The Role of SNCA Rep1 Microsatellite in Parkinson's Disease Progression

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Abstract

α-synuclein is a constituent of Lewy bodies and mutations of its gene cause familial PD. A previous study showed that a variant of α-synuclein gene (*SNCA*), namely the 263bp allele of Rep1 was associated to faster motor progression in PD. On the contrary, a recent report failed to detect a detrimental effect of Rep1 263 on both motor and cognitive outcomes in PD. Aim of this study was to evaluate the influence of the Rep1 variants on disease progression in Parkinson's Disease (PD) patients. We recruited and genotyped for *SNCA*-Rep1 426 PD patients with age at onset ≥40 years and disease duration ≥4 years. We then analyzed frequency and time of occurrence of wearing-off, dyskinesia, freezing of gait, visual hallucinations and dementia. *SNCA* Rep1 263 carriers showed increased risk of both dementia (HR=3.03) and visual hallucinations (HR=2.69) compared to 263 non-carriers. In conclusion, *SNCA* Rep 1 263 allele is associated to a worst cognitive outcome in PD.

1. Introduction

Parkinson's disease (PD) is the second most frequent neurodegenerative disease [1], and is clinically defined by the presence of resting tremor, rigidity, and bradykinesia [2]. Nonetheless, non-motor features such as mood deflection, anosmia, and sleep disturbances can even pre-date motor impairment in PD [3]. As disease progresses, further disabling symptoms appear, such as posture and gait impairment on the motor side, and cognitive decline and hallucinations in the non-motor domain [4,5].

PD neuropathology is characterized by the loss of dopaminergic neurons and the presence of Lewy bodies (LBs) in surviving neurons [6,7]. LBs contain fibrils composed of alpha-synuclein, a small protein involved in synaptic vesicle trafficking and neurotransmitter release [7] but also in more widespread functions, including the triggering neuroinflammatory processes [8]. Duplication and triplication of alpha-synuclein gene (*SNCA*) cause dominant early-onset PD, suggesting that overexpression of wild-type alpha-synuclein is sufficient to cause the disease [9,10,11]. The amount of alpha-synuclein is relevant in sporadic disease as well, since its expression is higher in brain tissue from PD patients compared to control tissue [12]. Furthermore, genome wide association studies revealed that *SNCA* variations are associated with sporadic PD development [13].

Variations in the complex microsatellite D4S3481 (called Rep-1), located approximately 10kb upstream of the translational start of *SNCA*, have been reported to increase PD risk. *SNCA* Rep1 is essentially triallelic (259, 261, and 263 base pairs in length) and a meta-analysis of association studies showed higher frequency of 263bp allele in cases than in controls [14]. Furthermore, the 259bp allele was found to be associated to a decreased risk of PD, being more frequent in controls than in cases, whereas no relevant effect was observed for the 261bp allele [14]. To date, data on the role of *SNCA* common variants in PD progression are conflicting. Ritz et al. analyzed 232 PD patients and found that the risk of faster motor decline was 4-fold increased in carriers of the Rep-1 263bp promoter variant [15]. Thereafter, a study in which outcome was measured in terms of life expectancy, did not detect any association between *SNCA* Rep-1 genotypes and risk of death in

6154 PD cases [16]. More recently, Markopoulou et al. [17] analyzed the correlation between *SNCA* Rep-1 genotypes and both motor and cognitive outcomes in a large cohort of PD patients. Surprisingly, they found an opposite role of the microsatellite variants, with shorter alleles providing worst outcomes.

On this background, the aim of our study was to investigate whether *SNCA* Rep-1 may influence the progression of Parkinson's disease in a cohort of Italian PD patients, clinically characterized trough the collection of solid and reliable milestones of disease evolution [Williams-Gray CH, Mason SL, Evans JR, Foltynie T, Brayne C, Robbins TW, Barker RA. The CamPaIGN study of Parkinson's disease: 10-year outlook in an incident population-based cohort. J Neurol Neurosurg Psychiatry. 2013 Nov;84(11):1258-64..

2. Patients and Methods

2.1. Patients

426 patients (249 males) with PD [2] were enrolled according to the following inclusion criteria: age at onset ≥40 years, longitudinal follow-up ≥ 4 years. All patients were of Italian origin and were enrolled at the following Movement Disorders Centers: 1) University of Piemonte Orientale, Novara, 2) University of Insubria, Varese, 3) IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Dino Ferrari Center, Neuroscience Section, University of Milan 4) C. Mondino National Institute of Neurology Foundation, IRCCS, Pavia 5) Parkinson Institute, ASST Gaetano Pini-CTO, Milan. The Parkinson Institute cohort (144 patients) had been previously analyzed in both a case-control study assessing the role of *SNCA* variants in PD susceptibility [18] and in the collaborative GEO-PD study on survival [16].

Patients who had at least one 1st or 2nd degree relative with a diagnosis of primary parkinsonism, and/or age at onset ≤ 50 years and/or peculiar clinical features had been previously analyzed to exclude pathogenic mutations on known PD-genes (*SNCA, LRRK2, Parkin, PINK1, DJ-1*) according to EFNS guidelines [19].

This study was approved by the local Ethics Committee. Patients were included in the study after having read and signed an informed consent form for research purpose.

2.2. Assessment of PD progression

All recruited patients had been longitudinally followed-up by neurologists expert in movement disorders at each center. The mean follow up duration was 11.3 years. Clinical records were retrospectively analyzed taking into consideration only variables that had been assessed in each center. Agreement was found on the following parameters: gender, age at onset, disease duration, and family history. Furthermore, the presence and time of onset of the following motor and non-motor complications was recorded: wearing-off, dyskinesia, freezing of gait, visual hallucinations and dementia. Dementia was diagnosed according to MDS criteria [20].

2.3. Determination of *SNCA* Variants

Genomic DNA extraction was performed from peripheral blood using standard procedures. We analyzed the microsatellite *SNCA* Rep1 in the entire cohort (426 subjects). The *SNCA* Rep1 region was PCR amplified from genomic DNA using the primer couple: 5′-GACTGGCCCAAGATTAACCA-3′ (fluorescently labeled with 6-FAM) and 5′-CCTGGCATATTTGATTGCAA-3′ PCR products were resolved by capillary electrophoresis on an ABI-3130XL DNA Analyzer (Applied Biosystem, Foster City, CA, USA) using GeenScan-500 ROX (Applied Biosystem) as molecular weight marker Allelic sizes were assessed using the GeneMapper 4.0 software.

To confirm the accuracy of the genotyping method, we sequenced several individuals representative of each genotype as standard samples.

2.4. Statistical Analysis

Allelic distribution was assessed for Hardy-Weinberg equilibrium (HWE) with Fisher's exact test.

SNCA Rep1 was analyzed using a dominant genetic model (e.g. subjects 263-carriers vs 263-non carriers) as previously described [15]

Continuous variables were reported as median and 25th and 75th percentile, categorical variables were presented as frequencies (counts) and percentages. Allelic frequencies were compared with the χ2 test, differences between age at onset and disease duration were assessed with Mann-Whitney U Test.

The influence of *SNCA* variant carrier status on disease natural history was investigated performing time-to-event analysis: wearing-off, dyskinesia, freezing of gait, visual hallucinations and dementia were considered as separate outcomes. Every patient contributed time of observation from disease onset to complication under study or the last assessment. Individual analyses were performed for each complication under study and cumulative incidences were estimated using the Kaplan-Meier (KM) methods. Log-rank test was used to examine univariable association with outcome. Multivariable Cox's proportional hazards regression model was fitted to obtain the hazard ratio (HR) for *SNCA* variant carrier status adjusted by gender, age at PD onset and recruitment center.

Proportional hazard (PH) model was assessed by regression scaled Schoenfeld residuals against the log time. All P values are 2-tailed and the significance cut-off was P < 0.05. Statistical analysis was performed using STATA v14.

3. Results

Demographic and clinical features of the 426 PD patients are summarized in Table 1.

Observed frequencies of *SNCA* Rep1 genotypes were in HWE (p=0.19)(Supplementary Table 1).

Disease duration did not differ significantly between Rep1 263 carriers and 263 non-carriers. On the contrary, the former group showed significantly earlier age at onset compared to the latter (p=0.016, and p= 0.088 respectively, Mann-Whitney U Test; supplementary Table 2).

To compare the probability to develop each complication over time in 263 carriers vs 263 non-carriers, we performed a Kaplan-Meyer survival analysis. (Figure 1 and Supplementary Figure 1).

At ten years from onset, cumulative incidence of dementia was higher in 263 carriers than in 263

non-carriers (26.39 vs 13.70%, log-rank test p=0.001; Table 2). Similar findings were also observed for visual hallucinations and wearing-off (24.48 vs 15.01% and 65.33 vs 53.02%, log-rank test p=0.0037 and p=0.028 respectively; Table 2).

Finally, after applying a multivariable Cox regression model adjusting by gender, age at PD onset and recruitment center, a statistically significant difference persisted for dementia and visual hallucinations but not for wearing-off. In detail, we found that 263 carriers had 3.03 higher risk of dementia, 2.69 higher risk of visual hallucinations, and 1.26 higher risk of wearing-off compared to 263 non-carriers (p<0.001, =0.001, and 0.236 respectively; Table 3).

Table 1. Demographic and clinical features of study population.

	n=426
Male, n, (%)	249 (58.5%)
Age at onset, median (25th-75th)	62 (55-68)
Age at assessment, median (25th-75th)	74.0 (68-79)
Disease duration, median (25th-75th)	11.0 (8-14)
Familiy history, n, (%)	81 (19.0%)
Wearing-off, n, (%)	231 (54.2%)
Dyskinesia, n, (%)	196 (46.0%)
Freezing of gait, n, (%)	169 (39.7%)
Visual hallucinations, n, (%)	85 (20.0%)
Dementia, n, (%)	77 (18.1%)

Table 2. Probability of complications at 10 years from disease onset.

	Rep 1 263 carriers % at 10 years (95% CI)	Rep 1 263 non carriers % at 10 years (95% CI)	P*
Wearing-off	65.33 (50.03 - 80.14)	53.02 (47.47– 58.59)	0.028
Dyskinesia	44.10 (29.81 – 61.55)	44.05 (38.67 – 49.81)	0.360
Freezing of gait	33.19 (21.07 – 49.72)	34.25 (29.18 – 39.92)	0.621
Visual hallucinations	24.48 (13.88 – 40.97)	15.01 (11.50 – 19.47)	0.004
Dementia	26.39 (15.50 – 42.72)	12.94 (9.72 – 17.13)	0.001

*Log-rank test

Table 3 Multivariable Cox's regression model on all study population.

	HR (95%CI)*	P
Wearing-off	1.26 (0.86 - 1.86)	0.236
Dyskinesia	0.94 (0.62 - 1.45)	0.794
Freezing of gait	0.97 (0.61 - 1.55)	0.901
Visual hallucinations	2.69 (1.57 - 4.60)	0.001
Dementia	3.03 (1.74 – 5.30)	<0.001

*Hazard Ratio (HR) of Rep 1 263 carriers versus 263 non carriers.
Data are adjusted by gender, age at PD onset and recruitment center.

Supplementary Table 1. Genotype distribution frequencies.

		N(%), n=426
Rep 1	257/259	1 (0.23%)
	259/259	22 (5.16%)
	259/261	147 (34.51%)
	259/263	7 (1.64%)
	261/261	212 (49.77%)
	261/263	35 (8.22%)
	263/263	2 (0.47%)

Supplementary Table 2. Age at PD onset and disease duration for 263 carriers and 263 non-carriers.
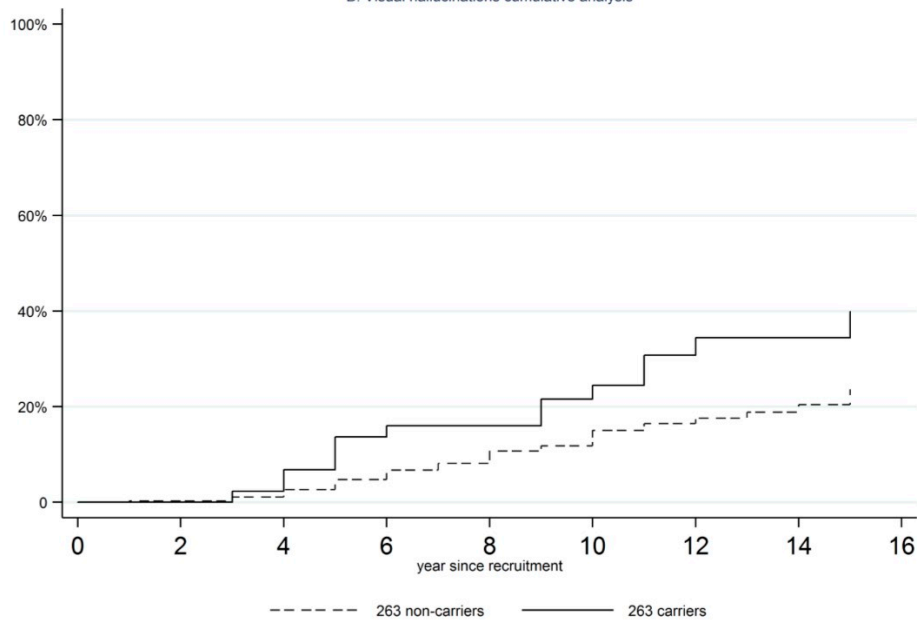
	N(%), n=426		
	263 carriers	263 non carriers	P Mann-Whitney U Test
Age at PD onset median (25th-75th)	56 (53-64)	62 (55 -68)	0.016
Disease duration	12.5 (9.5 - 17)	11 (8-14)	0.088

Figure 1. Kaplan-Meyer survival analysis of complications (dementia panel A; visual hallucinations panel B; wearing-off panel C).
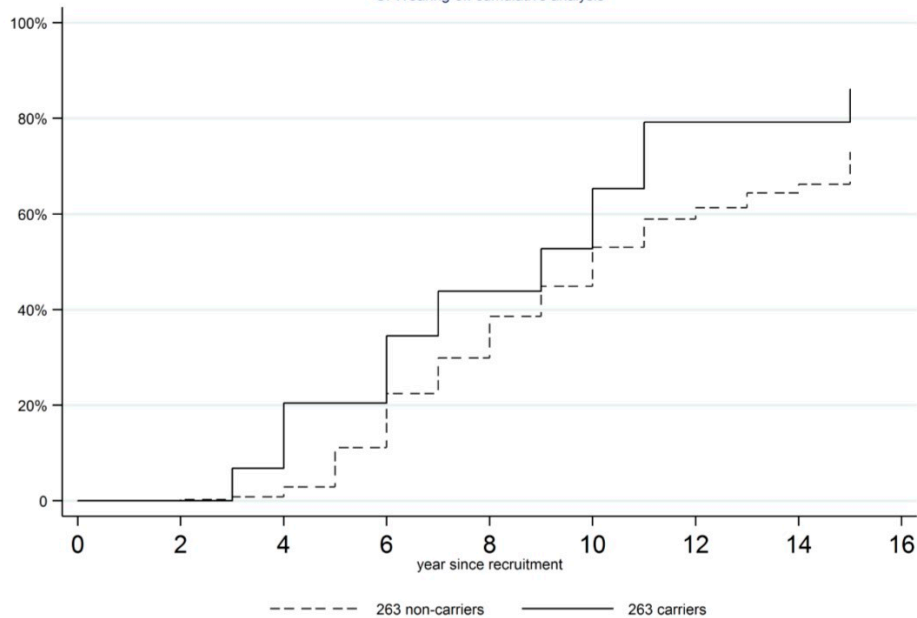
A. Dementia cumulative analysis
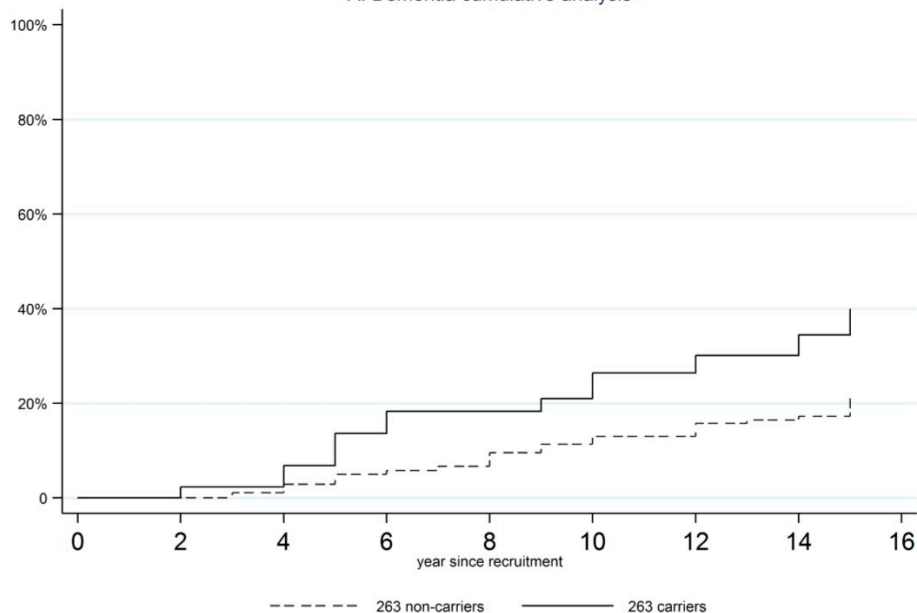
B. Visual hallucinations cumulative analysis

C. Wearing-off cumulative analysis

Supplementary Figure 1. Kaplan-Meyer survival analysis of complications (dyskinesia panel B; freezing of gait panel A).

B. Dyskinesia cumulative analysis

263 non-carriers   263 carriers

A. Freezing of gait cumulative analysis

263 non-carriers   263 carriers
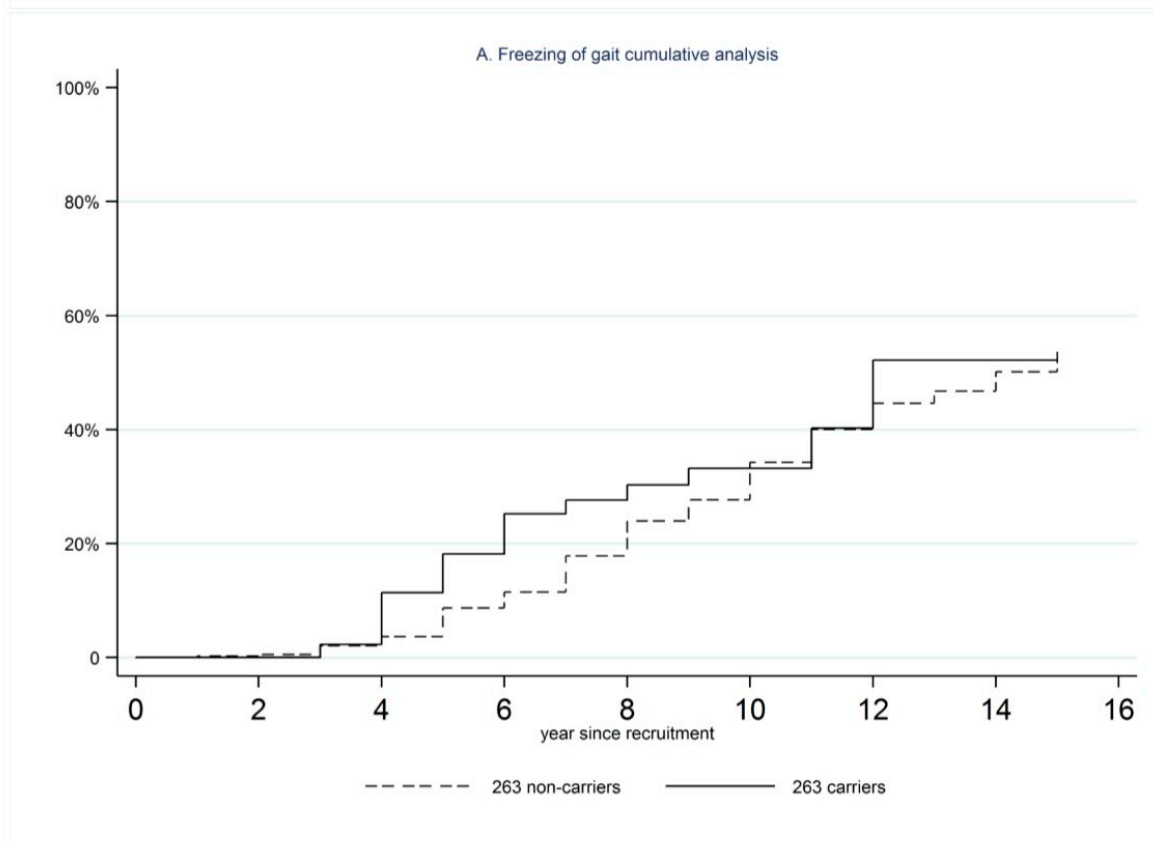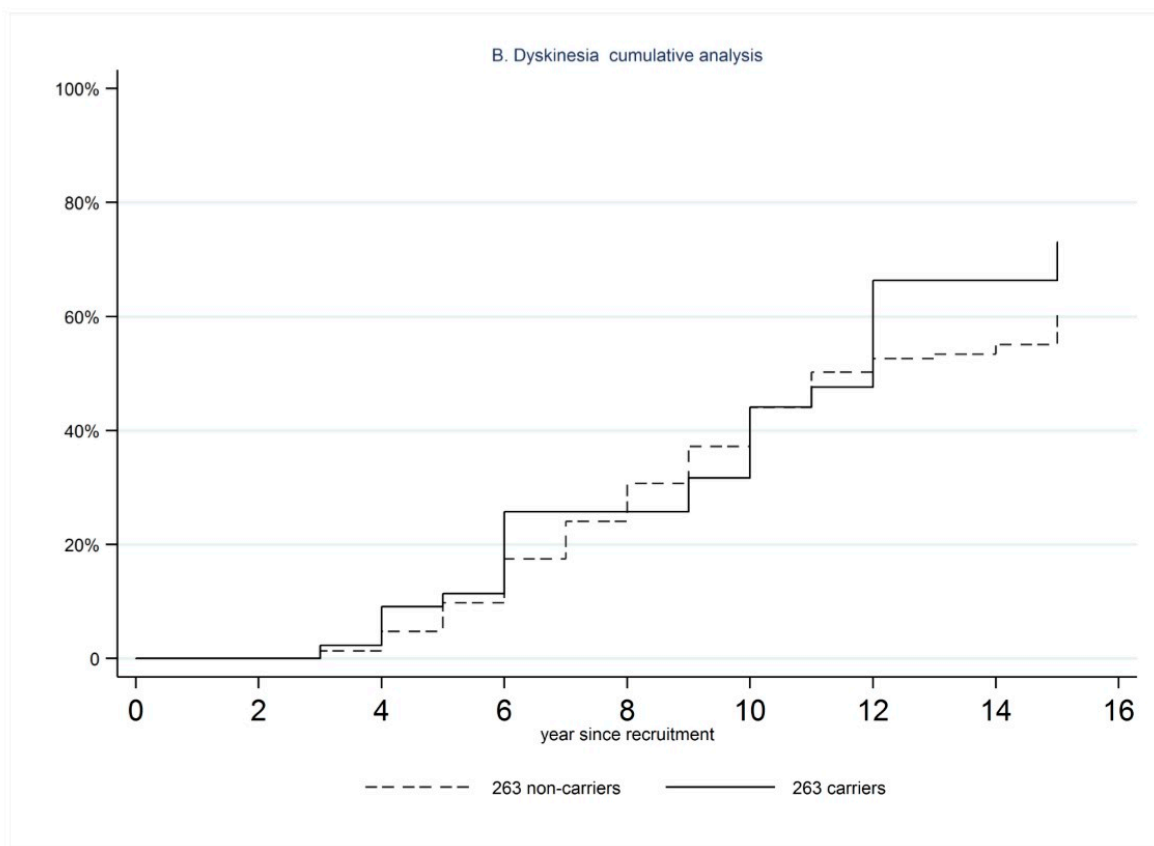
4. Discussion

This study showed for the first time that Rep1 polymorphic variants of *SNCA* influence non-motor progression in PD. PD patients who carry *SNCA* Rep1 263 allele have 3.03 increased risk of dementia and 2.69 increased risk of visual hallucinations compared to patients carrying shorter Rep1 variants. As regards motor progression, Rep1 263 carriers display slightly increased cumulative incidence of wearing-off, compared to 263 non-carriers, even though such difference was not statistically significant in multivariable regression.

To date, strong evidence supports a genetic component in PD susceptibility, whereas studies showing a relevant role for disease modifying genes are less represented [21, 22, 23]. This might be due, at least in part, to the difficulty in collecting data on disease evolution compared to recording disease development. Consistently, large collaborative studies aimed at tracking PD progression are ongoing [24, 25] In our study, we choose to include only patients with longitudinal follow-up and accurate information on the main motor and non-motor complications, putting together a very well characterized population of 426 patients.

The cognitive features of 263 carriers recalled that of familial PD caused by rare pathogenic *SNCA* multiplications, in which progression is faster compared to sporadic PD [26]. It is known that such mutations cause alpha-synuclein over-production through increased mRNA expression [27]. As regards the possible effect of the length of Rep1 microsatellite on alpha-synuclein expression, conflicting data were reported. Chiba-Falek et al. detected a 3-fold increase of alpha-synuclein expression with longer compared to shorter Rep1 alleles in a cellular model [28]. Furthermore, alpha-synuclein mRNA varied 1.7-fold in transgenic mice carrying the different Rep1 alleles [29]. These findings were further supported by Fuchs et al. who detected a correlation between protein level in blood and Rep1 genotypes [30]. On the contrary, a recent a report by Soldner et al. suggested that neither the deletion of the entire Rep1 repeat sequence nor the presence of the three different length alleles (including 263bp) were able to influence significantly *SNCA* expression. Nonetheless, the authors themselves underline that their findings might have been influenced by the

cellular model analysed, which allows to detect only early events [31]. For such reasons, a robust biologic relationship between the 263 allele and PD progression still has to be confirmed

Our study is the first to explore the correlation between *SNCA* polymorphisms and PD progression in the European population. On the contrary, two Northern American studies, the first on 233 patients and the second on 1098 patients, were published in 2012 and 2014 respectively [14,15].

In the first study, a faster motor progression in PD patients carrying at least one Rep1 263 was reported [15]. As regards motor progression in Rep1 263 carriers, our findings display a trend similar to Ritz et al. [15], even though our data lose statistical significance in the multivariable regression model. Nonetheless, it should be noted that discrepancies might be related to substantial methodological differences. In fact, Ritz et al [15] studied a cohort of 233 PD patients for a relatively short follow-up (5.1 years) and measured motor progression with the motor section of UPDRS. This score: a) does not weigh the different motor signs, b) does not evaluate cognitive and neuropsychiatric complications, c) is strongly influenced by treatment, d) shows a relevant inter-rater variability [32]. On the other hand, our study of Rep1 SNPs included a larger population (426 patients) with a follow-up of 11.3 years. Progression was measured through clinical milestones, which are reliable measures of patients' status, especially when multicentric studies are concerned.

A second report was published in 2014 by Markopoulou et al [17]. In this study, authors failed to detect a detrimental effect of Rep1 263 allele on PD progression. On the contrary, they found that patients carrying longer Rep1 alleles had a better motor outcome and a similar trend was observed for cognitive outcome, even though without statistical significance. Even in this case, important differences between the two studies might account for the discordant results. Firstly, our clinical data were collected through direct patients' examination whereas cognitive data included in the report by Markopoulou et al. [17] were collected through telephonic interviews. Secondly, the biological effect of Rep1 microsatellite might differ among subjects, especially when a different ethnic background is involved. In fact, Rep1 is a complex microsatellite characterized by a mixed dinucleotide composition. Alleles with the same size could have different composition and it was

reported that two alleles with the same length but different dinucleotide composition might have different functional effects on the promoter activity [28]. Finally, the way data are presented in the two studies makes results hardly comparable. In fact, Markopoulou et al. [17] aggregate patients carrying two different genotypes (261-261 and 259-263) in a unique very large group, thus losing the impact of 263 allele.

5. Conclusion

Our study found a significant and biologically plausible correlation between *SNCA* Rep1 263 allele and the risk of development of dementia and visual hallucinations in a cohort of Italian PD patients. Future studies on larger patients' populations are mandatory to replicate our findings. Early identification of patients at high risk of complications has relevant implications in terms of both prognosis and disease modifying therapy.

Author roles

(1) Research project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript: A. Writing of the First Draft, B. Review and Critique

C.L.: 1A, 1C, 3A

D.M.F.: 1B, 1C, 3A

Tu.S.: 2A, 2B

O.G.: 1C

C.M.: 1C

M.L.:1C

Te.S.: 1C

R.G.: 1C

D.F.A.: 1C, 3B

L.C.: 1C

T.I.: 1C

Z.R.: 1C

Ce.C.: 1C

D.A.S.: 3B

M.C.: 2A

C.G.P.: 3B

B.G.: 3B

P.C.: 1B, 3B

C.R.: 3B

G.S.: 1B, 3B

Co.C.: 1A, 1B, 2C, 3B

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