight of the 20 missense variants were predicted as possibly or probably damaging, while 12 were predicted to be benign (Table 2). There was no strict correlation between the degree of conservation of a given variant and bioinformatic prediction of its functional effect. One patient, a 75 years-old man with onset at age 70, carried two distinct heterozygous missense variants, c.770C>T and c.802C>G, resulting in p.T257I and p.L268V substitutions. However, both changes were inherited by two clinically asymptomatic daughters, aged 42 and 40 years, suggesting that the two variants were located in *cis* on the same chromosome and that the patient carried one wild-type copy of the *PINK1* gene.

Table 1. General demographic and clinical data of the probands included in the study and frequencies of heterozygous carriers of *PINK1* rare variants

		all patients	*PINK1* heterozygotes
Number of patients		1126	20/1126 (1.8%)
Gender M:F		686:440 (1.6:1)	9:11 (1:1.2)
Age at the study, yrs		61.4 (11.8; 21-95)	63.7 (8.9; 51-78)
Age at onset, yrs (n=1120)*		50.1 (11.3; 17-85)	52.2 (10.2; 37-70)
Disease duration, yrs (n=1120)*		11.3 (6.5; 3-57)	11.5 (7.0; 4-37)
Age at onset groups	<30 yrs	30 (2.7%)	0/30
	30-39 yrs	185 (16.5%)	2/185 (1.1%)
	40-49 yrs	304 (27.1%)	8/304 (2.6%)
	50-59 yrs	341 (30.4%)	5/341 (1.5%)
	60-69 yrs	260 (23.2%)	5/260 (1.9%)
Familiality for PD (n=1101)*		195 (17.7%)	5/195 (2.6%)
Inheritance (n=1101)*	dominant	70 (6.4%)	1/70 (1.4%)
	recessive§	83 (7.5%)	0/83
	unclear	61 (5.5%)	4/61 (6.6%)
	sporadic	887 (80.6%)	15/887 (1.7%)
Parental consanguinity (n=1101)*		26 (2.4%)	0/26

Age at the study, age at onset and disease duration are reported as means (SD, range); Age at onset groups, familiality, inheritance and consanguinity are reported as counts (%). *number of probands with available information; §including also 19 cases with negative family history but parental consanguinity.

Frequency of *PINK1* heterozygotes

The overall frequency of *PINK1* heterozygous rare variants was not significantly different in the patient and control groups (1.8% vs. 1.5%, OR = 1.2, 95%CI 0.47-2.98). Heterozygous patients were found in all age groups, with the highest rate (2.6%) in the fifth decade. Considering 50 years as a cut-off point, frequencies were not significantly different in early and late onset cohorts (10/519, 1.9% vs. 10/601, 1.7%). None of the probands from families consistent with recessive inheritance carried heterozygous rare variants, that were found in sporadic patients (1.7%), in one family with possible dominant transmission (1.4%) and with higher frequency among probands with positive family history but unclear inheritance (6.6%) (Table 1).

Phenotypes of *PINK1* heterozygotes

Detailed clinical features of heterozygous patients were compared to those of 10 patients carrying two *PINK1* mutations and to those of a subset of 320 wild-type cases (see Patients and Methods) (Table 3). The overall phenotype of heterozygotes was indistinguishable to that of wild-type patients, with no significant differences between the two groups. Conversely, several features were identified in carriers of two mutations that differed from both single heterozygotes and wild-type patients. These included a lower onset age with more frequent onset in a lower limb; a higher occurrence at the latest follow-up of gait impairment, urinary urgency and drug-induced dyskinesias; a longer disease duration, with slower progression (estimated by dividing the *off* UPDRS motor score

by the years of duration) and greater improvement of UPDRSIII values between *off* and *on* states. All other clinical features were comparable in the three groups, with rare occurrence of atypical signs at onset such as dystonia and diurnal fluctuations of symptoms.

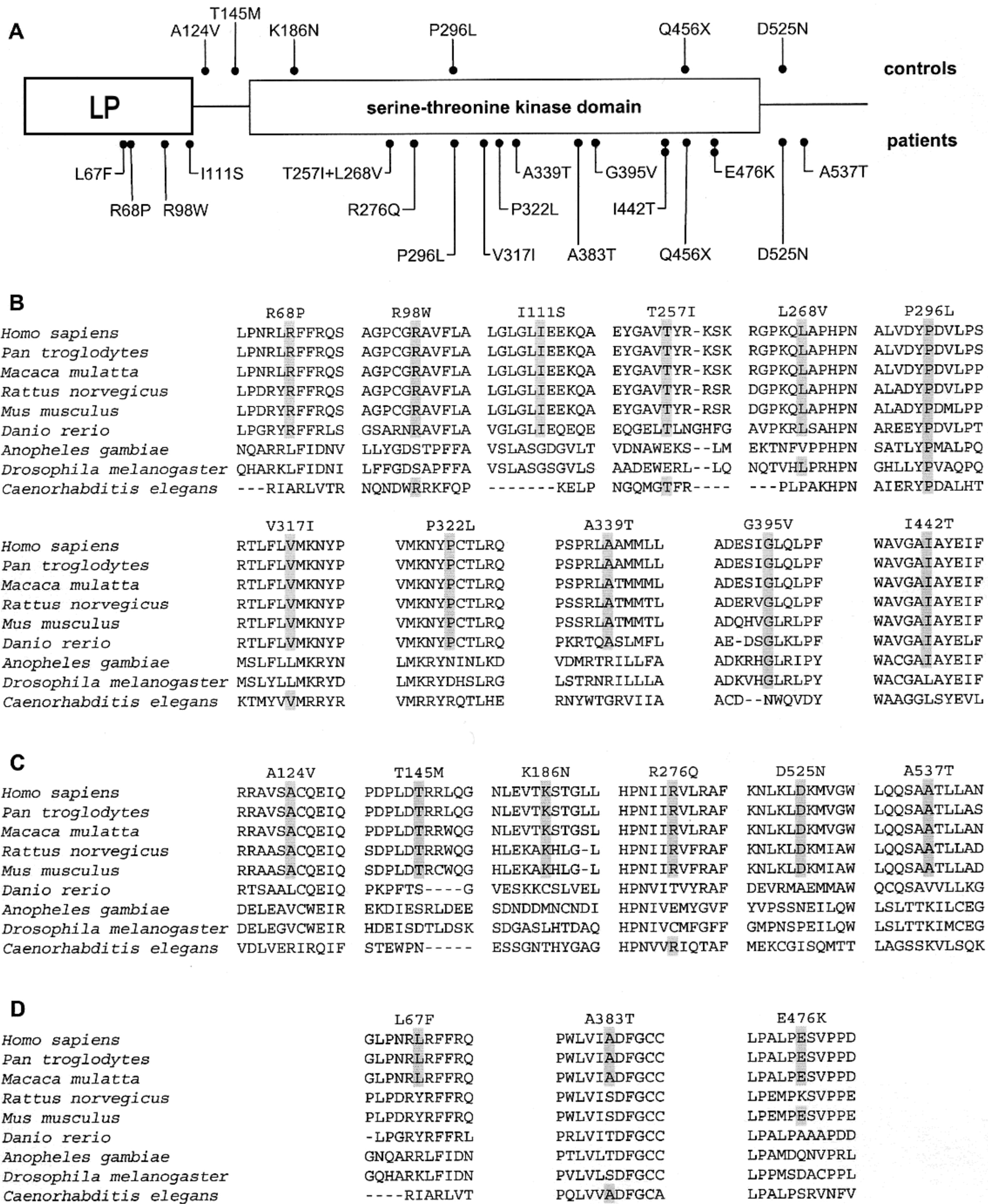

Figure 1. A) Schematic of the *PINK1* gene reporting heterozygous variants identified in patients and controls. The g.15445_15467del23 splice-site mutation (found in one patient) is not depicted. Black dots indicate the number of subjects carrying each variant. LP: leader peptide. B to D) Conservation across species (shaded in grey) of residues affected by missense variants: B) residues conserved in vertebrates; C) residues conserved in mammals; D) poorly conserved residues.

Table 2. Overview of *PINK1* heterozygous rare variants in the present study and published series

nucleotide change	protein change	exon	type	PolyPhen	probands	controls	references
c.199C>T	p.L67F	1	missense	benign	1	0	present*
c.203_204GC>CT	p.R68P	1	missense	benign	1	0	present
c.292C>T	p.R98W	1	missense	++	1	0	present*
c.332T>G	p.I111S	1	missense	+	1	0	present*
c.371C>T	p.A124V	1	missense	benign	0	1	present*
c.434C>T	p.T145M	2	missense	+	0	1	present*
c.440G>A	p.R147H	2	missense	+	1	0	1
c.558G>C	p.K186N	2	missense	++	1	1	present,2
c.587C>T	p.P196L	2	missense	++	1	0	3
c.626C>T	p.P209L	2	missense	benign	1	0	2
c.692A>G	p.E231G	3	missense	+	1	0	4
c.704A>T	p.N235I	3	missense	+	0	1	4
c.770C>T	p.T257I#	3	missense	benign	1	0	present*
c.787A>G	p.R263G	4	missense	benign	0	1	4
c.802C>G	p.L268V#	4	missense	benign	2	0	present,5
c.827G>A	p.R276Q	4	missense	benign	1	0	present*
c.836G>A	p.R279H	4	missense	+	1	0	6
c.838G>A	p.A280T	4	missense	benign	1	0	7
c.887C>T	p.P296L	4	missense	++	1	1	present
c.949G>A	p.V317I	4	missense	benign	2	0	present,8
c.952A>T	p.M318L	4	missense	+	2	0	2,4
c.965C>T	p.P322L	5	missense	++	1	0	present*
c.1015G>A	p.A339T	5	missense	benign	4	0	present,4,8
c.1084G>C	p.D362H	5	missense	++	0	1	4
c.1147G>A	p.A383T	6	missense	benign	3	0	present,8,9
c.1184G>T	p.G395V	6	missense	++	1	0	present*
c.1196C>T	p.P399L	6	missense	++	1	0	10
c.1220G>A	p.R407Q	6	missense	+	1	0	11
c.1231G>A	p.G411S	6	missense	benign	5	0	8,12,13
g.15445_15467del23		**7**	**splice**	**n.a.**	**1 (1)**	**0**	**present,14**
c.1273C>T	p.P425S	7	missense	+	1	0	4
c.1291T>C	p.Y431H	7	missense	+	1	0	8
c.1311G>A	**p.W437X**	**7**	**nonsense**	**n.a.**	**0 (4)**	**1**	**3,15,16**
c.1325T>C	p.I442T	7	missense	+	2	0	present
c.1352A>G	p.N451S	7	missense	+	1	0	8
c.1366C>T	**p.Q456X**	**7**	**nonsense**	**n.a.**	**4 (5)**	**1**	**present,3,8,9,12,17**
c.1382T>G	p.L461R	7	missense	+	0	1	8
c.1426G>A	p.E476K	7	missense	benign	4	2	present,3,4,8
c.1493C>T	p.P498L	8	missense	benign	1	0	13
c.1502G>A	p.R501Q	8	missense	+	0	1	8
c.1573G>A	p.D525N	8	missense	benign	1	1	present
c.1602_1603insCAA	**p.534_535insQ**	**8**	**insertion**	**n.a.**	**1 (1)**	**0**	**6**
c.1609G>A	p.A537T	8	missense	benign	1	0	present*
c.1723T>C	p.C575R	8	missense	++	1	0	8

+, possibly damaging; ++, probably damaging; n.a., not applicable; *unpublished; #in *cis* in 1 proband. Bold: variants also found in homozygous/compound heterozygous state (probands). References: 1, Healy 04; 2, Djarmati 06; 3, Bonifati 05; 4, Rogaeva 04; 5, Tan 05; 6, Klein 05; 7, Tan 06; 8, Abou-Sleiman 06; 9, Ibanez 06; 10, Tang 06; 11, Fung 06; 12, Zadikoff 06, 13, Toft 07; 14, Marongiu 07; 15, Valente 04a; 16, Criscuolo 06; 17, Hedrich 06.

Table 3. Comparison of clinical features in single heterozygotes, carriers of two mutations and wild-type patients

Clinical feature	Number of *PINK1* rare variants (number of patients)			P
	0 (n=320)	1 (n=20)	2 (n=10)	
M:F ratio	171:149 (1.1:1)	9:11 (1:1.2)	5:5 (1:1)	n.s.
Onset				
age	50.4 (11.5; 22-78)	52.2 (10.2; 37-70)	41.0 (9.9; 30-67)	0.031 (2-0); 0.035 (2-1)
asymmetry	304 (95.0)	19 (95)	8 (80)	n.s.
site:				
- upper limb(s)	237 (74.8)*	16 (80.0)	3 (30.0)	0.005 (2-0); 0.015 (2-1)
- lower limb(s)	49 (15.5)*	2 (10.0)	7 (70.0)	<0.001(2-0); 0.002(2-1)
- both	31 (9.8)*	2 (10.0)	0	n.s.
symptom:				
- tremor	145 (45.7)*	9 (45.0)	2 (20.0)	n.s.
- rigidity/akinesia	152 (47.9)*	9 (45.0)	6 (60.0)	n.s.
- both	20 (6.3)*	2 (10.0)	2 (20.0)	n.s.
dystonia	19 (5.9)	0	0	n.s.
sleep benefit	5 (1.6)	0	1 (10.0)	n.s.
Most recent follow-up				
disease duration	11.6 (6.7; 4-45)	11.5 (7.0; 4-37)	17.8 (9.4; 7-35)	0.020 (2-0); 0.048 (2-1)
rest tremor	227 (70.9)	18 (90.0)	9 (90.0)	n.s.
akinesia	310 (96.9)	20 (100)	10 (100)	n.s.
rigidity	304 (95.0)	20 (100)	10 (100)	n.s.
gait impairment	183 (57.4)	10 (50.0)	9 (90.0)	0.050 (2-0); 0.049 (2-1)
orthostatic hypotension	29 (9.1)	1 (5.0)	1 (10.0)	n.s.
urinary urgency	67 (20.9)	7 (35.0)	6 (60.0)	0.010 (2-0)
urinary incontinence	19 (5.9)	3 (15.0)	2 (20.0)	n.s.
hyperreflexia	55 (17.2)	3 (15.8)	3 (30.0)	n.s.
psychiatric disorders	91 (28.4)	6 (30.0)	4 (40.0)	n.s.
dementia	20 (6.3)	1 (5.0)	0	n.s.
UPDRS III:				
-*on*	20.6 (10.9;4-76) n265	18.2 (11.2; 4-43) n19	16.2 (11.2; 6-37) n9	n.s.
-*off*	35.3 (17.1; 7-82) n132	42.9 (10.6; 24-55) n8	42.8 (15.6; 21-72) n8	n.s.
- % improvement	48.1 (16.6; 26-88) n87	46.8 (18.3; 27-80) n7	70.3 (12.9; 49-82) n6	0.006 (2-0); 0.036 (2-1)
- *off*/duration	4.6 (3.3; 0.5-16.7)	3.5 (1.1; 1.7-5.0)	2.4 (0.9; 1.3-3.6)	<0.001 (2-0)
Hoehn-Yahr score	2.5 (0.9; 1.0-5.0)	2.8 (0.6;2.0-4.0)	2.5 (0.8; 1.0-4.0)	n.s.
Treatment				
motor fluctuations	177 (55.3)	15 (75.0)	7 (70.0)	n.s.
dyskinesias	151 (47.2)	11 (55.0)	9 (90.0)	0.009 (2-0)
LEDD	707 (420;60-2250)	820(297;200-1220)	599(323;264-1357)	n.s.

For categorical variables, percentage values are in brackets. Quantitative variables are described by the mean value, with standard deviation and range in brackets. *calculated on 317 patients. "n" followed by a number indicates the sample numerosity for that variable. LEDD: Levodopa Equivalent Daily Dose; % improvement = mean % improvement of UPDRSIII score between *off* and *on* state, calculated as follows: [(*off* – *on*)/*off*]; P values were obtained by comparing the three groups of patients for each variable. For significant values (p<0.05), corrected values obtained from pairwise comparisons between two groups are reported (type of comparison in brackets). n.s. non significant.

DISCUSSION

The prevalence of *PINK1* heterozygous rare variants in PD patients has been reported to vary considerably among distinct studies. This variability is likely due to the different recruitment criteria adopted by each study (i.e. early vs. late onset, sporadic vs. autosomal recessive) and the type of sequence changes considered as pathogenic. To our knowledge, this is the largest study analyzing the frequency of *PINK1* heterozygous rare variants in over 1000 patients. Moreover, the selective inclusion of variants predicted to affect the protein primary structure, both in the present study and in reviewing the literature, allowed us to consistently pool our results with published data, in order to obtain an estimate of the overall associated risk.

In our cohort, including sporadic and familial cases of all ages at onset, we identified 20 heterozygous patients, with an overall frequency of 1.8%. Similar studies of mixed patient populations reported variable frequencies of *PINK1* heterozygotes, ranging from 0.3 to 2.3% (Rogaeva et al., 2004; Healy et al., 2004; Abou-Sleiman et al., 2006; Toft et al., 2007). Heterozygous rare variants were not found in any of our families consistent with autosomal recessive inheritance or in other large series of recessive cases (Li et al., 2005; Ibanez et al., 2006). Considering only sporadic early onset patients (onset <50 years), we found a heterozygote frequency of 1.8%, that falls within the 1.2 - 3.4% range obtained in comparable cohorts (Bonifati et al., 2005; Klein et al., 2005; Fung et al., 2006, Tan et al., 2006). Of note, we identified a similar proportion of single heterozygotes (1.7%) also within late onset patients, confirming the report of seven heterozygotes with onset above 50 years by Abou-Sleiman and colleagues (2006). However, one study on 175 Norwegian and German patients with late onset PD failed to identify *PINK1* mutations or rare variants, possibly reflecting a lower mutation frequency of the *PINK1* gene in those populations (Schlitter et al., 2005).

The frequency of single heterozygous variants in the *Parkin* gene is generally higher than that found in *PINK1*, ranging between 2 and 15% EOP patients in molecular screens that employed both mutation analysis and exon dosage. Single heterozygous variants have also been reported in the *DJ-1* gene, with considerably lower frequency than in the other two genes (reviewed in Klein et al., 2007).

The role of such heterozygous changes is still controversial, with several open questions. A first major issue relates to the actual pathogenicity of at least some of the variants detected in heterozygous state. While the functional role of truncating or splice-site mutations is well established, this is not always apparent for missense variants resulting in the substitution of a single amino acid, and even more doubtful for variants resulting in silent changes or for intronic variants not obviously affecting splice sites or regulatory regions. For this reason, to minimize the chance of including harmless variants in calculations of frequencies and relative risks, we have chosen to consider only *PINK1* heterozygous rare variants predicted to affect the protein primary structure.

Including the present study, 44 such variants have been reported so far (Table 2). Interestingly, only four are non-missense (two nonsense, one splice-site mutation and one 3bp insertion) and these are the only ones to have been detected also in the homozygous or compound heterozygous state in autosomal recessive families (in bold in Table 2). Forty *PINK1* variants (91%) are missense and have been identified only in the heterozygous state either in patients, in controls or both. In the absence of functional assays, available tools to predict their pathogenicity are the degree of conservation of the mutated residue among PINK1 orthologues, the type of amino acid change (conservative vs. non-conservative) and the location within functional domains. Based on these data, bioinformatic tools such as PolyPhen can predict the possible impact of missense variants on the structure and function of a protein although, in the case of PINK1, the accuracy of such predictions can be hampered by the lack of crystal structure of the protein and the poor characterization of its functional domains. PolyPhen analysis for the 40 *PINK1* missense variants predicted that only 23 (57.5%) were possibly or probably intolerant thus expected to be pathogenic, while 17 of them (42.5%) were likely benign (Table 2). Several changes predicted as benign affect residues that are poorly or only partially conserved among orthologues, supporting the hypothesis that such variations do not substantially affect the protein's structure or function. Yet, in selected cases these predictions are challenged by *in vitro* functional studies. The most representative example is p.E476K, a missense variant that has been detected only in the heterozygous state both in patients and controls. This change is predicted to be benign and is poorly conserved even within mammals (see figure 1), yet it has been shown to severely impair mitochondrial membrane potential after cellular stress induced by proteasomal inhibition (Abou-Sleiman et al., 2006). Thus, *in silico* predictions of the pathogenic role of missense variants must be taken with caution, and in the absence of proper functional assays it is not possible to correctly evaluate the pathogenicity of such variants.

Another major issue to be considered is the possibility that in heterozygous patients the second mutation has been missed. The sensitivity of some published studies might indeed be low, especially those testing only for

variants in the coding sequence and intron-exon junctions, that would miss exon dose imbalances and possible pathogenic mutations within regulatory sequences or introns. Yet, carriers of a heterozygous variant have routinely been tested for exon rearrangements, even within studies not systematically adopting this strategy (such as most *PINK1* screens) and promoter or intronic mutations, although possible to occur, are not likely to explain all heterozygous cases. The hypothesis of a digenic inheritance, i.e. heterozygotes carrying a second mutation in a distinct gene, has been put forward by a single report describing a family carrying one *DJ-1* and one *PINK1* heterozygous variants (Tang et al., 2006), but was never confirmed in many subsequent studies - including the present one - that failed to identify mutations in other genes in heterozygous patients. Therefore, this mechanism is not likely to play a significant role, unless a major involvement of still unidentified genes is presumed.

An intriguing hypothesis suggests that single heterozygous variants, while not sufficient *per se* to determine the disease, may increase the risk to develop PD, interplaying with other genetic and environmental factors in a multifactorial model of disease pathogenesis. This is mostly supported by neurophysiological and functional neuroimaging studies, that showed significant albeit subclinical signs of dopaminergic dysfunction in healthy carriers of *Parkin* and *PINK1* single mutations (reviewed in Klein et al., 2007). A role for these variants as PD susceptibility factors would justify their higher frequency among our patients with positive family history of unclear inheritance (6.5%), and their occurrence also in healthy subjects and in individuals with very mild parkinsonian signs (Khan et al., 2005; Hedrich et al., 2006). Yet, it is presently unknown whether the subclinical abnormalities found in healthy heterozygotes are going to remain stable or to evolve towards a clinically manifest phenotype. In the lack of long term follow-up studies, one way to look at this problem is to study large recessive pedigrees with homozygous or compound heterozygous mutations segregating in affected subjects, in which several relatives are in fact heterozygous for one mutation. In published families, the vast majority of heterozygotes are healthy, often including subjects old enough to have manifested the disease (Wu et al., 2002; Munhoz et al., 2004; Valente et al., 2004a; Marongiu et al., 2007). Yet, few *Parkin* and *PINK1* pedigrees have been described in which some heterozygous relatives received a diagnosis of possible, probable or even definite PD (Pramstaller et al., 2005; Criscuolo et al., 2006; Hedrich et al., 2006). In these rare cases, however, other "private" genetic and/or environmental factors might concur to influence the disease risk.

Case-control studies also represent a valid research strategy to evaluate whether the risk to develop the disease is increased in heterozygous carriers. We have calculated the ORs in our cohort and in the few published studies that comprehensively screened the *PINK1* gene both in parkinsonian cases and healthy controls (Rogaeva et al., 2004; Bonifati et al., 2005; Abou-Sleiman et al., 2006), and have pooled data in a meta-analysis effort (figure 2). Single ORs varied widely among different reports, from values indicating an increased risk to protective figures. Overall, *PINK1* heterozygotes were more frequent among cases than in controls (1.7% vs. 1.0%), with an OR of 1.62 (95%CI 0.88 - 2.99) that did not reach statistical significance (p=0.121). We applied the same meta-analysis to published *Parkin* case-control screenings (Lincoln et al., 2003; Clark et al., 2006; Kay et al., 2007; Klein et al., 2007; Lesage et al., 2007), obtaining frequencies more than doubled in cases than in controls (4.5% vs. 1.8%) and a significant OR of 2.53 (95%CI 1.40-4.56, p=0.002). These values are broadly in line with those often obtained in multifactorial conditions, and in fact heterozygous rare variants in *Parkin* and *PINK1* seem to contribute only slightly to PD genetic susceptibility. Heterozygotes would have a risk increased about two folds compared to wild-type individuals, and the vast majority are likely to remain unaffected for all their life. Long-term follow-up studies of healthy carriers and functional assays are needed to better decipher the actual role of heterozygous rare variants, and at present genetic counseling in these cases should be performed with the utmost caution.

Some studies have suggested that heterozygous variants in ARP genes might influence the age at onset of PD (Bonifati et al., 2005; Sun et al., 2006). In our cohort, the mean onset age of heterozygotes was indeed a decade higher than that of patients with two mutations, but did not differ to that of mutation-negative cases. However, cases with onset over 60 years were relatively underrepresented in our cohort. This resulted in a mean onset age of wild-type cases about a decade younger than the average for idiopathic PD, hindering an accurate estimate of the effect of heterozygous variants.

A detailed comparison of clinical features revealed that the phenotype of *PINK1* heterozygotes was indistinguishable from that of wild-type patients. This is in line with the hypothesis that heterozygous variants could act as minor risk factors in a multifactorial model of disease, eventually resulting in the commonest PD phenotype. Conversely, a few clinical features were identified that could help distinguish carriers of two mutations. In particular, the detection of a significantly higher frequency of onset in lower limbs and gait impairment has not been reported before and deserves further evaluation. In this study, the retrospective model of analysis and the small size of the two carrier groups represent two obvious limits of the statistical analysis, that holds a power

≥75% only for differences with ORs >3 or <0.33 at $\alpha = 0.05$. Hence, we cannot exclude that some smaller differences between patient groups might have been missed. For these reasons, it would be useful to replicate these comparisons in other large cohorts, preferably in prospective clinical studies.

In conclusion, we think it is essential to keep a clear-cut distinction between the occurrence of *PINK1* (and other ARP genes) homozygous or compound heterozygous mutations and heterozygous rare variants, especially when counseling patients and their relatives. The first condition is fully penetrant and causative of ARP that is inherited as a classical monogenic trait, although still unknown modifier factors are likely to influence the age at onset and clinical presentation of the disease. The second condition is yet to be fully understood, but several experimental evidences candidate ARP heterozygous rare variants as minor risk factors contributing to the genetic susceptibility to multifactorial, “idiopathic” PD.

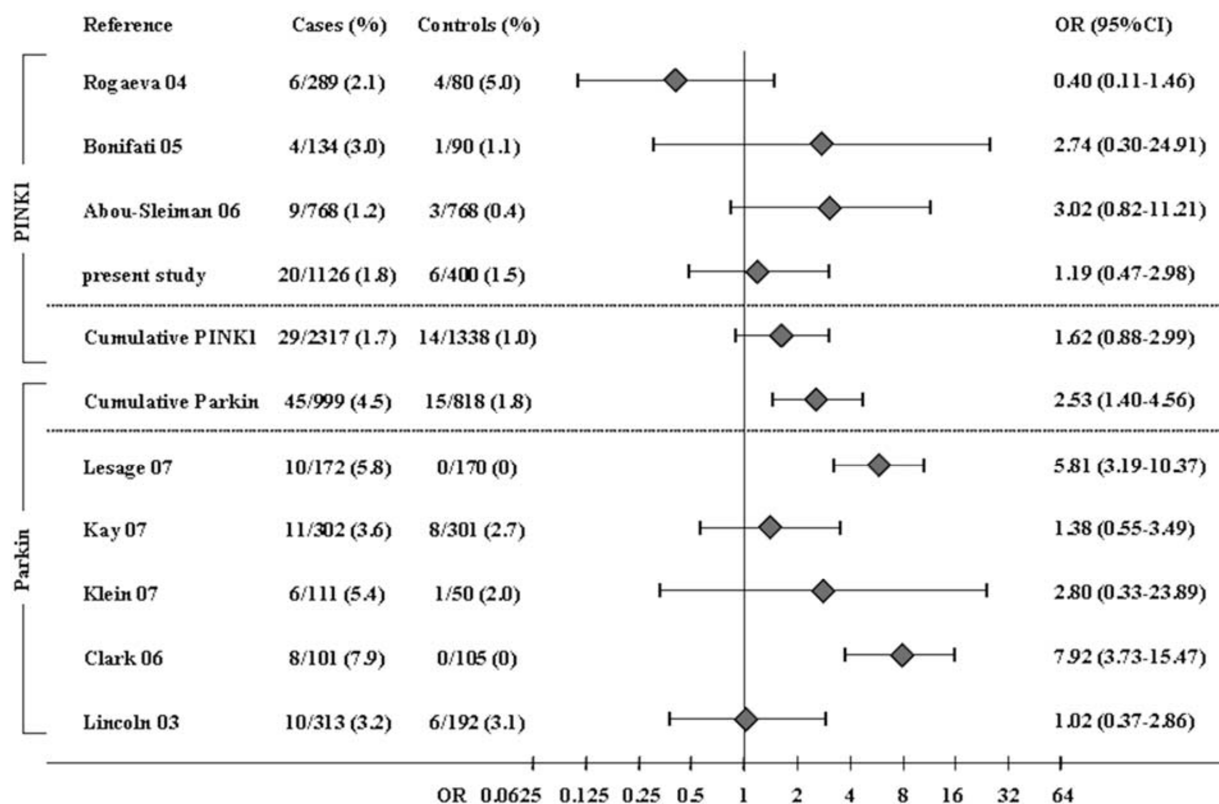

Figure 2. Diagram of odds ratios (ORs, diamonds) and 95% confidence intervals (CI, vertical bars) calculated for heterozygous rare variants in the *PINK1* and *Parkin* genes from selected mutation screenings of cases and controls (see Methods). In the studies by Clark et al. (2006) and Lesage et al (2007), ORs cannot be calculated thus the reported figures represent the frequency of heterozygous patients (CI in brackets).

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