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Parkinson Disease subtype: morphostructural and neurobiological correlates

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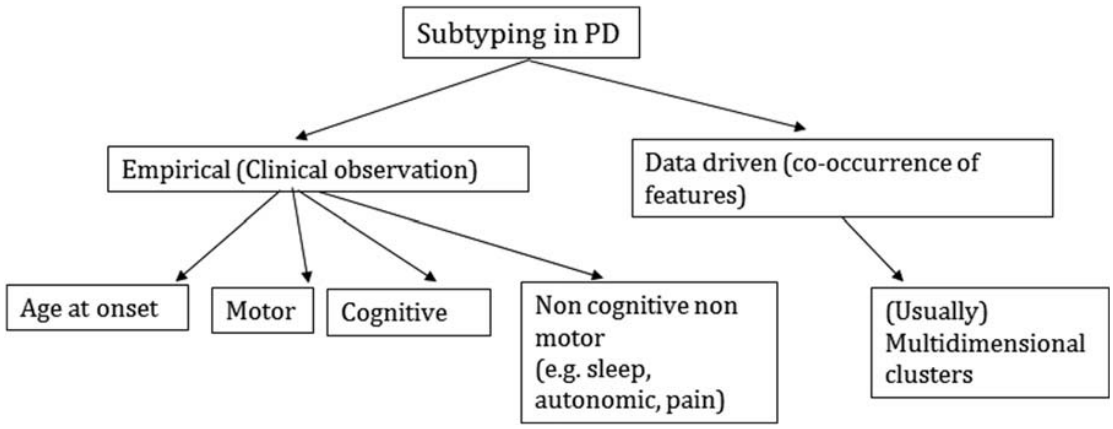
Background

The widely used term of Parkinson's Disease (PD) refers to a progressive motor syndrome, classically associated with neuronal loss in the substantia nigra and inclusions containing the synaptic protein α synuclein (α syn) in the cell bodies and processes of surviving neurons (known as Lewy bodies and Lewy neurites, respectively) in this region. However, the current view of malady reconsider PD as a complex clinicopathological entity, where the "proteiform" multiorgan involvement results from convergent multifactorial biomolecular substrates **(Di Battista, 2014).** It follows that, from a clinical point of view, PD presents as an extremely heterogeneous disorder with a wide range of symptoms and scarcely predictable progression trajectories. Therefore, it become necessary subtyping PD in order to better define the pathogenic process, the clinical progression and the response to treatment. In this term, the National Institutes of Health delineated subtype identification as one of the top 3 clinical research priorities in PD **(Sieber 2014)**. The research efforts on this topic has been directed into the study that empirically classify PD from clinical observation and those that categorize PD without aprioristic assumptions, using a data-driven approach. **(Marras 2016)**

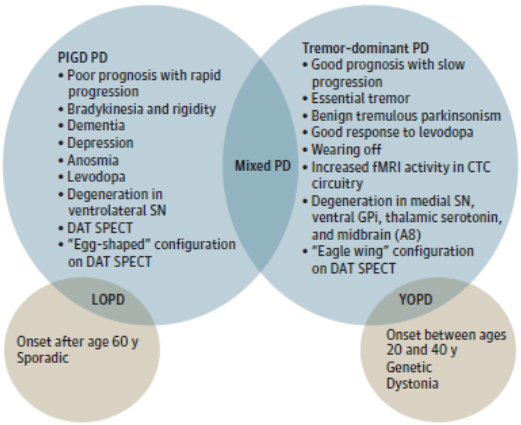


Approaches to clinical subtyping in Parkinson's disease. Subtyping approaches can be divided into those that are empirically derived, from clinicalobservation, and data-driven based on co-occurrence of clinical features with no a priori hypothesis about the way clinical features will cluster together. ***(Marras 2016)***

One of the earliest empirical approach for subtyping PD was based on motor phenotype, categorizing the disease into (1) tremor dominant (TD) and (2) non tremor dominant (NTD) (lately specified as PIGD, postural instability and gait disorder) **(Jankovic 1990)**. Because its unidimensional nature and easily application, this classification has influenced several research focusing on PD subtype, including neuroimaging, genetics and clinical studies. Although numerous

reports converge to define TD subtype as a more benign form **(Figure, Thenganatt 2013)**, the biological basis that underlie the difference between the two phenotype remain tendentially incomplete.

Figure. Parkinson Disease (PD) Subtypes



Subtypes include postural instability and gait difficulty (PIGD) and tremor-dominant PD. CTC indicates cerebellothalamocortical; DAT, dopamine transporter; fMRI, functional magnetic resonance imaging; GPi, globus pallidus interna; LOPD, late-onset PD; SN, substantia nigra; SPECT, single-photon emission computed tomography; YOPD, young-onset PD.

Considering that PD subtypes likely represent the resulting from variable contributions of a number of simultaneous pathological processes, the contribute of genetic become determinant for understanding the pathological basis that impact clinical variability.

Experimental setting: Genetic Architecture of *MAPT* Gene Region in Parkinson Disease Subtypes.

Introduction

The microtubule-associated protein tau (MAPT) is a phosphorylated protein primarily expressed in the brain, where it assists in stabilisation of the cytoskeleton and axonal transport in neurons. The human gene encoding MAPT lies on chromosome 17q21, within a ~900 kb ancestral genomic inversion that generates a ~1.8 megabase (Mb) region of linkage disequilibrium (LD) defined by two extended haplotypes, referred to as H1 and H2 (Baker et al., 1999). In contrast to H2, the H1 haplotype is evolutionarily dynamic and contains a number of variation composed of single nucleotide polymorphisms (SNP) highly correlated with each other (Pittman et al., 2005).

Dominantly inherited mutations in *MAPT* were formerly associated with forms of frontotemporal dementia and parkinsonism linked to chromosome 17, first providing evidence of a link between tau dysfunction and neurodegeneration (Hutton et al., 1998; Spillantini et al., 1998). Beside dominantly inherited diseases, the most common H1 haplotype has been linked not only to tauopathies such as Alzheimer's disease (AD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Myers et al., 2005; Pittman et al., 2006) but also to the most common synucleinopathy such as Parkinson disease (PD), providing mostly positive albeit partially conflicting results (see Zabetian et al., 2007 for summary). However, the impact of H1 haplotype as a risk factor for PD has been confirmed in GWAS studies (Simon-Sanchez et al.,2009; Edwards et al.,2010) and meta-analysis by the PDGene forum (http://www.pdgene.org/) (Lill et al., 2012).

Given the convergent data indicating a role of *MAPT* locus in PD, recent lines of research have moved to refine the potential effect of H1 haplotype into phenotypic traits of the malady (Huang et al. 2015; Davis et al., 2016; Wang et al., 2016), such as the progression of cognitive deficits (Williams-Gray et al., 2009; Seto-Salvia et al., 2011) and motor clinical subtype (Di Battista et al., 2014).

Moreover, taking into account that the true allele risk associated with neurodegenerative disorders could reside at any position within an approximately 900kb region, that includes genes other than *MAPT*, the H1 haplotype has been partitioned into several H1-specific subhaplotypes in order to more precisely map the disease-associated region. Currently fine-mapping studies of the *MAPT* H1/H2 clades have identified specific subhaplotypes associated with AD (Myers et al., 2005) and PSP (Pittman et al., 2005), while mostly conflicting results have been described with PD (Fung et al., 2006; Vandrovcova et al., 2009; Seto-Salvia et al., 2011).

Therefore, we sought to analyze the genetic architecture of *MAPT* in a cohort of PD patients where we had formerly observed an association between H1 homozygosity and non-tremor dominant PD subtype, and whether specific variants of the H1 clade were linked with clinical phenotypes.

Methods

Subjects

A total of 197 unrelated control subjects (mean age: 68.2 ± 18.8, 67% male) and 181 unrelated sporadic PD patients (mean age: 70.9 ± 8.4 years, 56% male, mean age of diagnosis: 60.7 years) were consecutively recruited from March 2011 to July 2012 for the present study. This study cohort has been formerly described in a published study (Di Battista et al., 2014) that analyzed PD motor subtypes in H1 homozygote vs. H2 carriers, no control subjects were enrolled for our previous report. All patients were of European ancestry and originated from the regions of Central and Southern Italy. Patients were selected from the Parkinson outpatient centre of the Sapienza University of Rome and fulfilled the UK Brain Bank criteria for PD. Patients with signs of atypical parkinsonism, doubtful response to dopaminergic replacement therapy, dementia (Mini-Mental State Examination score < 24) or unreliable clinical data (disease duration ≤ 3 years, less than 3 clinical assessments) were not included.

Clinical assessment

For the assignment of clinical subtype, the patients underwent at least three clinical assessments by two expert neurologists in movement disorders during the study period, moreover, clinical notes were reviewed to obtain retrospective data on clinical onset. The UPDRS III performed in the last visit is reported. The patients were classified into two clinical subtypes, by applying the criteria published in a previous study (Selikhova et al., 2009):

(a) tremor dominant (TD), i.e. patients with tremor as the only motor sign at onset or tremor as the prominent motor symptom according to the UPDRS part III;

(b) non-tremor dominant (NTD), i.e. patients with predominant rigidity and bradykinesia but no tremor or only mild tremor at rest. Only patients with at least three years of disease duration were enrolled for the study in order to obtain a reasonable depiction of the clinical subtype.

Clinicians were blinded of patients *MAPT* background during the study examinations.

The patients' clinical and demographic data are shown in Table 1. The controls were selected among blood donors. Written informed consent to the study was obtained from all the PD patients and control subjects. The study was approved by the local ethics committee of Sapienza University of Rome.

Genetic analyses

DNA from peripheral blood was isolated using standard procedures. *MAPT* haplotype was determined by testing for the presence of a 238bp deletion between exons 9 and 10 (*del-In9)*, which is characteristic of the H2 haplotype. The *del-In9* polymorphism was amplified by polymerase chain reaction (PCR) and separated on 6% native polyacrylamide gel and then visualized after ethidium bromide staining by UV transillumination. The amplification reaction was set to a volume of 25 µl containing 1.5mM $MgCl_2$, 200 µM dNTP, 50mM KCl, 10 mM Tris-HCl pH 8.3, 0.25 µM of each primer and 1 U of Promega Taq DNA polymerase, 30 amplification cycles were performed. PCR conditions for *del-In9* were: 94°C for 30sec, 63°C for 30sec and 72°C for 30sec. The forward primer was 5'-GTTTCCACTGTTTCCAGAGTTCC and the reverse primer was 5'-TTTTACAATCTCAGCCCCTAGC. H1 yields a 574bp product and H2 a 336bp product under these conditions.

The SNPs rs1467967, rs242557, rs3785883, rs2471738, rs7521 were detected by means of the restriction fragment length polymorphism PCR (PCR-RFLP) method. To amplify by PCR the *MAPT* haplotype SNPs of interest, oligonucleotide primer pairs were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A volume of 10 µl of PCR product for each SNPs was digested by 1 U of the corresponding restriction endonuclease: rs1467967 (*DraI*); rs242557 (*ApaHI*); rs3785883 (*BsaHI*); rs2471738 (*BstEII*); rs7521 (*PstI*). All nucleases were from New England Biolabs and the reaction volume of 20 µl was incubated for 2h at the temperature indicated by the manufacturer. The sequences of primers for PCR reactions, restriction enzymes used for RFLPs and size of fragments are shown in Table 2. For the SNPs rs1467967, rs242557 and rs2471738 a multiplex PCR reaction was performed in a volume of 50 µl, at the following settings: 94°C for 30sec, 60°C for 30sec and 72°C for 30sec. PCR conditions for the rs3785883 were: 94°C for 30sec, 54°C for 30sec and 72°C for 30sec. The SNP rs7521 was amplified using the following parameters: 94°C for 30sec, 62°C for 30sec and 72°C for 30sec.

Genotyping accuracy was confirmed randomly by DNA sequencing.

Statistical analysis

We assessed each SNP for Hardy-Weinberg equilibrium in cases and control subjects. For all markers, Fisher's exact test was used to test for allele frequencies association between cases (PD, NTD-PD, and TD-PD) and controls. *P*-values were considered significant at $P < 0.05$.

The program FAMHAP Ver.19 (http://famhap.meb.uni-bonn.de/) (Herold and Becker, 2009) was used to reconstruct H1 subhaplotypes and calculate haplotypes frequencies. For comparison reasons, subhaplotypes with frequencies <5%

were included in the analysis only if observed at a higher frequency (>5%) in one of the other groups. We first performed a global likelihood ratio test to assess whether the overall subhaplotype frequency distribution differed between cases and control subjects. In instances in which the overall distribution significantly differed we examined the effect of each individual subhaplotype compared with all others. We calculated pairwise LD (measured as D') between subhaplotypes in cases and control subjects, and created a graphic representations of the data using Haploview 4.1 (https://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/downloads) (Barrett et al., 2005). A Bonferroni correction was used to take into account multiple testing.

Results

Following the classification procedure, we found 46 Tremor Dominant (TD-PD) and 135 Non Tremor Dominant (NTD-PD) PD patients. Patient groups (NTD-PD vs. TD-PD) were comparable with regard to age, age at onset and disease duration. A statistically significant difference in motor performance assessed with UPDRS part III at last visit was observed between the two PD groups (NTD-PD 20.6±9.9 vs. TD-PD 16.5±8.1; $p<0.05$) (Table 1).

Six *MAPT* htSNPs were tested for association in all patients and control group. Allele frequencies for all SNPs were in Hardy-Weinberg equilibrium, except for rs2471738, for which a marginally significant deviation was seen in cases (p=0.02) but not in control subjects (p=0.9).

To test the relation between H1-SNPs and PD risk, we first performed a single-locus analysis of the *MAPT* genetic variants. Results are shown in Table 3. A significant overrepresentation of the H1 allele in the entire PD group (comprising both NTD-PD and TD-PD) compared with controls (79.5 vs. 69.5%; χ^2=9.9; OR, 1.7; 95% CI, 1.2-2.4; p=0.002) was detected. The association was greater in the PD subgroup of NTD patients compared with controls (82 vs. 69.5%; χ^2=13.6; OR, 2.03; 95% CI, 1.4-3; p=0.0003) and remained significant after correction for multiple testing ($p_{correct}$=0.008), while no statistically significant difference was disclosed between PD subgroup of TD patients and control subjects (72 vs. 69.5%; χ^2=0.17; OR, 1.1; 95% CI, 0.7-1.9; p=0.7).

Among the other SNPs, only the rs3785883 was marginally statistical significant in the NTD-PD subgroup compared with control subjects (χ^2=4.3; OR, 1.5; 95% CI, 1.02-2.3; p=0.044), but this difference did not remain significant after statistical correction considering the number of SNPs analyzed.

To clarify the association found between PD and the *MAPT* H1 variation and to assess whether any of the H1 subclades previously described (Myers et al., 2005; Pittman et al., 2005) could be influencing PD risk or motor phenotypes, we performed a haplotype association study comparing *MAPT* subhaplotype frequencies between the

different group of patients and controls. On the H1 background, 20 subhaplotypes were identified with a frequency of ≥1% and only those with frequencies >5% in at least one of the groups in study were considered for the analysis. A total of seven subhaplotypes were selected (Table 4). Significant overall differences in selected subhaplotype frequencies (defined by the tagging SNPs rs1467967, rs242557, rs3785883, rs2471738, *del-in9*, rs7521) were found between PD patients and controls (p=0.014), between NTD-PD and controls (p=0.0005) and between TD-PD vs. NTD-PD (p=0.035), while no statistically significant difference was detected between controls and TD-PD patients (p=0.35).

The *MAPT* H2a haplotype was significantly underrepresented in the NTD-PD subgroup compared with controls (p=0.024; OR, 0.6) and with TD-PD patients (p=0.018; OR, 0.5), suggesting a protective effect.

Detailed analyses showed a significant difference in the frequency of the H1h subhaplotype in the PD group (p=0.013; OR, 2.6) compared with controls. However, the difference was greater in the subgroup of NTD-PD patients (p=0.007; OR, 2.9). After correction for the number of haplotypes analyzed only this last difference remained statistically significant.

The pairwise linkage disequilibrium analysis (LD) for all SNPs was performed in the control and PD groups. The LD plot showed in the Fig 1, indicates in PD patients, that alleles at rs242557, rs3785883, rs2471738 and rs7521 are in strong LD with the *del-in9* (marker 5), but are not in strong linkage with each others, indicating that these markers are H1 specific.

Discussion

Analyzing the role of *MAPT* locus in neurodegenerative disorders, such as PD, represents an effort to elucidate the interaction between genetics and functional disease outcomes. Although several studies including ours have found an association between the H1 haplotype and PD, the functional role of this variant still remains to be identified.

This paper aimed at refining the *MAPT* role in PD by examining the architecture of the entire gene in order to determine its possible associations with PD, and PD motor phenotypes, in a cohort of patients of Italian ancestry. Our results are consistent with the growing body of evidence that supports the *MAPT* H1 haplotype as a risk factor for sporadic PD. Moreover, we observed a peculiar risk distribution of *MAPT* haplotype, and H1 subhaplotype, according to PD motor phenotype. Indeed, while NTD-PD subgroup showed a statistically significant overrepresentation of H1 clade, no differences were observed between TD-PD subgroup and control subjects. Among the other SNPs analyzed, the rs3785883 polymorphism was nominally significant, exclusively in the NTD-PD subgroup compared with controls. This finding is consistent with a previous study where a moderate association at SNP rs3785883 was also found in a

Greek cohort of PD patients (Fung et al., 2006). With regard to MAPT subhaplotype, we found that H1h was associated with PD and this association remained statistically significant after correction for multiple testing when NTD-PD subgroup was considered. To our knowledge this is the first study comprehensively assessing *MAPT* locus in PD according to clinical motor subtype and the strong points of this study are the regular motor clinical assessments for all patients included in the cohort.

The results of our study potentially raise two orders of consideration: the first report of a significant PD risk of the H1 haplotype in a PD subgroup, namely NTD-PD and the original finding of an association between the H1h subhaplotype and the same PD clinical subtype, in a cohort of Caucasian European ancestry.

At present, phenotype-genotype association studies that analyze the role of *MAPT* haplotypes on PD are mostly focused on cognitive profiling. These researches overall indicate an involvement of H1 haplotype on specific cognitive domains such as memory and visuo-spatial functions (Williams-Gray et al., 2009). According to these studies, *MAPT* variation influences cognition and the function of specific brain circuitry even in early phases of PD (Nombela et al., 2014) and even in healthy control subjects (Winder-Rhodes et al., 2015). Although the relationship between *MAPT* haplotype and cognitive functions remains to be determined since no specific regional degeneration or neurochemical alterations have been provided, the effect seems to be detectable even in relative small number of subjects.

Unlike many other quantitative phenotypic traits, there are now evidences suggesting that clinical motor phenotype of PD may not represent a mere semiological matter. Indeed, while the
TD-PD patients could be considered a subgroup with a benign clinical course and a slower process of degeneration at least for the most part of the disease course (Selikhova et al., 2009; Eggers et al., 2012; Deuschl 2013; Selikhova et al., 2013), NTD-PD are likely more prone to develop a series of motor and non motor complaints, inherent to the spreading of the degenerative process (Rosenberg-Katz et al., 2013; Zhang et al., 2013; Herman et al., 2014; Solla et al., 2015).

Interestingly, the contribution of *MAPT* gene in motor impairment has been described in a large community-based cohort of neurologically healthy aging individuals (Shulman et al., 2014), where an association between H1 haplotype and mild parkinsonian signs, especially bradykinesia, has been observed without evidence of PD hallmark at pathological assessment. The authors speculated that neuroanatomical dysfunction of cortico-nigro-striatal pathways, different from those classically observed in PD, may contribute to the development of parkinsonian signs. Therefore, given the prevailing view of H1 haplotype as a genetic risk factor for neurodegeneration, we can speculate that H1 background may partake to the expression of a PD clinical motor subtype associated with increased functional disability.

On the other hand, given the overall underrepresentation of H2 haplotype in a range of neurodegenerative disorders and assuming that this haplotype is associated with more efficient brain function, our observation of a significant overrepresentation of H2 in TD-PD may indicate a protective role of the H2 haplotype. Some studies

assessed the role of MAPT variants in gene expression (Hutton et al., 1998; Spillantini et al., 1998; Caffrey et al., 2006; Pittman et al., 2006). Some authors reported that the disease risk conferred by MAPT variants could be related to a higher total or 4R tau levels. Additionally, a protective effect of MAPT H2-haplotype due to an increase espression in N-terminal exon-containing MAPT transcripts has been speculated. …..

Although several lines of evidence, including basic researches, pathological findings and genotype-phenotype association support the role of *MAPT* haplotypes in PD, the exact mechanistic model of this link remains to be determined. Moreover, the pathological findings related to *MAPT* background in neurodegenerative disorders are partially conflicting. Indeed, while some studies conducted on PSP human brain indicate that H1 haplotype does not affect the pathological or biochemical phenotypes (Liu et al., 2001), other found an higher expression of 4R-tau from the H1 haplotype compared to H2. Other authors observed that the *MAPT* H1 haplotype enhances the overall α-synuclein deposition type pathology in dementia with Lewy Body (Colom-Cadena et al., 2013) and Alzheimer Disease (Wider et al., 2012).

Nevertheless, no comprehensive assessments of α-synuclein burden or Alzheimer-like pathology according to *MAPT* haplotype are currently available in PD; therefore, systematic and well-powered analysis exploring the functional outcomes of *MAPT* region variations remain mandatory.

Moreover, we found that a specific subhaplotype, the H1h variant, was overrepresented in our PD population, more significantly in NTD-PD patients.

A number of studies have been performed for association of *MAPT* subhaplotypes variability and PD with inconsistent results (Fung et al., 2006; Vandrovcova et al., 2009; Seto-Salvia et al., 2011). Moreover, other studies analyzed just few of the SNPs described to characterize the H1 specific subhaplotypes (Fidani et al., 2006; Winkler et al., 2007; Das et al., 2009; Refenes et al., 2009; Huang et al., 2015; Wang et al., 2016).

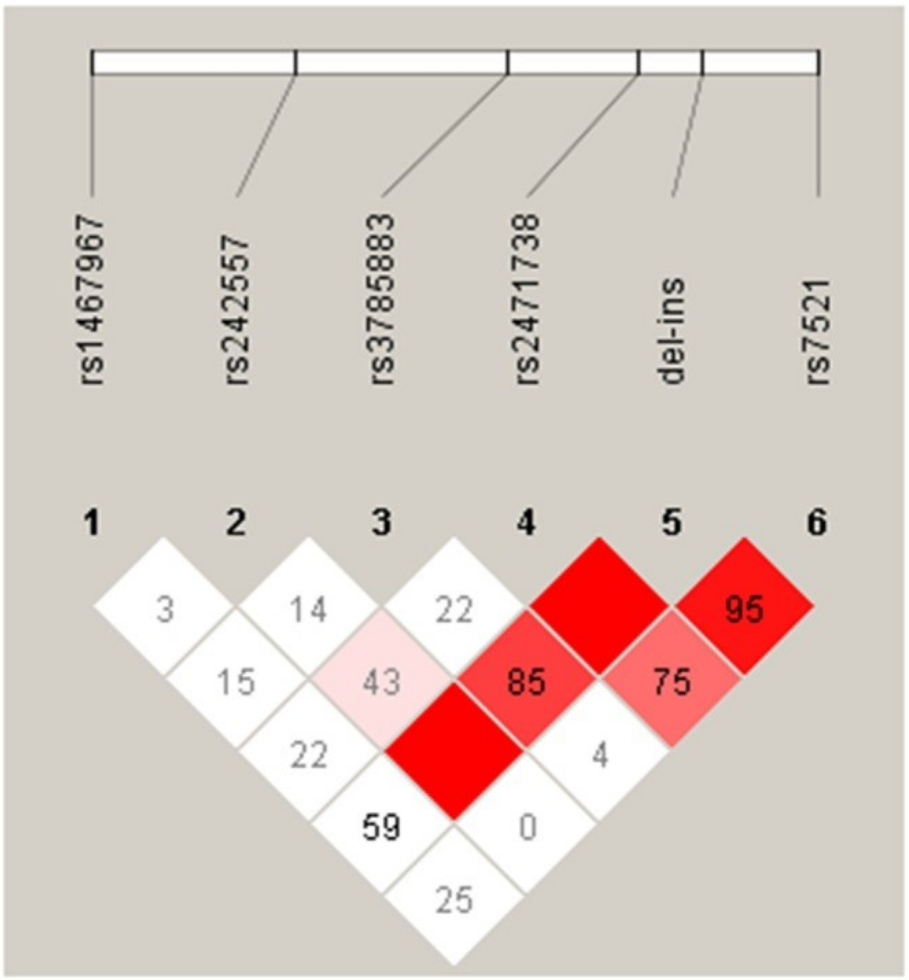
Given the concern that H1h variants may be driven by the genetic architecture related to ethnicity (Fung et al., 2006), it is worth considering that a biological link between such variant and neurodegenerative process has been formerly reported. Indeed, it has been found in Alzheimer's disease (AD) that the A-allele of the rs3785883 SNP is associated with increased cerebrospinal fluid (CSF) tau levels and tau mRNA expression (Kauwe et al., 2008). Moreover, the authors observed that the *MAPT* genetic variation defined as H1h subhaplotype showed significant elevation of CSF tau compared with the H2 haplotype. The contribution of the A-allele of the rs3785883 to tau expressions in AD has been subsequently confirmed in a larger series (Allen et al., 2014). Therefore, our findings may be congruent with, and complementary to these reports considering that NTD-PD patients showed significantly higher levels of CSF tau protein and tau/beta index if compared to TD-PD (Jellinger 2012; Prikrylova et al., 2012).

If confirmed, this result may indicate that a specific H1 subhaplotype increases the risk of developing a NTD-PD disease, at least in populations of South European ancestry.

Our study supports the hypothesis that genetic variability in the *MAPT* region is involved in PD susceptibility and may contribute to PD phenotypic expression, confirming that large-scale evaluation in different populations could be relevant to understand the role of population-specific heterogeneity.

Given the findings that H1 may act synergistically with other gene variants in determining risk for PD, future researches should concern with gene–gene interactions to provide critical insights into mechanisms of disease susceptibility.

Nevertheless, the genetic architecture of *MAPT* in determining PD phenotypic expression as well as the possible functional effect of H1h subhaplotype deserves attention



Legend to Figure

Figure 1. Linkage disequilibrium (LD) between the MAPT H1 genotyped SNPs in our PD group. The relative position of the MAPT H1 tagging Single-Nucleotide Polymorphisms, SNPs, is shown (top). Within each diamond the pairwise standardized coefficient of LD (D′ values x 100) are presented.

Table 1. Demographic and clinical characteristics of patients and controls.

Characteristic	**Controls** N=197	**PD** N=181	**NTD-PD** N=135	**TD-PD** N=46
Male sex, No. (%)	67% (132)	56% (102)	58% (78)	52% (24)
Age, (Means +/-S.D.)	68.2+/-18.8	71.8+/-8.6	71.7 +/-8.5	71.9+/- 9.2
Age at onset, (Mean+/-S.D.)	NA	60.5+/-8.9	60.4 +/- 9.2	61.0 +/- 7.8
Disease duration (Mean+/-S.D.)	NA	10.4+/-4.8	10.4 +/- 5.1	10.3 +/- 4.1
UPDRS III score	NA	19.5+/-9.6	20.6 +/- 9.9	16.5 +/- 8.1

Table 2 Primer sequences and restriction enzymes used for detection of the investigated polymorphisms.

Gene Polymorphism (alleles)	**PCR primers (5'- 3')**	**Enzyme (fragment bp)**
rs1467967 (G/A)	Fwr: CACAGCCACCCTCCCTCTAAC	*DraI*(267/ 186, 81)

	Rev: GGCTCCACCCTTCAGTTTTGGA	
rs242557 (A/G)	Fwr: CTTGATGATGCATGGACCTCTC	*ApaHI* (211/ 139, 72)
	Rev: TTGACAGTACCCACGACACGTG	
rs3785883 (A/G)	Fwr: CCATCACCTTGTCAGAAACTC	*BsaHI* (277/ 164, 113)
	Rev: AGCCATGTGGTAGCCTCAG	
rs2471738 (T/C)	Fwr: CTCTCTGGACCCTCATCCACC	*BstEII* (170/ 104, 66)
	Rev: GAGAACCGAATGAGGACTGGAA	
rs7521 (G/A)	Fwr: ACCTCTGTGCCACCTCTCAC	*PstI* (231/ 160, 71)
	Rev: AGGTGAGGCTCTAGGCCAGT	

Fwr: forward primer; Rev: reverse primer.

Table 3. MAPT single SNPs association results.

			Major Allele Frequency			
Variant	**Location in MAPT**	**Major Allele**	**CO**	**PD (p-value) OR (95% CI)**	**NTD-PD (p-value) OR (95% CI)**	**TD-PD (p-value) OR (95% CI)**
rs1467967	5'Exon 1	A	59	60 (0.88)	63 (0.37)	51 (0.16)
				1.02 (0.7-1.3)	1.16 (0.8-1.6)	0.7 (0.41-1.1)
rs242557	5'Exon 1	G	68	66 (0.53)	63 (0.2)	74 (0.31)
				0.9 (0.67-1.2)	0.8 (0.58-1.1)	1.3 (0.8-1.2)
rs3785883	Intron 3	G	83.5	78 (0.064)	77 (0.044)	81.5 (0.64)
				0.7 (0.5-1.02)	0.6 (0.45-0.98)	0.87 (0.5-1.6)
rs2471738	Intron 9	C	80	77 (0.4)	76 (0.25)	81.5 (0.77)

				0.87 (0.6-1.2)	0.8 (0.5-1.2)	1.12 (0.6-2)
del-in9	Intron 9	H1	69.5	**79.5 (0.002)**	**82 (0.0003)**	72 (0.7)
				1.7 (1.2-2.4)	2.03 (1.4-3)	1.1 (0.7-1.8)
rs7521	3'exon 14	G	55	53 (0.6)	52 (0.47)	55 (1)
				0.92 (0.7-1.2)	0.89 (0.6-1.2)	1.03 (0-6-1.6)

Abbreviations: CO, controls; PD Parkinson Disease; PD-NTD, Parkinson Disease Non Tremor Dominant; PD-TD, Parkinson Disease Tremor Dominant; OR, odds ratio; 95% CI, Confidence Interval. Boldface values indicate significant results after Bonferroni correction for multiple testing ($p_{correct}$=0.008).

Table 4. MAPT haplotype association results.

		Haplotype Frequency			
Haplotype ID	**Haplotype Variants[a]**	**CO**	**PD**	**NTD-PD**	**TD-PD**
H2a	AGGCdelG	21	16.7	13[b]	26[c]
H1b	GGGCinsA	17	17	16	24
H1c	AAGTinsG	7	8.8	10.5	5
H1d	AAGCinsA	3	5.3	5.6	5.3
H1e	AGGCinsA	6.3	8.2	9.3	3.7
H1h	AGACinsA	2.6	6.7[d]	**7.4[e]**	5.3
H1i	GAGCinsA	8	5	4.6	5.3
			0.015	0.0005	0.35 (0.035)

Abbreviations: CO, controls; PD, Parkinson Disease; PD-NTD, Parkinson Disease Non Tremor Dominant; PD-TD, Parkinson Disease Tremor Dominant; MAPT, microtubule-associated protein tau. OR, odds ratio; 95% CI, Confidence Interval.

[a]Alleles for the SNPs defining the haplotype are given in the 5' to 3' order as follows: rs1467967, rs242557, rs3785883, rs2471738, *del-In9*, and rs7521.

[b]0.024, OR 0.6; 95%CI 0.4-0.9: NTD-PD vs. CO.

[c]0.018, OR 0.5; 95%CI 0.28-0.87: NTD-PD vs. TD-PD.

[d]0.013, OR 2.6; 95%CI 1.2-5.5: PD vs. CO.

[e]0.007, OR 2.9; 95% CI 1.3-6.3; NTD-PD vs.CO. (Boldface value indicate significant result after Bonferroni correction for multiple testing, $p_{correct} = 0.00714$).

Numbers on the last line indicate p values that result from global haplotype frequency comparison between each group of patients and controls, while the p value in brackets results from comparison between TD-PD and NTD-PD patients.

Clinical setting: Intercepting Parkinson disease nonmotor subtypes: a proof-of-principle study in a clinical setting.

Background

Parkinson's disease (PD) is a progressive neurodegenerative disorder, with proteiform clinical spectrum and scarcely predictable evolution (Di Battista, 2014). A great effort has been engaged to define homogenous groups in order to intercept a pathophysiological coherence and prognostic trajectories of the disease. To this purpose, the identification of non-motor domains remains a priority for evaluation and managing of PD patients. First, non-motor symptoms (NMS) are integral to the disease and complementary to the diagnostic procedure. Second, identifying NMS subtypes may have a possible prognostic value. Third, from a research point of view, NMS are significant determinants of etiopathological studies. Finally, and most important, the burden of NMS highly affects the health-related quality of life of PD patients. Different models of PD non-motor subtyping have been recently proposed in literature. Some authors have postulated that certain symptoms tend to aggregate in specific clusters following an anatomo-clinical correlation model (Marras 2016). According to this view, the occurrence of NMS clusters may be explained by the presence of different stages which underlie the pathological process observed in PD patients. Based on this "anatomo-clinical model" Chaudhuri and Marras (2016) have identified three main subtypes of nonmotor profile: a brainstem subtype characterized by the prevalence of sleep and autonomic dysfunctions, a limbic variant with depression, fatigue and weight loss, and, a cognitive subtype, with a particular predominance of cholinergic dysfunctions such as memory impairment, apathy and anxiety.

a. *a brainstem subtype characterized by a prevalence of sleep dysfunction and autonomic control,*
b. *a limbic variant with depression and fatigue ad weight loss*
c. *a cognitive subtype, with a particular predominance of cholinergic dysfunctions as memory impairment, apathy and anxiety.*

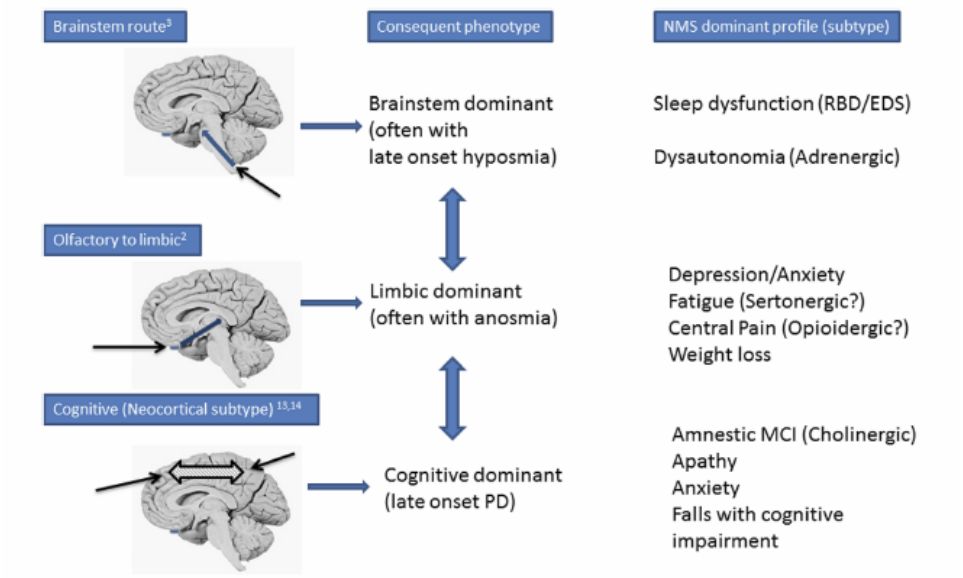


FIG. 2. Possible routes of spread of pathology in PD as described from pathophysiological studies and consequent phenotypic expression and non-motor subtypes. RBD, rapid eye movement behavior disorder; EDS, excessive daytime sleepiness; MCI, mild cognitive impairment. In some subtypes specific neurochemical deficits such as adrenergic, serotonergic, opioidergic, and cholinergic pathways are preferentially involved, sometimes greater than dopaminergic involvement. ↕, overlap between the phenotypes are possible. Hatched arrow in cognitive phenotype diagram indicates frontal and parietal involvement with underpinning cholinergic dysfunction. Figure adapted from Sauerbier et al.[1] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Marras and Chaudhuri, 2016)

Using another approach, other groups investigated the correlation between motor phenotypes and the overall burden of NMS. However, the results of these studies based on empirical "motor model" of NMS profile are partially conflicting (Kadastik-Eerme, 2016). Khoo et al found that a greater number of NMS is associated with the PIGD phenotype in newly diagnosed nondemented PD patients (2013). Constipation, autonomic and sensory symptoms were found prevalent in the non-tremor dominant (NTD) subtype by Muller et coll. (2011). In contrast, other authors did not find a higher overall burden of NMS in the akinetic-rigid phenotype compared with tremor dominant (TD) individuals within an incident PD cohort.

Another aspect of great interest is the potential contribution of the genetic background to the prevalence and progression of NMS in PD; to date, previous researches have mostly focused on cognitive decline, which is one of the most disabling non-motor complication of latest stages of PD. Convergent data suggest a role for MAPT genotype in Parkinson's disease dementia (PDD) (Robakis 2016). However, the possible association of MAPT genotype (in particular H1/H1 homozygous vs H2 carriers) to non-motor phenotypes has not yet been investigated; this finding could support a "genetic model" of NMS subtypes.

Whatever the NMS model ("anatomo-clinical", "motor" or "genetic"), it should be based on its relevance in common clinical practice.

Our study aims to investigate whether in a sample of PD patients a genetic model according to the genotype MAPT distribution of NMS subtypes can be argued; moreover, we proposed to conduct a cluster analysis on NMS pattern of our cohort.

Methods

Subjects

We included in our cohort PD patients consecutively afferent to the outpatients Parkinson's Disease Unit of Sapienza University of Rome. All subjects fulfilled the UK Brain Bank criteria for idiopathic PD (ref). Individuals were excluded if they had signs of atypical parkinsonism, dementia and/or doubtful response to dopaminergic replacement therapy.

The study protocol was approved by the XXX Hospital Ethics Committee and all patients gave their informed written consent.

Patients assessment

We collected demographic data (age, gender and education), neurological history (age at onset of PD, duration of disease and specific treatments); the levodopa equivalent daily dose (LEDD) was also calculated.

All patients underwent a clinical and a neuropsychological examination and a genetic analysis.

Clinical evaluation. Three neurologists with expertise in movement disorders (MD, AR, XX) investigated PD patients using motor scales, such as Unified Parkinson's Disease Rating Scale (UPDRS) part III and V (Hoen and Yahr Scale [H&Y]), freezing of gait questionnaire (FOGq) and non motor scales, such as Non Motor Symptoms Scale (NMSS) to assess frequency and severity of a wide range of NMS (ref NMSS+ ref NOSTRO ARTICOLO!!), Autonomic Scale for Outcomes in Parkinson's disease-Motor (SCOPA-Aut) for evaluate dysautonomic dysfunctions and Epworth Sleepiness Scale (EPSS) for sleep disturbances evaluation. Three main motor phenotypes have been considered: tremor-dominant subtype (TD), non-tremor dominant subtype (NTD) and postural gait and instability disorder (PIGD) subtype

Genetic analyses.

MAPT haplotype was determined in this cohort as described above.

Data analysis

Descriptive statistics were used for the characterization of the sample, by means of the univariate ANOVA test for continuous variables and the Pearson's $\chi 2$ test for categorical variables.

To explore currently proposed NMS clusters of PD (ref), we performed a correlation analysis by means of Pearson's correlation coefficient (r) of the following NMS: dysautonomic symptoms (total score of SCOPA aut) with respect to sleep disorders and to fatigue (sleep/fatigue domain of NMSS) in order to investigate a "brainstem variant", depression (total score of BDI) with respect to anxiety and apathy (subscores of NPI) to investigate a "limbic variant", cognitive functions (total score of MOCA) with respect to anxiety and apathy (subscores of NPI) to investigate a "cognitive variant".

We explored the relationship among the different NMSS domains with other measures for the same construct (or other related constructs) and motor scores, respectively,

In order to reduce data originating from raw scores of several NMS scales (investigating some overlapping symptoms), we performed a factor analysis with varimax rotation; we have excluded scores with a poor dispersion of the values. We performed then a hierarchical cluster analysis by using the factors identified as parameters.

Standard values for acceptability and reliability were established based on previous studies (ref). Statistical analyses were performed by means of the Statistical Package for the Social Sciences (SPSS 23).

Results

One hundred and thirty-seven patients with PD were included in the study. Table 1 shows demographic, clinical characteristics and scores distribution of motor and non-motor scales of the whole sample.

Homozygous and heterozygous H1 PD patients did not differ with respect to age of onset, duration of disease, severity of motor symptoms investigated by UPDRS part III and H&Y scale, motor phenotypes, total score of all the scales used to assess NMS and the subitems of NMSS.

Considering motor phenotypes, we did not found any significant difference between motor and non-motor scales, except for a lower total score of MOCA (F 13.317, $p< 0.001$) and a higher FOG score (F 8.908, $p< 0.001$) in PIGD group with respect to other phenotypes (TD and NTD).

In the attempt to verify the anatomo-clinical model proposed by Chaudhuri et al. , we have observed a significant positive relation between sleep/fatigue domain of NMSS and dysautonomic symptoms ($R = 0.452$, $p < 0.001$) and depression ($R = 0.620$, $p < 0.001$), and between cognitive functions and apathy ($R = -0.289$, $p = 0.001$).

We have checked this last finding by mean of an univariate ANOVA test using total score of MoCA as a continuous variable and the subscore for apathy of NPI as a factor: this further analysis confirmed previous results ($F = 3.546$, $p = .017$).

Factor analysis has identified six factors, as shown in table 2, which can be summarized into: 1= cognitive functions 2= mood disorders 3= sleep dysfunctions 4= gastrointestinal disorders 5 = urological disorders 6= concern of their own cognitive impairment. No statistically significant differences has been found between these six factors and aplotype.

Through hierarchical cluster analysis using the 6 above-mentioned factors as parameters, we sought to identify cluster of at least 10 people; we have explored the possibility of generating from 3 to 20 clusters. Only the choice to divide the sample in 10 clusters followed 3 groups of 83 (cluster 1), 13 (cluster 4) and 11(cluster 7), subjects; the other 7 clusters consisted in a small number of individuals.

No statistically significant differences in aplotype has been found in the 10 clusters explored.

The three main clusters were similar in terms of age, sex, disease duration and cognitive functions (assessed by MoCA total score),

Statistically significant differences in NMS scales (total and subitems scores) among the three main clusters are reported in table 3. Overall, cluster 1, which is the most numerous group, showed a less severe motor, neuropsychiatric and functional impairment; cluster 4 had a greater motor and non-motor impairment and cluster 7 was found to be more depressed with respect to the other main clusters.

We performed then a sensitivity analysis with non parametric tests (Kruskal Wallis) given the small number of subjects in cluster 4 and 7 with respect to cluster ; differences encountered in BDI and NPI scores between the groups 4 and 7 were lost; significant statistically differences remain in memory / attention and urological subitems of NMSS, where cluster 7 achieved intermediate scores between cluster 1 and 4. Therefore, cluster 7 seems to be more concerned about his own difficulties of memory and concentration than cluster 4, while no significant differences have been found in real cognitive performances at MoCA. Moreover, total score of MoCA does not correlate with the score of memory / subitem attention of the NMS.

Table 1 Demographic data and assessment of motor and non-motor symptoms in patients with Parkinson's disease.

	N	Value
Age	137	69.1 ± 7.4 (47 – 86)
Age of onset	134	61.0 ± 7.5 (40 – 77)
Duration of disease	134	8.2 ± 4.5 (1 – 19)
Sex: F (%)	137	50 (36.5 %)
Genetic profile: H1 homozygous (%)	129	82 (63.6 %)
UPDRS III	129	17.9 ± 7.6 (4 – 44)
FOG	131	8.4 ± 6.2 (0 – 24)
H&Y	129	2.1 ± 0.6 (1 – 3)
- 1		19 (14.7 %)
- 1.5		1 (0.8 %)
- 2		72 (55.8 %)
- 2.5		8 (6.2 %)
- 3		29 (22.5 %)
Tremor dominant (%)	133	45 (33.8 %)
MOCA	127	22.6 ± 4.8 (9 – 30)
BDI	133	10.6 ± 8.3 (0 – 44)
NPI	133	11.6 ± 12.0 (0 – 64)
SCOPA aut	133	15.0 ± 8.2 (1 – 40)
ESS	134	6.3 ± 5.0 (0 – 24)
NMSS	133	44.9 ± 32.8 (1 – 193)

Data are expressed as mean ± standard deviation (range) except where indicated

UPDRS III, Unified Parkinson's Disease Rating Scale part III; FOG, Freezing Of Gate scale; H & Y, Hoen & Yahr scale; MOCA, Montreal Cognitive Assessment; BDI, Beck Depression Inventory; NPI, Neuropsychiatric Inventory ; , SCOPA-Aut, Autonomic Scale for Outcomes in Parkinson's disease-Motor; ESS, Epworth Sleepiness Scale; NMSS, Non Motor Symptoms Scale.

Table 2 Factor analysis with varimax rotation of raw scores of different non-motor scales used to assess non-motor symptoms.

	1	2	3	4	5	6
MoCA (Attention)	0.761					
MoCA (Executive)	0.750					
MoCA (Visuospatial)	0.701					
MoCA (Abstraction)	0.658					
MoCA (Language)	0.595					
MoCA (Delayed recall)	0.590					
MoCA (Memory)	0.559					
MoCA (Orientation)	0.540					
BDI (Psychiatric)		0.803				
NPI (Depression)		0.801				
NMSS (Mood/Apathy)		0.796				
NPI (Anxiety)		0.668				
NPI (Sleep)			0.775			
NMSS (Sleep/Fatigue)		0.442	0.682			
BDI (Somatic)			0.636			
NMSS (Miscellaneous)			0.426			
ESS total score			0.422			
NMSS (Gastrointestinal)				0.892		
SCOPA Aut (Gastrointestinal)				0.843		
NMSS (Urinary)					0.909	
SCOPA Aut (Urinary)					0.900	
NMSS (Attention/Memory)						0.710

1= cognitive functions 2= mood disorders 3= sleep dysfunctions 4= gastrointestinal disorders 5 = urological disorders 6= concern of their own cognitive impairment.

MOCA, Montreal Cognitive Assessment; BDI, Beck Depression Inventory; NPI, Neuropsychiatric Inventory ; , SCOPA-Aut, Autonomic Scale for Outcomes in Parkinson's disease-Motor; ESS, Epworth Sleepiness Scale; NMSS, Non Motor Symptoms Scale.

Table 3 Comparative statistics for different non-motor symptoms scales (total and subitems scores) among the three main clusters

	1	4	7	F	p	Post-hoc
BDI						
- Total	7.1 ± 4.6	17.8 ± 7.5	17.4 ± 8.4	33.481	< .001	1<4, 1< 7
- Psychiatric	6.3 ± 5.8	13.3 ± 5.6	13.8 ± 7.0	46.665	< .001	1<4, 1< 7
- Somatic	3.0 ± 1.9	4.5 ± 2.6	3.6 ± 2.3	3.161	0.47	**1<4**
NPI						
- Total	6.6 ± 6.7	19.6 ± 10.3	18.9 ± 10.0	25.235	< .001	1<4, 1< 7
- Depression	1.0 ± 1.6	3.8 ± 3.1	6.9 ± 2.5	51.845	< .001	**1 < 4 < 7**
- Anxiety	1.5 ± 2.2	3.7 ± 3.1	4.7 ± 3.3	11.329	< .001	1<4, 1< 7
- Apathy	0.3 ± 0.8	2.1 ± 2.5	0.4 ± 0.7	13.979	< .001	**1<4, 7<4**
- Irritability	0.5 ± 0.8	1.7 ± 1.5	1.7 ± 2.9	7.963	.001	1<4, 1< 7
- Sleep	2.0 ± 3.1	4.7 ± 3.3	2.4 ± 3.1	3.875	.024	**1<4**
SCOPA						
- Total	11.9 ± 3.4	27.5 ± 6.3	11.8 ± 3.4	36.575	< .001	**1<4, 7<4**
- Urinary	4.8 ± 3.1	13.0 ± 2.6	3.8 ± 2.4	45.628	< .001	**1<4, 7<4**
- Thermoregulatory	1.2 ± 1.5	3.3 ± 2.4	1.1 ± 1.3	9.529	< .001	**1<4, 7<4**
- Sexual	1.3 ± 2.3	3.5 ± 2.4	1.8 ± 2.2	4.876	.010	**1<4**
NMSS						
- Total	29.8 ± 20.8	86.1 ± 22.6	48.4 ± 20.1	41.228	< .001	**1 < 7 < 4**
- Sleep/Fatigue	6.3 ± 6.9	14.8 ± 6.4	12.8 ± 4.5	11.507	< .001	1<4, 1< 7
- Mood/Apathy	3.4 ± 5.3	18.4 ± 10.6	17.9 ± 13.0	37.477	< .001	1<4, 1< 7
- Attention/Memory	2.7 ± 4.2	8.6 ± 7.0	2.4 ± 2.9	9.643	< .001	**1<4, 7<4**
- Urinary	5.8 ± 5.5	25.6 ± 6.6	1.8 ± 2.7	81.328	< .001	**1<4, 7<4**
- Sexual	1.7 ± 3.5	5.2 ± 4.5	1.9 ± 2.3	5.355	.006	**1<4**

Table provides only subitems which result statistically significant different among the three main clusters (1, 4, 7)

Discussion.

In our cohort of incident unselected PD patients, we were not able to intercept some of the clusters of PD nonmotor subtypes recently proposed in literature. None of the models tested in our cohort exhibit statistical evidence of a cluster coherence within the nonmotor symptoms spectrum.

Overall, these results likely confirm the complex pathophysiology of PD where no single biological mechanism is the solely determinant.

Although the discrete clusters described in literature are not recognizable, in our population some symptoms of the anatomical model tend to correlate; indeed, we observed a significant correlation between fatigue/excessive sleepiness and depression or cognitive functions and apathy. It is worth considering that these associations have been frequently reported in literature, possibly reflecting a phenotypic trait rather than the expression of pathogenicity in the anatomical model.

Moreover, the lack of association between MAPT haplotypes and a specific profile in the whole spectrum of nonmotor symptoms may suggest that MAPT background partake to the "cortical side" of PD. Indeed, MAPT H1 haplotype, and in some cases specific subhaplotypes, have been associated with dementia, motor subtypes and recently with cortical load of Lewy body, which are cortical pertinent aspects.

When we analyzed the correlation between motor phenotype (Tremor dominant, mixed forms, and PIGD patients) and the nonmotor profile we were not able to find any significant correlation. In our cohort no specific nonmotor profile was found in association with the empirical model of PD motor syndromes. Although PD motor phenotypes probably present different biological basis and are relevant in terms of prognostic value, it is possible to speculate that the route of progression of motor disability follows degenerative trajectories that are only partial superimposable to those of nonmotor phenomenology. Furthermore, the motor subtypes attribution may not take into account for the instability of these subtype, especially when patients are classified following an adequate observational period. Finally, it is worth to consider that after the initial and intermediate stages of the disease duration the majority of cases tend to converge in a more complex motor syndrome.

The cluster analysis performed in our cohort identified three main groups of patients, of different numerosity, that share a similar nonmotor symptoms profile:

group number 1, the vast majority of patients, that express a moderate burden of all nonmotor symptoms,

group number 4, patients with severe dysautonomic symptoms, apathy, excessive sleepiness, urologic and sexual dysfunctions;

and finally group number 7, with a prominent depressive phenotype despite a relative benign form of PD.

The three groups are comparable in terms of age, disease duration and age at onset, patients have also similar disease motor impairment.

Given that these nonmotor phenotypic traits tend to aggregate in patients with similar disease duration, we may assume that they clinically reflect the dysfunction of the neurotrasmettitorial networks involved beside the ineluctable dopamine depletion.

In our "network-based model", patients of group 1, which are the majority of PD patients and represent he main phenotypic PD trait, have a classical dopamine related syndrome or are in a state of the malady were dopaminergic depletion still underlies for the majority of symptoms.

In group 4, the presence of prominent autonomic symptoms, apathy, sleep dysfunctions, urologic and sexual dysfunctions may be the expression of a cholinergic and autonomic systems failure attendant to the dopaminergic syndrome. This minority of patients display also display a statistically significant presence of FOG, which can be considered a straddling cognitive-motor symptom. Most of these symptoms are potentially treatable and the identification of autonomic, cognitive or FOG related may yield to a better treatment.

Patients in phenotypic trait 7 present an overall intermediate burden of nonmotor symptoms but a higher degree of depression, fatigue and sleep disturbances. Such phenotypic trait may be ascribable, according to our network based model, to a louder monoaminergic depletion. Indeed, findings from clinical, neuroimaging, and animal studies shows that dopaminergic system is the major contributor to the pathophysiology of these symptoms; nonetheless, the degeneration of noradrenergic and serotonergic projection systems also has an impact on psychiatric symptoms of PD. Whether depressive symptoms are at risk for subsequent cognitive collapse or motor worsening remains a matter of debate, with current available studies showing partially conflicting results.

In conclusion, the overall results of our study suggest that nonmotor subtyping may not be feasible in a real life clinical setting and the vast majority of patients with intermediate stages of disease do not correspond to a specific cluster or a clinico-pathological subsyndrome of PD. However, searching for autonomic-cognitive dysfunctions (trait number 4) and depression (trait number 7) may allow to intercept phenotypic traits of the disease with potentially treatable complaints that juxtapose motor impairment.

We are aware of the relative small numerosity of our cohort, however, the lack of stringent selection criteria and the incident quality of the cohort probably resemble a real life ambulatory population of PD patients. Furthermore, our patients were assessed with a comprehensive battery of scales and questionnaire to examine the whole spectrum of nonmotor symptoms.

Given the potential relevance of nonmotor subtypes, a possible perspective is to standardize a clinical and instrumental work-up to define uniformly nonmotor PD subtypes.

Experimental setting II: DAT Methylation in Parkinson Disease: Correlation with peripheral Dopamine Transporter gene expression and central activity

The role of epigenetic mechanisms in the function and homeostasis of the central nervous system and their participation in the neurological disorders remain an intriguing feature. The epigenetic phenomenon has been defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that does not entail a change in DNA sequence". DNA methylation is the best-understood epigenetic modification modulating transcriptional plasticity. It refers to binding a methyl group to a CpG islands in the genomic sequence. Methyl groups bound to the genomic sequence reduce the DNA binding capacity for transcription factors. However, in some cases methyl groups are able to enhance transcription factors attachment to promoter regions.

The correlation between DNA methylation and neurodegenerative diseases, and in particular in PD, was pointed out by some studies. (table, Haoyang Lu et al. 2013)

SNCA	*SNCA* (intron1 and promoter)	Hypermethylation	Decreased expression of *SNCA*
		Hypomethylation	Overexpression of *SNCA*
Inflammatory cytokines	*TNF-α*	Hypomethylation	Increased risk of apoptosis in dopaminergic neurons
Clock genes	*CRY1 NPAS2*	Devoid of methylation	Disorder of circadian rhythms
Telomere	Subtelomeric region	Constant methylation	Telomere shortening
Other genes	*PARK16/1q32 GPNMB STX1B*	Methylation alteration	PD risk
	Cytochrome P45 2E1	Hypomethylation	Increased PD susceptibility

Although these findings indicate the potential role of DNA methylation in the neurodegenerative diseases, the mechanisms remain inadequately characterized.

PD is a complex multiorgan clinicopathological entity, typically characterized by progressive degeneration of dopaminergic nigro-striatal pathways.

Considering the prominent role of dopaminergic system dysregulation in Parkinson Disease, the epigenetic derangement of the dopamine metabolism determinants deserves a peculiar attention. In this term, Dopamine Transporter (DAT1) exerts a prominent role in the process of dopaminergic striatal signalling. Given the evidence that molecular DAT imaging provides in vivo a measure of dopamine terminal depletion in PD, we sought to explore the potential influence of DAT gene epigenetic modification on central and peripheral expression.

THE ROLE OF DAT1 IN PARKINSON'S DISEASE

The Dopamine Active Transporter-1 (DAT1), also known as SLC6A3, is a membrane-spanning protein that re-uptakes Dopamine (DA) from the synaptic cleft into the presynaptic neurons (Uhl, 2003).

This process is driven by the ion-gradient created by the Na+/K+-ATPase, two Na+ ions and one Cl- ion are transported with the substrate (Torres, 2003). The result of this process is the decrease of DA concentration in the synaptic cleft and and increase in the presynaptic neuron. DAT-KO mice were found to have a 300-fold increase in time of DA in the striatal extracellular space, a 5-fold increase of extracellular DA concentration and a 95% decrease in striatal intracellular DA concentration (Torres, 2003). As every monoamine transporter, DAT is expressed almost only in cells that contain its cognate neutrotransimetter. In DAT's case, the highest concentrations of this protein are found in the cell bodies of neurons of the Ventral Tegmental Area (VTA) and pars compacta of the Substantia Nigra (SNpc) of the mesencephalic brain steam.

Psychostimulants that enhance locomotor activities interact with DAT. Cocaine and Amphetamine act has competitive inhibitors of DA uptake (Uhl, 2003; Torres 3003).

DAT has also been found to be a transporter for 1-methyl-4-phenylpyridinium ion (MPP+), a product of the extracellular metabolisation of N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MTPT). MPP+ is Parkinsonism inducing neurotoxin that causes death specifically in Dopaminergic neurons.

Kitayama et al studied the correlation between MPP+ toxicity and MPP+ transport in cell-lines expressing wild-type and mutant DAT1 genes. They discovered not only that cell-lines insensitive to high concentrations of MPP+ became susceptible when expressing DAT, but also that the sensibility to MPP+'s toxicity was parallel to an increase in DA

uptake velocity. Analyzing MPP+ toxicity among the different DAT mutants, Kitayama and colleagues concluded that the toxicity was directly correlated to the affinity of the transporter (Km) for MPP+, rather than the velocity of uptake.(Kitayama 1998)

It has also been suggested that DAT interacts with alpha-synucleine, increasing the activity of the transporter (Uhl,2003).

The human DAT/SLC6A3 gene is located in the chromosome 5p15.3 and is divided in 15 exons. In the 3' untranslated region of the gene there is a Variable Number of Tandem Repeat (VNTR) of approximately 40 bp. The VNTR copy number can be between three and eleven, with the most frequent VNTR copy number being nine (9R) and ten (10R) (Vanderbergh, 1998). At least fifty Single Nucleotide Polymorphisms (SNPs) have also been discovered in the 5' regions of the gene and, in vivo , two haplotypes were found that distinguish different levels of [11 C] cocaine binding (T-841C/rs2652511/C-839T and A11821G/rs2937639/G11736A) (Drgon,2006).

Moreover, the 10R VNTR was associated with a higher gene expression in vitro (Fuke, 2006). It has been, therefore, argued that the polymorphism in 3' and 5' could influence the gene expression and DAT availability in the Striatum.

Van de Giessen and coll. measured striatal DAT availability using 123I-(2-b-carbomethoxy-3-b(4-iodophenyl)-tropane) (123I-b-CIT). High affinity for DAT-SPECT in healthy subjects that expressed the 40 bp VNTR and specific SNPs in the 5' end of the gene. Further studies observed that the 9R allele repeat was associated with a higher striatal DAT concentration compared to the 10R homozygotes; on the other hand, in this report did not emerge any association bewteen the 5' SNPs and DAT availability. (Drgon,2006)"Haplotype analysis revealed that the identified effect of the 9R allele seems to be caused mainly by a subgroup of 9R carriers, the T-A-9R (rs2652511– rs2937639–VNTR) " (van de Giessen, 2009).

A meta-analysis, reviewing studies on striatal availability of DAT through SPECT both in healthy controls and in neuro-psychiatric patients, moderated previous results. The author observed that while there was an increase in DAT availability in certain haplotypes, this was not statistically significant and that the majority of the previous studies were underpowered, concluding that while there might be a correlation between VNTR and 5' polymorphisms and DAT availability in the striatum, this needs to be further investigated (Costa, 2011).

The selectivity of the expression of DAT coupled with the death of DA neurons that strongly express DAT has led to the hypothesis of DAT's involvement in the pathogenesis of PD. If we compare the DAT concentrations in three areas of the brain that contain dopaminergic neurons: arcuate nulceus of the hypothalamus, ventral tegmental area, Substantia Nigra pars compacta, we can see that it correlates with the degree of neuronal loss in PD "(SNpc > VTA > arcuate nucleus)" (Guzey, 2012; Uhl, 2003).

Consequently, polymorphisms in DAT/SLC6A3 might be involved in the pathogenesis of PD. Several studies have been conducted in order to observe a correlation between the 3' VNTR and the 5' SNPs and PD (Arai,1995; Le Couteur, 1997;Kim, 2000;Morino, 2000).

Le Couteur and colleagues, in a case-control study, found that the 11R allele was present in circa the 2,5% of patients and only 0,5% of the controls. This allele was therefore associated with a ten-fold increase in risk of developing PD between the two populations and could explain up to 5% of cases (Le Couteur, 1997). Kim and colleagues, in a similar study in a Korean population, partially confirmed previous results (2,5 fold increased risk) (Kim, 2000). Neither of these studies attempted to explain the involvement of DAT in the pathogenesis of PD.

Morino et al investigated the role of a specific SNP (exon 9 1215 A/G) in a PD Japanese coorte. The report suggested that the 1215 A/G SNP was associated with a decrease in susceptibility to PD (Morino, 2000). However further studies failed to replicate the results (Liu 2001,Kimura 2002).

In 2014 Zhai and colleagues reviewed 18 studies on 3' VNTR and 11 on SNP. The meta-analysis proved conclusively only that the 10R allele conferred protection against PD only in East-Asian populations but not in Caucasians. Other results were found to be the product of either statistically biased or underpowered studies. The review also analysed the combined effects of the four SNPs (rs6347, rs3756450, rs2652510 and rs2550956) in the SLC6A3 gene locus on risk for PD. The results showed that only rs2652510 genotype GG is associated with a higher PD risk in all population (Zhai,2014).

Several approaches confirmed that the dopaminergic neurons loss correlate decrease in DAT concentration in PD.

Harrington et al (1996) and Counihan & Penney (1998) have examined DAT mRNA expression in both healthy controls and in PD patients in the Ventral Mesencephalon from post mortem samples. Both reports underline increase DAT mRNA expression in the SNpc of healthy donors if compared to PD patients. The reserchers detected also a marked decrease in DAT mRNA concentrations in the surviving neurons in PD patients, especially in the SNpc. Results that are consistent with the scientific literature on the matter. The marked reduction of DAT could be ascribable either 1) a compensatory mechanism for diminshed DA concentration in the synaptic cleft or 2) might just be the expression of a general decrease of overall activity in a dying cell (which parallels the decrease in level of transcription of TH, Tyrosine Hydroxylase, the rate limiting enzyme for the synthesis of DA) (Harrington,1996;Counihan & Penney,1998).

The laboratory findings are supported by neuroimaging reports. SPECT with [123-I]beta-CIT and [123-I]FP-CIT, radioligands that selectively bind the dopamine transporter, are routinely used for detecting the carrier-protein decrease in vivo, corroborating the diagnosis of PD when non dopamine-deficient syndromes are considered (i.e. dystonic and severe essential tremors), or for early PD detection (Innis,1993;Booij,1997, Brooks 2010).

The study of DAT, its polymorphisms and its involvement in the pathogenesis of PD has led to hypothesise its contribution also for PD treatment and, in particular, for the common disabling motor complication of this as levodopa-induced dyskinesia (LID).

A PET study suggested that LID development was associated with low levels of DAT expression in pre-synaptic terminals. These levels were significantly lower than DAT expression levels in patients with Motor Fluctuations (MF), suggesting an association between dyskinesias and DAT expression. Moreover the study suggested that the known risk factors for LID (age, disease duration, UPRDS) are "effect modifiers [...] of a main determinating factor, that is, the downregulation of putaminal DAT"(Troiano,2009). A subsequent retrospective cohort study, that enrolled 127-drug naïve de novo patients with PD and conducted 18F-FP-CIT PET scanning, monitored the PD progression for a mean follow-up period of 3.4 years. The study demonstrated that patients with low baseline DAT levels have a significantly higher risk of developing LID, confirming the hypothesis that pre-synaptic dopaminergic denervation in the putamen, but not in the caudate, is crucial in the development of LID (Jin Yong Hong, 2014;Troiano 2009).

Much effort has been made in investigating the extent to which the various polymorphisms (VNTR and SNP) of DAT influence the response to levodopa and LID time latency (Altmann 2016; Kaplan, 2014; Contin 2004).

M. Contin et al. (2004) examined through [123I]-FP-CIT SPECT a cohort of PD patients that expressed 9R and 10R homozygotes VNTR and not only it did not find differences in DAT expression in the striatum, it did not also find that these polymorphisms influenced the response to levodopa nor levodopa-linked dyskinesias.

Another retrospective study tried to identify genetic factors associated with LID. The study analysed 14 SNP and VNTR in SLC6A3. After statistical analysis it was proved a significant association between the intronic SNP rs393795 and a higher latency to LID onset (time ratio = 4,96) (Kaplan,2014).

A recent paper addressed the issue of the high inter-individual fluctuation of the response to levodopa, trying to determine how much of it was ascribable to "biological, pharmacological and genetic factors" (Altmann,2014). The study analyzed the 3' UTR VNTR and tried to assess the response to levodopa of the various genotypes and found the minimum levodopa dosage at which every allele responds. The subset of patients with 9R/9R VNTR genotype needed less levodopa than any other allele. This seems consistent with previous studies that showed that the age-related DAT expression decreased was higher in 9R/9R. It was therefore hypothesised that, expressing lower levels of DAT, these patients would have higher DA concentration in the synaptic cleft and need less levodopa. The authors concluded that their paper has to be considered a first attempt and not a definitive guide towards a "pharmacogenetic algorithm for levodopa dose prediction" (Altmann,2014).

Methods

Subjects. Unrelated sporadic PD patients are consecutively recruited from the Parkinson outpatient centre of the Sapienza University of Rome and fulfilled the UK Brain Bank criteria for PD. Inclusion criteria included: 1) stable dopaminergic treatment 2) Mini Mental State Examination (MMSE) score >26; 3) no dementia according to the Movement Disorder Society (MDS) clinical diagnostic criteria (Emre et al, 2007) using an extensive neuropsychological battery; and 4) suitability for DAT-SCAN and MIBG. Exclusion criteria included: 1) history of neurological diseases other than idiopathic PD; 2) unclear history of chronic dopaminergic treatment responsiveness; 3) presence of major non stabilized medical illnesses (i.e. non stabilized diabetes, obstructive pulmonary disease or asthma, hematologic/oncologic disorders, vitamin B12 or folate deficiency, pernicious anemia, clinically significant and unstable active gastrointestinal, renal, hepatic, endocrine or cardiovascular disorders and recently treated hypothyroidism); 4) known or suspected history of alcoholism, drug dependence and abuse, head trauma and mental disorders (apart from mood or anxiety disorders) according to the DSM-IV TR criteria; and 5) presence of vascular brain lesions, brain tumor and/or marked cortical and subcortical atrophy on MRI scan.

The sample's clinical records were reviewed and patients were assessed by neurologists who are experts in movement disorders; the patients' clinical and demographic data are shown in Table below. The variables collected included: demographic data, age at onset, disease duration, familiarity for PD, total levodopa equivalent dose (LED) and UPDRS III at the time of last visit. The patients were finally classified in two clinical subtypes, by using criteria published in previous studies (Selikhova et al. 2009):

(a) tremor dominant (TD), i.e. patients with tremor as the only motor sign at onset or tremor as the prominent motor symptom according to the UPDRS part III

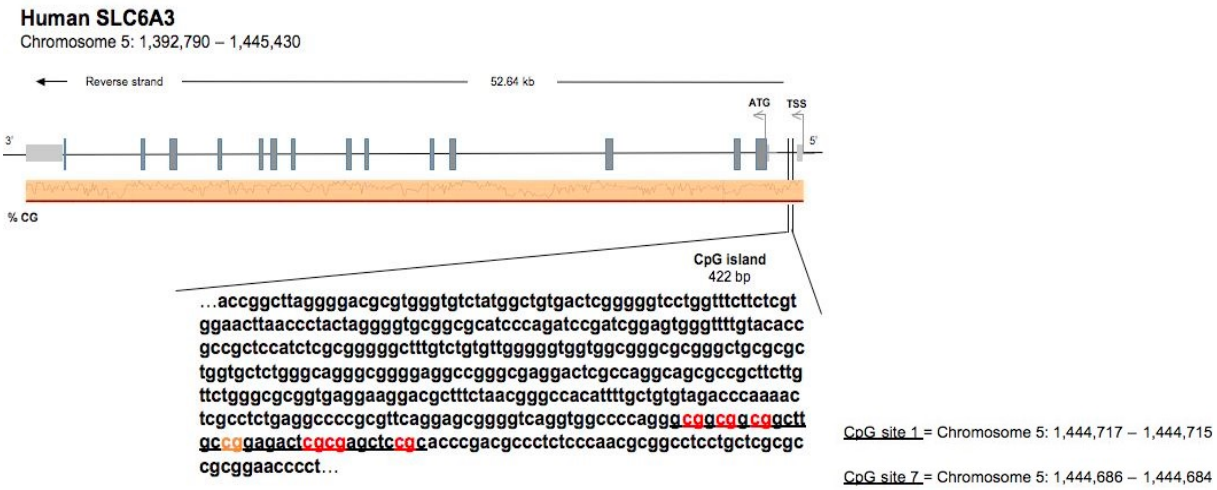
(b) non-tremor dominant (NTD), i.e. patients with predominant rigidity and bradykinesia but no tremor or only mild tremor at rest.

Genotyping

10 ml of blood samples in EDTA were taken from the participants and used for DNA and RNA extractions.

Pyrosequencing has been used as a standard technique to detect amount of methylation using bisulfite converted DNA. Details of pyrosequencing assay (hsl_SLC6A3_01PM Pyromark CpG assay (PM00022064)) including primer sequences are provided at dealer website (www.qiagen.com). After DNA extraction, 0.5 µg of DNA from each sample was treated with bisulfite, using a DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen) in according to the manufacturer's protocol. PCR conditions were as

follows: 95°C for 15 minutes, followed by 45 cycles of 94°C for 30s, 56°C for 30s, 72°C for 30s, and, finally, 72°C for 10 minutes. PCR products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analysed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage (mC/(mC+C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine).



Imaging study

Patients underwent two sequential nuclear medicine investigations: 123I-FP-CIT and 123I-MIBG. 123I-FP-CIT execution: after thyroid blocking with Lugol solution, an intravenous injection of 185 MBq of 123I-FP-CIT will be performed and brain SPECT images will be obtained 3 hours after injection. Semiquantitative analysis was performed by selecting three consecutive slices with the highest striatal uptake. Regions of interest of a fixed size was bilaterally drawn over the striatum (caudate nucleus and putamen); the occipital cortex will be used as the reference region and Striatum/occipital lobe ratio (R/L) will be calculated. 123I-MIBG execution: after thyroid blocking with Lugol solution, at 20 and 240 minutes after 185 MBq of MIBG administration, planar and SPECT cardiac images will be obtained. Planar MIBG images will be analyzed by means of heart to mediastinum ratio obtained drawing region of interest (ROI), to achieve semi-quantitative parameters relative to tracer distribution, early HM and late HM will be obtained. The planar SPECT cardiac imaging will be acquired at 15 and 255 minutes after tracer administration and LSS will be obtained by MIBG SPECT myocardial receptorial images.

Statistical analysis

Methylation data for each CpG site were first collected from the sequencing results. Data will be summarized by descriptive statistics: means and standard error of mean for continuous variables.

Data were analyzed using T-student test, with $p<0.05$ being considered as statistically significant.

The correlation between methylation status and were evaluated by Pearson correlation coefficient.

Results

No statistical differences in the general frequency of DAT CpG islands was revealed between patients (PD n=69 mean methylated islands 15,3±4,8) and age matched control subjects (Ctrl n=30 mean methylated islands 15,8± 5,5).

Univariate analysis intrapopulation of PD patients (n=69) [Table 1] showed significant methylation status frequency in PD patients in clinical variables were considered.

	mean	sd
Age	68,68116	7,199862649
Gender (m/f)	40/29	
Age at onset	62,44927536	7,79806958
LED	504,4927536	368,6513427
Disease Duration (y)	6,231884058	4,576618735
Onset th	63,85714286	7,731079178
LD	311,057971	305,8385716
LD duration	14,16666667	5,865945254
UPDRS	14,16666667	5,865945254
Axial-score	3,101449275	1,933793343
Subtype(TD/NTD)	28/40	
H&Y	1,789855072	0,564942716

[Table 1]

In particular in site 3, 6 and 7 were observed an increase of methylation status in PD patients with higher score of motor scale (UPDRS above median value, Axial score with clinical impairment of gait) and LD assumption (above 400mg).

	UPDRS		
	<14	>=14	sign
num	34	34	
site 1	12,45629	14,05724	0,140072
site 2	11,19771	13,80767	0,046067
sito 3	14,91857	18,84469	**0,011548**

	Axial Score		
	<4	>=4	sign
num	40	28	
site 1	12,65205	14,008	0,221917
site 2	12,02075	13,0128	0,465473
sito 3	15,356	18,9237	**0,024953**

sito 5	31,39257	34,18818	0,421585	31,39675	34,68143	**0,352043**
sito 6	5,625429	7,53697	**0,017433**	5,8625	7,539643	**0,04109**
sito 7	11,86371	16,4197	**0,033694**	12,27125	16,65107	**0,044776**
mean	14,57571	16,27728	0,159577	14,951	16,01713	0,392135

	LD		
	<400	>400	sign
num	43	25	
site 1	12,84976	13,81545	0,398574
site 2	11,96558	13,25591	0,355219
site 3	15,48767	19,13375	**0,025105**
site 5	32,08651	33,8892	0,617634
site 6	5,873488	7,722	**0,026991**
site 7	12,3207	17,0916	**0,031851**
mean	15,11926	15,83364	0,577687

Site 7 resulted with increase methylation level also when PD were categorized on basis of subtype motor phenotype.

	Motor Phenotype		
	NTD	TD	
num	40	28	sign
site 1	13,5802778	12,6692857	0,405582
site 2	13,2232432	11,3175	0,151051
sito 3	17,1694872	16,2703571	0,576875
sito 5	32,80375	32,6714286	0,970193
sito 6	7,052	5,84035714	0,14309
sito 7	16,11725	11,1567857	**0,022431**
mean	15,6436486	14,987619	0,592641

Moreover, although small sample size (n=15) significant methylation status difference was observed when balance impairment was present (H&Y≥2.5). It was revealed that DAT gene site explored were significantly more often methylated in PD patients with postural instability.

	H&Y		
	<=2	>2,5	
num	43	15	sign
site 1	12,6011765	15,4592308	**0,031553**
site 2	11,7001923	15,2107692	**0,030874**
site 3	15,6522642	21,115	**0,00396**
site 5	31,5509434	36,9833333	0,193319
site 6	5,96113208	8,64466667	**0,005249**
site 7	12,7830189	18,6386667	**0,023205**
mean	14,6957372	18,0223077	**0,025563**

Site 3 and 5 showed increased methylation in patients older than 70 year-old.

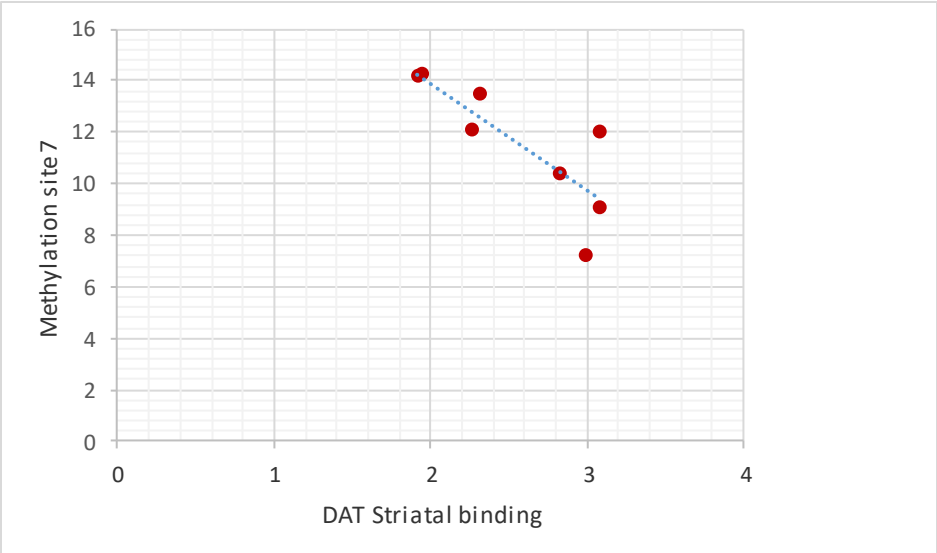
	Age		
	<70yo	>70yo	sign
num	32	36	

site 1	12,4609375	13,7851515	0,216195
site 2	11,4390625	13,2164706	0,171367
sito 3	14,97625	18,2427778	**0,035577**
sito 5	28,243125	36,3545946	**0,016541**
sito 6	5,873125	7,07216216	0,137757
sito 7	12,124375	15,577027	0,107189
mean	14,1861458	16,3252941	0,072584

No significant difference emerged when Gender, Disease Duration (cut-off five year), treatment duration and LED (although in site 3 was observed higher level of methylation in PD with more 500mg assumption daily, p=0.07)

Furthermore, eight PD de novo patients performed nuclear imaging studies [table below]; the results of this examination showed significant association in striatal DAT dopamine binding with methylation status at site 7 (P pearson= - 0,82311, p<0.05)

Pat.	Gend	Age	MIBG	DAT – Scan			Methylation						
			H/M	Striat	Caud	Put	site 1	site 2	site 3	site 5	site 6	site 7	mean
S P	M	69	1,64	2,27	2,6	1,95	11,74	10,16	15,05	27,97	5,46	12,11	13,75
P M	F	63	1,37	1,92	2,32	1,53	17,03	13,12	21,48	41,06	7,9	14,22	19,14
T B	F	70	1,03	2,99	2,26	2,32	10,03	9,26	10,8	21,95	4	7,25	10,55
N I	F	63	1,7	2,31	3	1,63	17,71	13,6	17,85	32,3	6,08	13,46	16,83
T C	F	62	1,26	3,08	4,18	1,97	10,03	9,58	13,01	22,17	6,28	9,08	11,69
O S	M	70	1,04	1,94	2,04	1,84	11,5	8,07	15,41	31,02	5,31	14,29	14,27
F M	F	63	1,12	3,08	3,27	2,9	15,49	15,58	19,87	39,19	5,89	12	18,00
P E	F	59	1,41	2,83	2,97	2,69	10,71	8,85	13,37	23,78	5,35	10,38	12,07



Discussion

Given the concern that small sample size examined cannot allow conclusive evaluations, these results may support the hypothesis that epigenetic phenomenons in the DAT gene partake in malady progression. It has been observed that hypermethylation in CpG sequence might induce conformational modification in chromatin, thereby inhibiting the access of transcriptional machinery to the promoter regions, with consequent reduction of genetic expression. Therefore, taking into account that it will be relevant to obtain the transcriptional effect in order to elucidate the biological implication, we can speculate that increased methylation status in DAT gene may be involved in decreased availability peripheral DAT.

In fact, current evidences indicate a reduction of DA markers, and DAT immunoreactivity in particular, in peripheral blood not only from PD patients but also in subjects suffering other neurodegenerative disorders (es. MSA, ALS); this reduction is frequently observed already in the early stages of these disorders, suggesting that alterations of DA system in peripheral blood are a potential marker of neurodegenerative disorders and that may theoretically represent a reasonable interpretative key of pathophysiological mechanisms (Buttarelli,2011).

In line with this, in our preliminary report, although no statistical differences in the general frequency of DAT CpG islands was revealed between patients and control group, PD patients showed consistent methylation status frequency in specific site when clinical variables were considered. In particular, we observed an increased methylation in PD population in specific site when prominent involvement of motor performance (including axial score) was reported and when balance impairment was clinical evident. These results could suggest that dynamic changes in methylation patterns may emerge during the disease progression. Additionally, the same sites tend to be hypermethylated when higher Levodopa doses were assumed. Therefore, although this hypemethylation status of DAT1 may be likely related to disease severity, we cannot exclude that our observations may reflect a pharmacological effect induced by levodopa.

Taking into account that methylation is a tissue-specific modification, SPECT study, nuclear imaging evaluation of pre-synaptic dopamine transporters in vivo, may contribute to understand the possible link between peripheral epigenetic modification and central dopaminergic mechanism.

Indeed, the correlation in our PD population between the methylation status of site 7 and DAT binding level in striatal structures in PD de novo patient support the hypothesis that peripheral epigenetic modification detected may reflect the central pathogenic process.

It worth to consider that previous studies have suggested that there is differential pathophysiological regulation of DAT expression at the central rather than the peripheral level in PD (Buttarelli, 2009); in fact the combined

immunocytochemistry for DAT on peripheral blood and [123I]-fluopane binding to the striatum showed lack of significant correlation in PD de novo patients. On the other hand, a highly significant correlation between peripheral blood and striatal DAT expression in PD patients treated with dopaminergic therapy (L-DOPA and/or dopamine agonists) was reported (Buttarelli, 2011). However, the studies focused on this topic remain anecdotical and the mechanistic interpretation of the few data available are not univocal.

Furthermore, the observation of different site 7 methylation level according to motor phenotype rises two order of hypothesis:

1) The intrinsic difference in dopaminergic system reflected by DAT methylation status may contribute to clinical motor expression.
2) Given the concern that TD patients present a milder form of PD, the observed results depend on secondary changes of the DAT system primarily related to disease severity.

Finally, the finding of increased methylation status in site 5 and 3 according to age in PD older than 70 year-old allows further consideration. Age is notoriously the most important "environmental" factor in PD development and per se associated with reduction in DAT availability. The age related DAT gene methylation observed might suggest that "age susceptibility" exerts its effect in a different way from the other variables considered.

The potential of DAT1 gene methylation as a biomarker in PD and, in particular, in PD progression and clinical expression warrants further investigations, including longitudinal sampling of the same individuals integrated with detailed clinical and neuroimaging evaluation.

General view

The construct of Parkinson's subtypes likely represents a paradigm where both genetic and epigenetic mechanism partake to pathogenesis and connect to environmental aspects (as dopaminergic treatment) yielding to the relevant heterogeneity of the phenotypic traits that is the clinical hallmark of this malady.

The fact that also monogenic form of PD presents intrinsic heterogeneous clinical and neuropathological phenotype (Pushmann 2013) strongly suggests the occurrence of multifactorial aspects in determining clinical features of the disease.

Assuming that MAPT wedge exerts its effect contributing to the progression of cognitive deficits and the expression of motor phenotypes, theoretical implications on MAPT in PD could define a model of complex pathophysiology related to aetiology, phenotype and progression. In this term, a MAPT contribution could be expected for non motor profile. However, the lack of association observed in our cohort between MAPT haplotype and non motor features may be ascribable to the specific role of MAPT in cortical route in PD degeneration. Nevertheless, given the concern that evaluation with structured questionnaires may be underpowered for detecting subtle dysfunctions, it is worth considering that transversal studies design *per se* may not confidently analyse trajectories of malady. Indeed, in our reports pertaining to nonmotor subtype conducted in a real life clinical setting, it emerged that the majority of patients feature intermediate stages of the disease, which cannot be associated with a specific cluster or a clinico-pathological subsyndrome of PD, thus dispersing the predictive value of the discrete variables.

Moreover, we found that dopaminergic system presents epigenetic modification that are significantly associated not only with PD motor phenotypes, but also with markers of severity (in particular when axial impairment were explored) and environmental factor (dopaminergic treatment), deemphasizing motor phenotype as a trait maker. Therefore, it is conceivable that motor phenotype partially depends on secondary changes of the DAT system primarily related to disease severity.

This hypothesis stresses the concept of axial and balance impairment as evocative measures of disease burden in all individuals affected by PD, a continuous marker of disease progression more than part of a discrete clinical phenotype.

In consideration of the concept above, PD subtypes could be considered as a dynamic clinical entities.

In line with this, the recent paper of Simani et al (2016) point to "instability" of motor subtype classification, suggesting caution when establishing correlations on the basis of motor phenotype.

To date, genome-wide association studies have identified several genetic variants associated with complex diseases. Moreover, epidemiological studies have identified numerous environmental factors that contribute to diseases

expression. However, the precise mechanistic insights are hindered by the lack of understanding of how risk variants and environment functionally connect to the pathogenesis and progression of PD.

In this term, the subtypes in Parkinson disease could not be discrete entities, but rather the resultant of a multifactorial continuum influenced by several genetic, epigenetic, age-related and environmental factors.

REFERENCES

Background

Sieber BA, Landis S, KoroshetzW, et al; Parkinson's Disease 2014: Advancing Research, Improving Lives Conference Organizing Committee. Prioritized research recommendations from the National Institute of Neurological Disorders and Stroke Parkinson's Disease 2014 conference. Ann Neurol. 2014;76(4):469-472.

Marras C, Chaudhuri KR.
Nonmotor features of Parkinson's disease subtypes.
Mov Disord. 2016 Aug;31(8):1095-102. doi: 10.1002/mds.26510. Review.

Jankovic J, McDermott M, Carter J, et al; Parkinson Study Group.
Variable expression of Parkinson's disease: a base-line analysis of the DATATOP cohort.
Neurology. 1990;40(10):1529-1534.

Thenganatt MA, Jankovic J.
Parkinson disease subtypes.
JAMA Neurol. 2014 Apr;71(4):499-504. doi: 10.1001/jamaneurol.2013.6233. Review.

Experimental setting I: Genetic Architecture of MAPT Gene Region in Parkinson Disease Subtypes.

Allen, M., Kachadoorian, M., Quicksall, Z., Zou, F., Chai, H.S., Younkin, C., et al. (2014). Association of MAPT haplotypes with Alzheimer's disease risk and MAPT brain gene expression levels. Alzheimers Res. Ther. 6:39. doi: 10.1186/alzrt268.

Baker, M., Litvan, I., Houlden, H., Adamson, J., Dickson, D., Perez-Tur, J., et al., (1999). Association of an extended haplotype in the tau gene with progressive supranuclear palsy. Hum. Mol. Genet. 8, 711–15. 10.1093/hmg/8.4.711.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J. (2005). Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263-5. doi: 10.1093/bioinformatics/bth457.

Caffrey, T.M., Joachim, C., Paracchini, S., Esiri, M.M., Wade-Martins, R. (2006). Haplotype-specific expression of exon 10 at the human MAPT locus. Hum Mol Genet. 2006 15(24):3529-37. doi: 10.1093/hmg/ddl429.

Colom-Cadena, M., Gelpi, E., Martí, M.J., Charif, S., Dols-Icardo, O., Blesa, R., et al., (2013). MAPT H1 haplotype is associated with enhanced α-synuclein deposition in dementia with Lewy bodies. Neurobiol. Aging 34, 936-42. doi: 10.1016/j.neurobiolaging.2012.06.015.

Das, G., Misra, A.K., Das, S.K., Ray, K., Ray, J. (2009). Microtubule-associated protein tau (MAPT) influences the risk of Parkinson's disease among Indians. Neurosci. Lett. 460, 16-20. doi: 10.1016/j.neulet.2009.05.031.

Davis, A.A., Andruska, K.M., Benitez, B.A., Racette, B.A., Perlmutter, J.S., Cruchaga, C. (2016). Variants in GBA, SNCA, and MAPT influence Parkinson disease risk, age at onset, and progression. Neurobiol. Aging 37, 209.e1-209.e7. doi: 10.1016/j.neurobiolaging.2015.09.014.

Deuschl G. (2013). Benign tremulous Parkinson's disease: a misnomer? Mov. Disord. 28, 117-9. doi: 10.1002/mds.25317.

Di Battista, M.E., Pascale, E., Purcaro, C., Passarelli, F., Passarelli, E., Guglielmi, R., et al., (2014). Clinical subtypes in Parkinson's disease: the impact of MAPT haplotypes. J. Neural. Transm. 121, 353-6. doi: 10.1007/s00702-013-1117-7.

Edwards, T.L., Scott, W.K., Almonte, C., Burt, A., Powell, E.H., Beecham, G.W., et al., (2010). Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Ann. Hum. Genet. 74, 97-109. doi: 10.1111/j.1469-1809.2009.00560.

Eggers, C., Pedrosa, D.J., Kahraman, D., Maier, F., Lewis, C.J., Fink, G.R. et al., (2012). Parkinson subtypes progress differently in clinical course and imaging pattern. PLoSOne 7:e46813. doi: 10.1371/journal.pone.0046813.

Fidani, L., Kalinderi, K., Bostantjopoulou, S., Clarimon, J., Goulas, A., Katsarou, Z., et al., (2006). Association of the Tau haplotype with Parkinson's disease in the Greek population. Mov. Disord. 21, 1036-39. doi: 10.1002/mds.20864.

Fung, H.C., Xiromerisiou, G., Gibbs, J.R., Wu, Y.R., Eerola, J., Gourbali, V., et al., (2006). Association of tau Haplotype-tagging polymorphisms with parkinson's disease in diverse ethnic parkinson's disease cohorts. Neurodegener. Dis. 3, 327-33. doi:10.1159/000097301.

Herman, T., Weiss, A., Brozgol, M., Giladi, N., Hausdorff, J.M. (2014). Gait and balance in Parkinson's disease subtypes: objective measures and classification considerations. J. Neurol. 261, 2401-10. doi: 10.1007/s00415-014-7513-6.

Herold, C., Becker, T. (2009). Genetic association analysis with FAMHAP: a major program update. Bioinformatics 25, 134-6. doi: 10.1093/bioinformatics/btn581.

Huang, Y., Wang, G., Rowe, D., Wang, Y., Kwok, J.B.J., Xiao, Q., et al., (2015). SNCA gene, but not MAPT, influences onset age of parkinson's disease in Chinese and Australians. Biomed. Res Int., Article ID 135674. doi: 10.1155/2015/135674.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., et al., (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702– 5. doi:10.1038/31508.

Jellinger, K.A. (2012). CSF biomarkers in different phenotypes of Parkinson disease. J. Neural Transm. 119, 455-6. doi: 10.1007/s00702-011-0736-0.

Kauwe, J.S., Cruchaga, C., Mayo, K., Fenoglio, C., Bertelsen, S., Nowotny, P., et al., (2008). Variation in MAPT is associated with cerebrospinal fluid tau levels in the presence of amyloid-beta deposition. Proc. Natl. Acad. Sci .U. S. A. 105, 8050-4. doi: 10.1073/pnas.0801227105.

Lill, C.M., Roehr, J.T., McQueen, M.B., Kavvoura, F.K., Bagade, S., Schjeide, B.M., et al., (2012). Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: The PDGene database. PLoS Genet. 8 (3), e1002548. doi: 10.1371/journal.pgen.1002548.

Liu, W.K., Le, T.V., Adamson, J., Baker, M., Cookson, N., Hardy, J., et al., (2001). Relationship of the extended tau haplotype to tau biochemistry and neuropathology in progressive supranuclear palsy. Ann. Neurol. 50, 494-502. doi: 10.1002/ana.1159.

Myers,A.J., Kaleem, M., Marlowe, L., Pittman, A.M., Lees, A.J., Fung, H.C., et al., (2005). The H1c haplotype at the MAPT locus is associated with Alzheimer's disease. Hum. Mol. Genet. 14, 2399-2404. doi: 10.1093/hmg/ddi241.

Nombela, C., Rowe, J.B., Winder-Rhodes, S.E., Hampshire, A., Owen, A.M., Breen, D.P., et al., (2014). Genetic impact on cognition and brain function in newly diagnosed Parkinson's disease: ICICLE-PD study. Brain 137, 2743-58. doi: 10.1093/brain/awu201.

Pittman, A.M., Myers, A.J., Abou-Sleiman, P., Fung, H.C., Kaleem, M., Marlowe, L., et al., (2005). Linkage disequilibrium fine mapping and haplotype association analysis of the tau gene in progressive supranuclear palsy and corticobasal degeneration. J. Med. Genet. 42, 837-46. doi:10.1136/jmg.2005.031377.

Pittman, A.M., Fung, H.C., de Silva, R. (2006). Untangling the tau gene association with neurodegenerative disorders. Hum. Mol. Genet. 15, R188-95. doi: 10.1093/hmg/ddl190.

Přikrylová Vranová, H., Mareš, J., Hluštík, P., Nevrlý, M., Stejskal, D., Zapletalová, J., et al., (2012). Tau protein and beta-amyloid(1-42) CSF levels in different phenotypes of Parkinson's disease. J. Neural Transm. 119, 353-62. doi: 10.1007/s00702-011-0708-4.

Refenes, N., Bolbrinker, J., Tagaris, G., Orlacchio, A., Drakoulis, N., Kreutz, R. (2009). Role of the h1 haplotype of microtubule-associated protein tau (MAPT) gene in Greek patients with Parkinson's disease. BMC Neurology 9: 26. doi: 10.1186/1471-2377-9-26.

Rosenberg-Katz K., Herman, T., Jacob, Y., Giladi, N., Hendler, T., Hausdorff, J.M. (2013).
Gray matter atrophy distinguishes between Parkinson disease motor subtypes. Neurology 80, 1476-84. doi: 10.1212/WNL.0b013e31828cfaa4.

Selikhova, M., Williams, D.R., Kempster, P.A., Holton, J.L., Revesz, T., Lees, A.J. (2009). A clinico-pathological study of subtypes in Parkinson's disease. Brain 132, 2947–57. doi: 10.1093/brain/awp234.

Selikhova, M., Kempster, P.A., Revesz,T., Holton, J.L., Lees, A.J. (2013). Neuropathological findings in benign tremulous parkinsonism. Mov. Disord. 28, 145-52. doi: 10.1002/mds.25220.

Seto-Salvia, N., Clarimon, J., Pagonabarraga, J., Pascual-Sedano, B., Campolongo, A., Combarros, O., et al., (2011). Dementia risk in Parkinson disease: disentangling the role of MAPT haplotypes. Arch. Neurol. 68, 359-64. doi: 10.1001/archneurol.2011.17.

Shulman, J.M., Yu, L., Buchman, A.S., Evans, D.A., Schneider, J.A., Bennett, D.A., et al., (2014). Association of Parkinson disease risk loci with mild parkinsonian signs in older persons. JAMA Neurol. 71, 429-35. doi: 10.1001/jamaneurol.2013.6222.

Simón-Sánchez, J., Schulte, C., Bras, J.M., Sharma, M., Gibbs, J.R., Berg, D., et al., (2009). Genome-wide association study reveals genetic risk underlying Parkinson's disease . Nat. Genet. 41, 1308-12. doi: 10.1038/ng.487.

Solla, P., Cadeddu, C., Cannas, A., Deidda, M., Mura, N., Mercuro, G., et al., (2015). Heart rate variability shows different cardiovascular modulation in Parkinson's disease patients with tremor dominant subtype compared to those with akinetic rigid dominant subtype. J Neural Transm. 122(10):1441-6. doi: 10.1007/s00702-015-1393-5.

Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A., Ghetti, B. (1998). Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. Proc. Natl. Acad. Sci. U. S. A. 95, 7737–41.

Tobin, J. E., Latourelle, J. C., Lew, M. F., Klein, C., Suchowersky, O., Shill, H. A., et al., (2008). Haplotypes and gene expression implicate the MAPT region for Parkinson disease: the GenePD Study. Neurology 71, 28-34. doi: 10.1212/01.wnl.0000304051.01650.23.

Vandrovcova, J., Pittman, A.M., Malzer, E., Abou-Sleiman, P.M., Lees, A.J., Wood, N.W., et al., (2009). Association of MAPT haplotype-tagging SNPs with sporadic Parkinson's disease. Neurobiol. Aging 30, 1477-82. doi:10.1016/j.neurobiolaging.2007.11.019.

Wang, G., Huang, Y., Chen, W., Chen, S., Wang, Y., Xiao, Q., et al. (2016). Variants in the SNCA gene associate with motor progression while variants in the MAPT gene associate with the severity of Parkinson's disease. Park. Relat. Disord. 24, 89-94. doi: 10.1016/j.parkreldis.2015.12.018.

Wider, C., Ross, O.A., Nishioka, K., Heckman, M.G., Vilariño-Güell, C., Jasinska-Myga, B., et al., (2012). An evaluation of the impact of MAPT, SNCA and APOE on the burden of Alzheimer's and Lewy body pathology. J. Neurol. Neurosurg. Psychiatry 83, 424-9. doi: 10.1136/jnnp-2011-301413.

Williams-Gray, C.H., Evans, J.R., Goris, A., Foltynie, T., Ban, M., Robbins, T.W., et al., (2009). The distinct cognitive syndromes of Parkinson's disease: 5 year follow-up of the CamPalGN cohort. Brain132, 2958–2969.

Winder-Rhodes, S.E., Hampshire, A., Rowe, J.B., Peelle, J.E., Robbins, T.W., Owen, A.M., et al., (2015). Association between MAPT haplotype and memory function in patients with Parkinson's disease and healthy aging individuals. Neurobiol. Aging 36, 1519-28. doi: 10.1093/brain/awp245.

Winkler, S., Konig, I.R., Lohmann-Hedrich, K., Vieregge, P., Kostic, V., Klein, C. (2007). Role of ethnicity on the association of MAPT H1 haplotypes and subhaplotypes in Parkinson's disease. Eur. J. Hum. Genet. 15, 1163-68. doi:10.1038/sj.ejhg.5201901.

Zabetian, C.P., Hutter, C.M., Factor, S.A., Nutt, J.G., Higgins, D.S., Griffith, A., et al., (2007).
Association analysis of MAPT H1 haplotype and subhaplotypes in Parkinson's disease. Ann. Neurol. 62, 137–44. doi: 10.1002/ana.21157.

Zhang, Y.H., Tang, B.S., Song, C.Y., Xu, Q., Lou, M.X., Liu, Z.H., et al., (2013). The relationship between the phenotype of Parkinson's disease and levodopa-induced dyskinesia. Neurosci. Lett. 556, 109-12. doi: 10.1016/j.neulet.2013.10.018.

<u>Clinical setting</u>: Intercepting Parkinson disease nonmotor subtypes: a proof-of-principle study in a clinical setting.

Marras C, Chaudhuri KR.
Nonmotor features of Parkinson's disease subtypes.

Mov Disord. 2016 Aug;31(8):1095-102. doi: 10.1002/mds.26510. Review.

Kadastik-Eerme L, Muldmaa M, Lilles S, Rosenthal M, Taba N, Taba P.
Nonmotor Features in Parkinson's Disease: What Are the Most Important Associated Factors?
Parkinsons Dis. 2016;2016:4370674. doi: 10.1155/2016/4370674.

Khoo TK, Yarnall AJ, Duncan GW, Coleman S, O'Brien JT, Brooks DJ, Barker RA, Burn DJ.
The spectrum of nonmotor symptoms in early Parkinson disease.
Neurology. 2013 Jan 15;80(3):276-81. doi: 10.1212/WNL.0b013e31827deb74.

Müller B, Larsen JP, Wentzel-Larsen T, Skeie GO, Tysnes OB; Parkwest Study Group.
Autonomic and sensory symptoms and signs in incident, untreated Parkinson's disease: frequent but mild.
Mov Disord. 2011 Jan;26(1):65-72. doi: 10.1002/mds.23387. Epub 2010 Oct 5.

Robakis D, Cortes E, Clark LN, Vonsattel JP, Virmani T, Alcalay RN, Crary JF, Levy OA.The effect of MAPT haplotype on neocortical Lewy body pathology in Parkinson disease.
J Neural Transm (Vienna). 2016 Jun;123(6):583-8. doi: 10.1007/s00702-016-1552-3.

Wang G, Huang Y, Chen W, Chen S, Wang Y, Xiao Q, Liu J, Fung VS, Halliday G, Chen S.
Variants in the SNCA gene associate with motor progression while variants in the MAPT gene associate with the severity of Parkinson's disease.
Parkinsonism Relat Disord. 2016 Mar;24:89-94. doi: 10.1016/j.parkreldis.2015.12.018

Davis AA, Andruska KM, Benitez BA, Racette BA, Perlmutter JS, Cruchaga C.
Variants in GBA, SNCA, and MAPT influence Parkinson disease risk, age at onset, and progression.
Neurobiol Aging. 2016 Jan;37:209.e1-7. doi: 10.1016/j.neurobiolaging.2015.09.014

Castrioto A, Thobois S, Carnicella S, Maillet A, Krack P.
Emotional manifestations of PD: Neurobiological basis.
Mov Disord. 2016 Aug;31(8):1103-13. doi: 10.1002/mds.26587.

Pirogovsky-Turk E, Moore RC, Filoteo JV, Litvan I, Song DD, Lessig SL, Schiehser DM.
Neuropsychiatric Predictors of Cognitive Decline in Parkinson Disease: A Longitudinal Study.
Am J Geriatr Psychiatry. 2016 Oct 12. pii: S1064-7481(16)30270-6. doi: 10.1016/j.jagp.2016.10.004.

Setó-Salvia N, Clarimón J, Pagonabarraga J, Pascual-Sedano B, Campolongo A, Combarros O, Mateo JI, Regaña D, Martínez-Corral M, Marquié M, Alcolea D, Suárez-Calvet M, Molina-Porcel L, Dols O, Gómez-Isla T, Blesa R, Lleó A, Kulisevsky J.
Dementia risk in Parkinson disease: disentangling the role of MAPT haplotypes.
Arch Neurol. 2011 Mar;68(3):359-64. doi: 10.1001/archneurol.2011.17.

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. doi: 10.1136/jnnp-2013-305277.

Martinez-Martin P, Leentjens AF, de Pedro-Cuesta J, Chaudhuri KR, Schrag AE, Weintraub D.
Accuracy of screening instruments for detection of neuropsychiatric syndromes in Parkinson's disease.
Mov Disord. 2016 Mar;31(3):270-9. doi: 10.1002/mds.26522.

Pandey S, Bajaj BK, Wadhwa A, Anand KS.
Impact of sleep quality on the quality of life of patients with Parkinson's disease: a questionnaire based study.
Clin Neurol Neurosurg. 2016 Sep;148:29-34. doi: 10.1016/j.clineuro.2016.06.014

Ylikoski A, Martikainen K, Sieminski M, Partinen M.
Sleeping difficulties and health-related quality of life in Parkinson's disease.
Acta Neurol Scand. 2016 Jun 10. doi: 10.1111/ane.12620. [Epub ahead of print

Kotagal V.
Is PIGD a legitimate motor subtype in Parkinson disease?
Ann Clin Transl Neurol. 2016 May 11;3(6):473-7. doi: 10.1002/acn3.312.

Simuni T, Caspell-Garcia C, Coffey C, Lasch S, Tanner C, Marek K; PPMI Investigators.
How stable are Parkinson's disease subtypes in de novo patients: Analysis of the PPMI cohort?
Parkinsonism Relat Disord. 2016 Jul;28:62-7. doi: 10.1016/j.parkreldis.2016.04.027.

Ba F, Obaid M, Wieler M, Camicioli R, Martin WR.
Parkinson Disease: The Relationship Between Non-motor Symptoms and Motor Phenotype.
Can J Neurol Sci. 2016 Mar;43(2):261-7. doi: 10.1017/cjn.2015.328.

Fitts W, Weintraub D, Massimo L, Chahine L, Chen-Plotkin A, Duda JE, Hurtig HI, Rick J, Trojanowski JQ, Dahodwala N.
Caregiver report of apathy predicts dementia in Parkinson's disease.
Parkinsonism Relat Disord. 2015 Aug;21(8):992-5. doi: 10.1016/j.parkreldis.2015.06.009.

Martínez-Horta S, Pagonabarraga J, Fernández de Bobadilla R, García-Sanchez C, Kulisevsky J.
Apathy in Parkinson's disease: more than just executive dysfunction.
J Int Neuropsychol Soc. 2013 May;19(5):571-82. doi: 10.1017/S1355617713000131

Monastero R, Di Fiore P, Ventimiglia GD, Camarda R, Camarda C.
The neuropsychiatric profile of Parkinson's disease subjects with and without mild cognitive impairment.
J Neural Transm (Vienna). 2013 Apr;120(4):607-11. doi: 10.1007/s00702-013-0988-y.

Winder-Rhodes SE, Hampshire A, Rowe JB, Peelle JE, Robbins TW, Owen AM, Barker RA.
Association between MAPT haplotype and memory function in patients with Parkinson's disease and healthy aging individuals.
Neurobiol Aging. 2015 Mar;36(3):1519-28. doi: 10.1016/j.neurobiolaging.2014.12.006

<u>Experimental setting II:</u> DAT Methylation in Parkinson Disease: Correlation with peripheral Dopamine Transporter gene expression and central activity - DAT

Uhl GR.
Dopamine transporter: basic science and human variation of a key molecule for dopaminergic function, locomotion, and parkinsonism.
Mov Disord. 2003 Oct;18 Suppl 7:S71-80. Review

Torres GE, Gainetdinov RR, Caron MG
Plasma membrane monoamine transporters: structure, regulation and function.
Nat Rev Neurosci. 2003 Jan;4(1):13-25.

Kitayama S, Mitsuhata C, Davis S, Wang JB, Sato T, Morita K, Uhl GR, Dohi T.

MPP+ toxicity and plasma membrane dopamine transporter: study using cell lines expressing the wild-type and mutant rat dopamine transporters.
Biochim Biophys Acta. 1998 Sep 16;1404(3):305-13.

Vandenbergh DJ, Persico AM, Hawkins AL, Griffin CA, Li X, Jabs EW, Uhl GR.
Human dopamine transporter gene (DAT1) maps to chromosome 5p15.3 and displays a VNTR.
Genomics. 1992 Dec;14(4):1104-6

Drgon T, Lin Z, Wang GJ, Fowler J, Pablo J, Mash DC, Volkow N, Uhl GR.
Common human 5' dopamine transporter (SLC6A3) haplotypes yield varying expression levels in vivo.
Cell Mol Neurobiol. 2006 Jul-Aug;26(4-6):875-89. Epub 2006 May 20.

Fuke S, Suo S, Takahashi N, Koike H, Sasagawa N, Ishiura S.
The VNTR polymorphism of the human dopamine transporter (DAT1) gene affects gene expression.
Pharmacogenomics J. 2001;1(2):152-6.

van de Giessen E, de Win MM, Tanck MW, van den Brink W, Baas F, Booij J.
Striatal dopamine transporter availability associated with polymorphisms in the dopamine transporter gene SLC6A3.
J Nucl Med. 2009 Jan;50(1):45-52. doi: 10.2967/jnumed.108.053652.

Costa A, Riedel M, Müller U, Möller HJ, Ettinger U.
Relationship between SLC6A3 genotype and striatal dopamine transporter availability: a meta-analysis of human single photon emission computed tomography studies.
Synapse. 2011 Oct;65(10):998-1005. doi: 10.1002/syn.20927. Review.

Uhl GR.
Hypothesis: the role of dopaminergic transporters in selective vulnerability of cells in Parkinson's disease.
Ann Neurol. 1998 May;43(5):555-60.

Counihan TJ, Penney JB Jr.
Regional dopamine transporter gene expression in the substantia nigra from control and Parkinson's disease brains.
J Neurol Neurosurg Psychiatry. 1998 Aug;65(2):164-9.

Güzey C, Allard P, Brännström T, Spigset O.
Radioligand binding to brain dopamine and serotonin receptors and transporters in Parkinson's disease: relation to gene polymorphisms.
Int J Neurosci. 2012 Mar;122(3):124-32. doi: 10.3109/00207454.2011.631716. Epub 2011 Dec 12.

Higuchi S, Muramatsu T, Arai H, Hayashida M, Sasaki H, Trojanowski JQ.
Polymorphisms of dopamine receptor and transporter genes and Parkinson's disease.
J Neural Transm Park Dis Dement Sect. 1995;10(2-3):107-13.

Le Couteur DG, Leighton PW, McCann SJ, Pond Sm.
Association of a polymorphism in the dopamine-transporter gene with Parkinson's disease.
Mov Disord. 1997 Sep;12(5):760-3.

Kim JW, Kim DH, Kim SH, Cha JK.
Association of the dopamine transporter gene with Parkinson's disease in Korean patients.
J Korean Med Sci. 2000 Aug;15(4):449-51.

A single nucleotide polymorphism of dopamine transporter gene is associated with Parkinson's disease.

Morino H, Kawarai T, Izumi Y, Kazuta T, Oda M, Komure O, Udaka F, Kameyama M, Nakamura S, Kawakami H.
Ann Neurol. 2000 Apr;47(4):528-31

Lin CN, Liu HC, Tsai SJ, Liu TY, Hong CJ.
Association study for Parkinson's disease and a dopamine transporter gene polymorphism (1215A/G).
Eur Neurol. 2002;48(4):207-9.

Kimura M, Matsushita S, Arai H, Takeda A, Higuchi S.
No evidence of association between a dopamine transporter gene polymorphism (1215A/G) and Parkinson's disease.
Ann Neurol. 2001 Feb;49(2):276-7.
Zhai D, Li S, Zhao Y, Lin Z.
SLC6A3 is a risk factor for Parkinson's disease: a meta-analysis of sixteen years' studies.
Neurosci Lett. 2014 Apr 3;564:99-104. doi: 10.1016/j.neulet.2013.10.060. Epub 2013 Nov 7.

Harrington KA, Augood SJ, Kingsbury AE, Foster OJ, Emson PC.
Dopamine transporter (Dat) and synaptic veside amine transporter (VMAT2) gene expression in the substantia nigra of control and Parkinson's disease.
Brain Res Mol Brain Res. 1996 Feb;36(1):157-62

Innis RB, Seibyl JP, Scanley BE, Laruelle M, Abi-Dargham A, Wallace E, Baldwin RM, Zea-Ponce Y, Zoghbi S, Wang S, et al.
Single photon emission computed tomographic imaging demonstrates loss of striatal dopamine transporters in Parkinson disease.
Proc Natl Acad Sci U S A. 1993 Dec 15;90(24):11965-9.

Booij J, Tissingh G, Boer GJ, Speelman JD, Stoof JC, Janssen AG, Wolters EC, van Royen EA.
[123I]FP-CIT SPECT shows a pronounced decline of striatal dopamine transporter labelling in early and advanced Parkinson's disease.
J Neurol Neurosurg Psychiatry. 1997 Feb;62(2):133-40.

Brooks DJ.
Imaging dopamine transporters in Parkinson's disease.
Biomark Med. 2010 Oct;4(5):651-60. doi: 10.2217/bmm.10.86. Review

Troiano AR, de la Fuente-Fernandez R, Sossi V, Schulzer M, Mak E, Ruth TJ, Stoessl AJ.
PET demonstrates reduced dopamine transporter expression in PD with dyskinesias.
Neurology. 2009 Apr 7;72(14):1211-6. doi: 10.1212/01.wnl.0000338631.73211.56. Epub 2008 Nov 19.

Hong JY1, Oh JS, Lee I, Sunwoo MK, Ham JH, Lee JE, Sohn YH, Kim JS, Lee PH.
Presynaptic dopamine depletion predicts levodopa-induced dyskinesia in de novo Parkinson disease.
Neurology. 2014 May 6;82(18):1597-604. doi: 10.1212/WNL.0000000000000385. Epub 2014 Apr 9.

Altmann V, Schumacher-Schuh AF, Rieck M, Callegari-Jacques SM, Rieder CR, Hutz MH.
Influence of genetic, biological and pharmacological factors on levodopa dose in Parkinson's disease.
Pharmacogenomics. 2016 Apr;17(5):481-8. doi: 10.2217/pgs.15.183.

Kaplan N, Vituri A, Korczyn AD, Cohen OS, Inzelberg R, Yahalom G, Kozlova E, Milgrom R, Laitman Y, Friedman E, Rosset S, Hassin-Baer S.
Sequence variants in SLC6A3, DRD2, and BDNF genes and time to levodopa-induced dyskinesias in Parkinson's disease.
J Mol Neurosci. 2014 Jun;53(2):183-8. doi: 10.1007/s12031-014-0276-9.

Contin M, Martinelli P, Mochi M, Albani F, Riva R, Scaglione C, Dondi M, Fanti S, Pettinato C, Baruzzi A.
Dopamine transporter gene polymorphism, spect imaging, and levodopa response in patients with Parkinson disease.
Clin Neuropharmacol. 2004 May-Jun;27(3):111-5.

Zhao Y, Xiong N, Liu Y, Zhou Y, Li N, Qing H, Lin Z.
Human dopamine transporter gene: differential regulation of 18-kb haplotypes.
Pharmacogenomics. 2013 Sep;14(12):1481-94. doi: 10.2217/pgs.13.141.

Lu H, Liu X, Deng Y, Qing H.
DNA methylation, a hand behind neurodegenerative diseases.
Front Aging Neurosci. 2013 Dec 5;5:85.

Moreau C, Meguig S, Corvol JC, Labreuche J, Vasseur F, Duhamel A, Delval A, Bardyn T, Devedjian JC, Rouaix N, Petyt G, Brefel-Courbon C, Ory-Magne F, Guehl D, Eusebio A, Fraix V, Saulnier PJ, Lagha-Boukbiza O, Durif F, Faighel M, Giordana C, Drapier S, Maltête D, Tranchant C, Houeto JL, Debû B, Azulay JP9, Tison F8, Destée A, Vidailhet M, Rascol O, Dujardin K, Defebvre L, Bordet R, Sablonnière B, Devos D; Parkgait-II Study Group.
Polymorphism of the dopamine transporter type 1 gene modifies the treatment response in Parkinson's disease.
Brain. 2015 May;138(Pt 5):1271-83. Epub 2015 Mar 23.

Brooks DJ.
Molecular imaging of dopamine transporters.
Ageing Res Rev. 2016 Jan 21. [Epub ahead of print] Review

Fanciulli A, Misasi R, Campanelli D, Buttarelli FR, Pontieri FR.
Dopaminergic drug-induced modulation of the expression of the dopamine transporter in peripheral blood lymphocytes in Parkinson's disease.
Pharmacol Rep. 2011;63(4):1056-60.

Buttarelli FR, Capriotti G, Pellicano C, Prosperi D, Circella A, Festa A, Giovannelli M et al.:
Central and peripheral dopamine transporter reduction in Parkinson's disease
Neurol Res, 2009, 31, 687–691

Buttarelli FR, Fanciulli A, Pellicano C, Pontieri FE.
The dopaminergic system in peripheral blood lymphocytes: from physiology to pharmacology and potential applications to neuropsychiatric disorders.
Curr Neuropharmacol. 2011 Jun;9(2):278-88.