UNIVERSITAT DE BARCELONA

Biomarker studies in LRRK2 associated Parkinson's disease

Alicia Garrido Pla

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Biomarker studies in LRRK2 associated Parkinson's disease

Directed by:

Eduard Tolosa Sarró, MD, PhD

Emeritus Professor

Universitat de Barcelona

Maria J Martí Domenech, MD, PhD

Consultant Neurologist

Parkinon's disease and other Movement Disorders Unit

Hospital Clínic Barcelona

Universitat de Barcelona

Tutor:

Francesc Valldeoriola Serra, MD, PhD

Associate Professor of Medicine

Consultant Neurologist

Director, Parkinon's disease and other Movement Disorders Unit

Hospital Clínic Barcelona

Universitat de Barcelona

Doctoral Program in Medicine and Translational Research

School of Medicine and Health Sciences. University of Barcelona

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Abbreviations and acronyms:

AD - Alzheimer's Disease

α-syn - α-synuclein protein

CSF - Cerebrospinal Fluid

DAT-SPECT - Dopamine Transporter Single Photon Emission Computed Tomography

DNM3 - Dynamin 3 gene

GWAS - Genome-Wide Association Study

IPD - Idiopathic Parkinson's disease

LRRK2 (gene) - Leucine-rich repeat kinase 2 gene

LRRK2 (protein) - Leucine-rich repeat kinase 2 protein

LRRK2-PD - LRRK2 associated Parkinson's disease

MDS - Movement Disorder Society

MRI - Magnetic Resonance Imaging

NMC- Non-manifesting carriers

PD - Parkinson's disease

PET - Positron Emission Tomography

PMCA - Protein Misfolding Cyclic Amplification

RBD - REM Sleep Behavior Disorder

RT-QuIC - Real-Time Quaking-Induced Conversion

SAA - Seeding Amplification Assay

SNCA - α-synuclein gene

SNP - Single Nucleotide Polymorphism

TDP-43 - TAR DNA-binding protein 43

UPSIT - University of Pennsylvania Smell Identification Test

Table of contents:

Thesis in compendium of publications format

This thesis is presented as a compendium of four articles on biomarker studies in LRRK2 associated Parkinson's disease (LRRK2-PD). The work is structured around four key objectives, each addressed in a published scientific article.

The first objective is to assess the impact of single nucleotide polymorphisms on the penetrance of LRRK2-PD. This is explored in Article 1: *α-synuclein (SNCA) but not dynamin 3 (DNM3) influences age at onset of leucine-rich repeat kinase 2 (LRRK2) Parkinson's disease in Spain.*

Article 1 - Garrido A*, Fernández-Santiago R*, Infante J, González-Aramburu I, Sierra M, Fernández M, Valldeoriola F, Muñoz E, Compta Y, Martí MJ, Ríos J, Tolosa E, Ezquerra M; Barcelona LRRK2 Study Group†. α-synuclein (SNCA) but not dynamin 3 (DNM3) influences age at onset of leucine-rich repeat kinase 2 (LRRK2) Parkinson's disease in Spain. Mov Disord. 2018 Apr;33(4):637-641. Rank: Q1 (D1). IF: 8.222

The second objective is to investigate the role of inflamation in the central nervous system during the pre-clinical stages of LRRK2-PD, using PET imaging. This is addressed in Article 2: *Imaging dopamine function and microglia in asymptomatic LRRK2 mutation carriers*.

Article 2 - Garrido A*, Gersel Stokholm M*, Tolosa E, Serradell M, Iranzo A, Østergaard K, Borghammer P, Møller A, Parbo P, Stær K, Brooks DJ, Martí MJ, Pavese N. Imaging dopamine function and microglia in asymptomatic *LRRK2* mutation carriers. J Neurol. 2020 Aug;267(8):2296-2300. Rank: Q1. IF: 3,956

The third and four objectives focus on the use of novel α-synuclein seeding amplification assays in *LRRK2* mutation carriers. First, by detecting α-synuclein aggregates in cerebrospinal fluid, and then applying the same assay to postmortem brain and cerebrospinal fluid samples of LRRK2-PD cases, to assess the test's ability to identify Lewy body pathology. These objectives are explored in Article 3: *α-synuclein RT-QuIC in cerebrospinal fluid of LRRK2-linked Parkinson's disease*, and Article 4: *α-synuclein Real-Time Quaking-Induced Conversion Identifies Lewy Body Pathology in LRRK2-PD.*

Article 3 - Garrido A, Fairfoul G, Tolosa ES, Martí MJ, Green A; Barcelona LRRK2 Study Group. α-synuclein RT-QuIC in cerebrospinal fluid of LRRK2-linked Parkinson's disease. Ann Clin Transl Neurol. 2019 May 9;6(6):1024-1032. Rank: Q1. IF: 3,660

Article 4 - Garrido A, Fairfoul G, Tolosa E, Marti MJ, Ezquerra M, Green AJE. Brain and Cerebrospinal Fluid α-synuclein Real-Time Quaking-Induced Conversion Identifies Lewy Body Pathology in LRRK2-PD. Mov Disord. 2023 Feb;38(2):333-338. Rank: Q1 (D1). IF: 10.338

Abstract:

Title: Biomarker studies in LRRK2 associated Parkinson's disease

Introduction: Mutations in the *LRRK2* (*leucine-rich repeat kinase-2*) gene are the most frequent cause of familial Parkinson's disease (PD) and are also responsible for a significant percentage of sporadic cases. The proportion of subjects with overt PD among carriers of the most frequent *LRRK2* pathogenic variants in our population (p.G2019S, and p.R1441G), has been reported from 20-40% at the age of 80 years, and the only well-established risk factor until date for an increased penetrance of *LRRK2* mutations is age. As in idiopathic PD (IPD), neurodegeneration in LRRK2-associated PD (LRRK2-PD) is thought to begin years before the onset of classical motor symptoms. Mechanisms triggering *LRRK2* disease development are unknown, and there are no biomarkers of ongoing disease in LRRK2-PD, nor for predicting the onset of manifest disease. The discovery of such biomarkers is an instrumental first step to understand the mechanisms underlying neurodegeneration in LRRK2-PD and eventual implementation of disease modification trials that could delay or prevent the development of manifest parkinsonism.

Hypothesis and objectives: Using an established cohort of *LRRK2* mutation carriers both symptomatic and asymptomatic, identified in our department, this PhD thesis aimed at identifying LRRK2-PD disease mechanisms and biomarkers of underlying pathology.

Methods: Our first objective was to assess genetic risk factors that could modulate penetrance of *LRRK2* mutations. We examined the impact of polymorphisms in the *DNM3* and *SNCA* genes on the age of onset of LRRK2-PD in a multicentre cohort of subjects carrying the G2019S mutation, with the results published in the first article of this thesis. Next, we investigated the potential role of central nervous system inflammation in LRRK2-PD, focusing on microglial activation in asymptomatic *LRRK2* mutation carriers through positron emitted tomography (PET) imaging, as detailed in the second article. Lastly, we aimed to assess the presence of α-synuclein (α-syn) aggregates in cerebrospinal fluid (CSF) and brain tissue of *LRRK2* mutation carriers using real-teal quacking induced conversion (RT-QuIC), a novel amplification assay to detect missfolded proteins. Two additional articles present the findings of this work.

Results: The key findings from these studies are as follows: (i) Polymorphisms within the *SNCA* gene, but not *DNM3* gene, contribute to penetrance of LRRK2-PD within the Spanish population. (ii) PET imaging in the preclinical stages of LRRK2-PD reveal activation of microglia, indicative of neuroinflammation, which may be preceded by dopaminergic degeneration. (iii) α-syn RT-QuIC detects abnormal aggregates of α-syn in CSF of patients with LRRK2-PD, albeit less frequently than in IPD. A subset of asymptomatic carriers with the G2019S *LRRK2* mutation exhibits abnormal aggregates of α-syn in CSF. These individuals present higher clinical scores for probable prodromal PD. α-syn RT-QuIC results in the postmortem CSF and brain tissue of LRRK2-PD patients reflect the underlying neuropathology in the cases studied. The lower frequency of positive α-syn RT-QuIC in CSF collected in vivo from G2019S LRRK2-PD patients can be attributed to the common absence of neuronal synuclein disease in this genetic form of PD.

Conclusions: Collectively, the information from these four articles, centered on LRRK2-PD, and which form the main body of this thesis, has advanced our understanding on the genetic factors involved in modulation of LRRK2-PD expression and on pathophysiological changes occurring in the early disease stages. Applying α-syn seeding amplification assay - RT-QuIC - we have identifiend a subgroup of mutation carriers, manifest and asymptomatic, that have α-syn aggregates in the CSF. In the final paper of this thesis, we demonstrate that RT-QuIC identifies with high sensitivity and specificity α-syn aggregates in CSF and brains of LRRK2-PD patients.

Abstract in Spanish:

Título: Estudios de biomarcadores en la enfermedad de Parkinson asociada a LRRK2

Introducción: Las mutaciones en el gen *LRRK2* (quinasa 2 con repeticiones ricas en leucina) son la causa más frecuente de enfermedad de Parkinson (EP) familiar y también son responsables de un porcentaje significativo de casos esporádicos. La proporción de sujetos con EP manifiesta entre los portadores de las variantes patogénicas más frecuentes de *LRRK2* en nuestra población (p.G2019S y p.R1441G) se ha reportado en un 20-40% a la edad de 80 años, y el único factor de riesgo bien establecido hasta la fecha para una mayor penetrancia de las mutaciones de *LRRK2* es la edad. Al igual que en la EP idiopática (EPI), se cree que la neurodegeneración en la EP asociada a LRRK2 (EP-LRRK2) comienza años antes del inicio de los síntomas motores clásicos. Los mecanismos que desencadenan el desarrollo de la enfermedad LRRK2 son desconocidos y no existen biomarcadores de la EP-LRRK2 en curso, ni biomarcadores para predecir la aparición de la enfermedad manifiesta. El descubrimiento de tales biomarcadores es un primer paso fundamental para comprender los mecanismos subyacentes a la neurodegeneración en la EP-LRRK2 y la eventual implementación de terapias modificadoras de la enfermedad que podrían retrasar o prevenir el desarrollo del parkinsonismo.

Hipótesis y objetivos: Utilizando una cohorte establecida de portadores de mutaciones *LRRK2* tanto sintomáticos como asintomáticos, identificados en nuestro departamento, esta tesis doctoral tuvo como objetivo identificar los mecanismos de la enfermedad EP-LRRK2 y los biomarcadores de la patología subyacente.

Métodos: Nuestro primer objetivo fue evaluar los factores de riesgo genéticos que podrían modular la penetrancia de las mutaciones *LRRK2*. Examinamos el impacto de los polimorfismos en los genes *DNM3* y *SNCA* sobre la edad de inicio de EP-LRRK2 en una cohorte multicéntrica de sujetos portadores de la mutación G2019S, con los resultados publicados en el primer artículo de esta tesis. A continuación, investigamos el papel potencial de la inflamación del sistema nervioso central en la EP-LRRK2, enfocándonos en la activación de la microglía en portadores asintomáticos de mutaciones *LRRK2* utilizando imágenes de tomografía por emisión de positrones (PET), como se detalla en el segundo artículo. Por

último, evaluamos la presencia de agregados de α-sinucleína (α-syn) en el líquido cefalorraquídeo (LCR) y el tejido cerebral de portadores de mutaciones *LRRK2* utilizando la técnica RT-QuIC (*Real-Time QUaking-Induced Conversion*), un nuevo método de amplificación para detectar proteínas mal plegadas. Dos artículos adicionales presentan los hallazgos de este trabajo.

Resultados: Los hallazgos principales de estos estudios son los siguientes: (i) Los polimorfismos dentro del gen *SNCA*, pero no del gen *DNM3*, contribuyen a la penetrancia de EP-LRRK2 en la población española. (ii) Las imágenes PET en las etapas preclínicas de EP-LRRK2 revelan activación de la microglía, indicativa de neuroinflamación, que puede ser precedida por la degeneración dopaminérgica. (iii) El test de amplificación de α-syn, α-syn RT-QuIC, detecta agregados anormales de α-syn en el LCR de pacientes con EP-LRRK2, aunque con menos frecuencia que en la EPI. Un subconjunto de portadores asintomáticos con la mutación *LRRK2* G2019S presenta agregados anormales de α-syn en el LCR. En estos individuos se observan puntuaciones clínicas más altas para la EP prodrómica probable. Los resultados de α-syn RT-QuIC en el LCR y tejido cerebral postmortem de pacientes con EP-LRRK2 reflejan la neuropatología subyacente en los casos estudiados. La menor frecuencia de un test α-syn RT-QuIC positivo en el LCR recogido in vivo en los pacientes con EP-LRRK2, puede atribuirse a frecuente ausencia de patología por sinuclena neuronal observada en esta forma genética de EP.

Conclusiones: En conjunto, la información de estos cuatro artículos, centrados en EP-LRRK2, y que forman el cuerpo principal de esta tesis, ha aumentado nuestro conocimiento sobre los factores genéticos involucrados en la modulación de la expresión de EP-LRRK2 y sobre los cambios fisiopatológicos que ocurren en las primeras etapas de la enfermedad. Aplicando el test de amplificación de α-syn - RT-QuIC - hemos identificado un subgrupo de portadores de mutaciones, tanto manifiestos como asintomáticos, que tienen agregados de α-syn en el LCR. En el último artículo de esta tesis, demostramos que RT-QuIC identifica con alta sensibilidad y especificidad agregados de α-syn en el LCR y en los cerebros de pacientes con EP-LRRK2.

Introduction

1.1 – Epidemiology and genetics of *LRRK2* mutations

Parkinson's disease (PD) is a chronic, incurable, neurodegenerative disorder that currently affects over 6 million people worldwide. The prevalence of PD has increased 2.5-times over the past three decades, establishing it as the second most prevalent neurodegenerative condition and a significant cause of neurological disability.(1) Advancing age is the primary risk factor for developing PD, and men are more likely to be affected than women, with a prevalence ratio of about 3:2.(1)

The cause of PD is complex and multifactorial and is only known in those cases where a pathogenic genetic mutation is detected. Since the description of the first mutations in the *SNCA* gene in 1997,(2) genetic studies have identified more than 20 genes associated with familial PD and 90 risk loci.(3–7) Among the genes with pathogenic mutations, one of the most relevant for its contribution to PD risk is the *leucine-rich repeat kinase 2* (*LRRK2*) gene.

The locus corresponding to *LRRK2*, was originally described in 2002 in the Sagamihara family in Japan,(8) followed in 2004 by the identification of causal mutations with Mendelian segregation of the disease in familial cases carrying the R1441C, Y1699C, and I2020T mutations.(9–11) Since then, six pathogenic mutations that co-segregate in an autosomal dominant fashion with the disease (N1437H, R1441G/H/C, Y1699C, G2019S, I2012T and I2020T) have been deciphered,(12–15) with reported frequencies of LRRK2-PD cases between 0.1 and 40% of all cases with PD, depending on the mutation and the population studied.(12,16–20) Among the pathogenic mutations of the *LRRK2* gene, the G2019S mutation, which is thought to originate from a common North African ancestor and is the most prevalent in the European population, and the R1441G mutation, commonly found in regions near the Basque Country, are the most frequent in the Spanish population.(21,22) In Spain, the reported prevalence of G2019S LRRK2-PD varies widely, ranging from 1 to 23% in familial cases to 1.7 to 6% in sporadic cases.(21–26) Additionally, the prevalence of R1441G mutations spans from 27.1% in familial cases to 4.6% in sporadic cases.(10,21,22,24,25,27,28) (**Table 1**) Apart from the known pathogenic mutations, genome-wide association studies (GWAS) have revealed other more frequent *LRRK2* variants considered risk factors for idiopathic PD (IPD).(3–6)

Table 1 | LRRK2 prevalence studies carried out in Spain.

Study	Patients with PD (All)		Patients with familial PD		Patients with sporadic PD		Mean age onset (+/- SD)
	n	Patients with *LRRK2* mutations (%)	n	Patients with *LRRK2* mutations (%)	n	Patients with *LRRK2* mutations (%)	
G2019S							
Gaig, 2006 (21)	302	13 (4.3)	94	6 (6.4)	208	7 (3.4)	57±13
González-Fernández, 2007 (22)	78	7 (8.9)	61	6 (1)	17	1 (5.8)	57.4±9
Gorostidi, 2009 (28)	418	16 (3.8)	181	10 (5.5)	237	6 (2.5)	58±11.5
Gao, 2009 (24)	187	3 (1.6)	15	0	172	3 (1.7)	52.2±12.6
Infante, 2006 (25) *Sierra, 2011* (26)	105 367	8 (7.6) 32 (8.7)	16 78	3 (18.8) 18 (23)	89 289	5 (6) 14 (4.8)	66.2±7.7 63.2
R1441G							
Paisán-Ruiz, 2004 (10)	137	11 (8)	30	6 (20)	107	5 (4.6)	NA
Mata, 2005 (27)	225	5 (2.2)	50	1 (2)	175	4 (2.3)	63.2 ± 9.8
Infante, 2006 (25)	98	0	16	0	82	0	-
Gaig, 2006 (21)	302	2 (0.7)	94	2 (2.6)	208	0	57±13
González-Fernández, 2007 (22)	78	15 (19.2)	61	15 (24.6)	17	0	54.6±11.7
Gorostidi, 2009 (28)	418	55 (13.1)	181	49 (27.1)	237	6 (2.5)	61.7±8.5
Gao, 2009 (24)	187	3 (1.6)	15	0	172	3 (1.7)	52.2±12.6

|Original Table. PD= Parkinson's disease

The discovery of *LRRK2* mutations as a cause of PD is relevant for several reasons, some of them we outline here:

(i) Similarities to IPD: LRRK2-PD typically manifest as a late-onset parkinsonism indistinguishable from IPD in its clinical manifestation and response to treatment.(12)

(ii) Prevalence and variable geographical distribution: Compared to other monogenic mutations causing PD, LRRK2-PD is common.(16–19) The distribution and frequency of different mutations are clustered on geographical and ethnic subgroups with an estimated prevalence of ~1 % in European familial PD populations, and of ~15-40% in North African Arab Berber or Ashkenazi Jewish populations.(18,19)

(iii) Incomplete penetrance: *LRRK2* mutations exhibit reduced penetrance, which varies across different pupulations.(29–40) The incomplete penetrance of LRRK2-PD suggests the involvement of additional risk factors that must play a role and require further investigation.

(iv) Heterogeneous neuropathology: Neuropathological studies of LRRK2-PD have shown heterogeneous histology, with nigral degeneration occurring with or without the deposition of various proteins. This pleomorphic pathology adds complexity to our understanding of the mechanisms underlying neurodegeneration in LRRK2-PD and, most important, questions the need to define PD based of the presence of α-synuclein (α-syn) aggregates in the brain.(41)

(v) Therapeutic target potential: Pathogenic *LRRK2* mutations increase kinase function,(42–44) making LRRK2 a modifiable therapeutic target.(45–47) The phenotypic and molecular similarities between LRRK2-PD and IPD(45) suggest that therapies targeting LRRK2 could be also effective for treating IPD.

Recent research has significantly enhanced our knowledge on the clinical features and natural history of LRRK2-PD through studies of large patient cohorts.(20) Research has also deepened our comprehension of the mutation's effect on cellular physiology,(48–51) and accelerated the development of promising therapies that target LRRK2.(45–47) At the Parkinson's Disease and Movement Disorders Unit of Hospital Clinic University in Barcelona, a group of approximately 100 LRRK2-PD patients and their relatives has been identified and studied, resulting in numerous scientific publications. These studies have provided information regarding the frequency and clinical characteristics of both manifest and premotor phases,(21,52–55) and provided insights as well into disease mechanisms,(56,57)

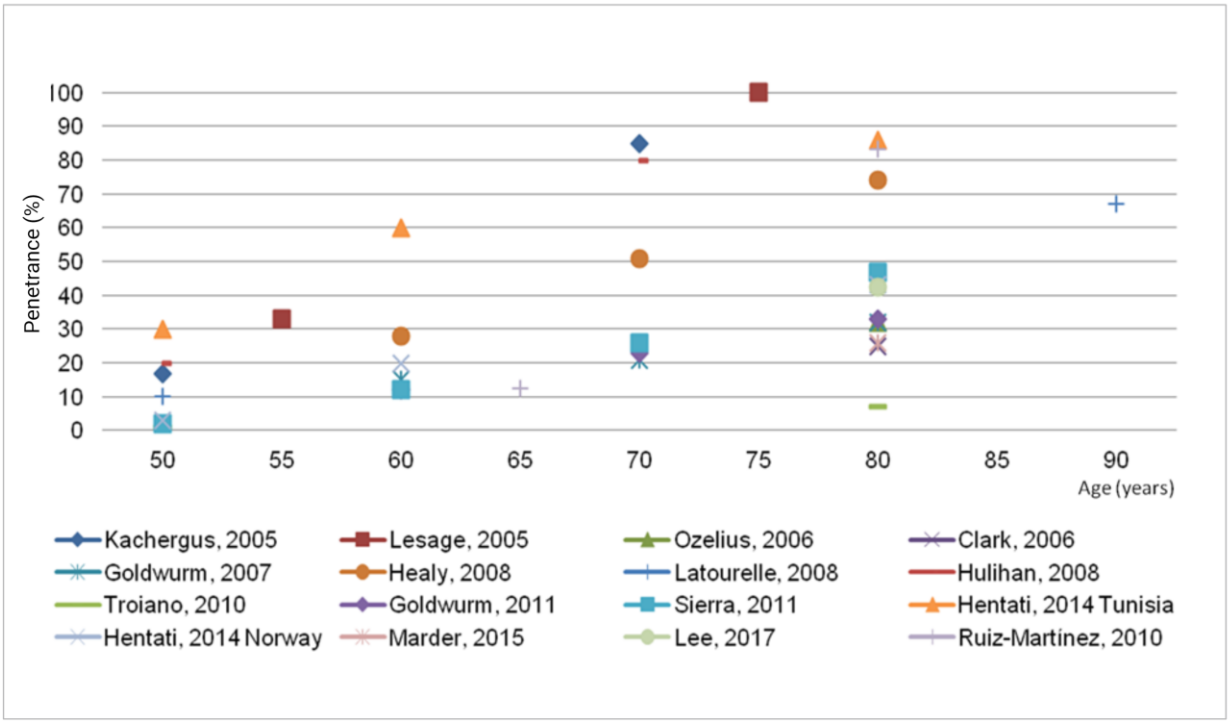
biomarkers,(55,57–61) and neuropathology.(62–65) Significant unanswered questions remain, though, that hinder the progression towards effective disease-modifying treatments. First, there is little information available on the various factors that influence the incomplete penetrance of *LRRK2* mutations. This lack of information prevents the identification of individuals who might be at potential premotor stages, making it challenging to enrroll them in disease modification trials aimed at early intervention. Second, the exact mechanisms that underlie neurodegeneration and the associated variable neuropathology are not well understood. This gap in knowledge makes predicting the potential effects of currently available interventions uncertain. Third, there is a significant abscence of a measurable marker for neurodegeneration in *LRRK2* carriers. This abscence makes it difficult to identify disease progression, at both early and more advanced stages. Moreover it limits the ability to monitor the effects of potential therapies accurately.

Penetrance, in the context of genetics, denotes the percentage of individuals carrying a pathogenic mutation associated with a particular disease who develop the condition. Factors that influence penetrance include both genetic and environmental elements.(40) Pathogenic mutations of *LRRK2* gene exhibit incomplete penetrance, indicating that not all carriers will express the disease. This phenomenon, among other factors, partly explains why a portion of LRRK2-PD cases presents as sporadic. Multiple studies have assessed the penetrance of LRRK2-PD(12,31–40) as summarized in **Figure 1, Table 2**. Age consistently increases the penetrance of LRRK2 across different populations. However, the variability in penetrance depending on ethnicity and the type of mutation suggests that other factors, apart from age, contribute to the risk of developing LRRK2-PD. The most recent studies on carriers of the G2019S mutation indicate that the likelihood of developing LRRK2-PD ranges from 25% to 42.5% by the age of 80, and the only study conducted in R1441G carriers suggests a higher penetrance rate for this mutation (83.4% at 80 years of age).(37–40) Based on this data, it is observed that over half of the carriers may either never manifest PD or do so at a considerably advanced age.

Single nucleotide polymorphisms (SNPs) in genes other than *LRRK2* have been identified in genome-wide association studies (GWAS) and in candidate gene studies, as possible modifiers of LRRK2-PD age of onset. Such gene-gene interactions have been described for SNPs at *SNCA*, *MAPT*, and *DNM3* genes.(66–69) Risk polymorphisms for these genes have been reported to decrease the age of onset of LRRK2-PD by 8-10 years. (66–69) Studying and confirming these genetic variants in specific populations will help determine the personalized risk of PD among *LRRK2* carriers and shed light on the genetic factors influencing the disease's development.

Figure 1 –

Studies of penetrance on LRRK2 G2019S and R1441G



|Original Figure

Legend to Figure 1:

This figure summarizes the results of various studies on the penetrance of *LRRK2* mutations, specifically G2019S and R1441G, in Parkinson's disease. Each marker represents data from a different study, showing the percentage of penetrance at various ages. More detailed information of population evaluated on each study can be found at Table 2.

Table 2 | Summary of studies of penetrance of LRRK2 G2019S and R1441G.

Study	Country of origin	PD Penetrance at 50-60 years	PD Penetrance at >70years
G2019S			
Kachergus, 2005 (30)	Norway; US; Ireland; 1 Poland	17% (50 years)	85% (70 years)
Lesage, 2005 (18)	Morocco; Algeria; France; Tunisia; Belgium; Portugal; Netherlands; US	33% (55 years)	100% (76 years)
Ozelius, 2006 (19)	US Ashkenazi Jewish	-	31.8%- 35.2% (lifetime)
Clark, 2006 (29)	US and US Ashkenazi Jewish	-	24% non-AJ (80 years) 26.2% AJ (80 years)
Goldwurm, 2007 (31)	Italy	15% (60 years)	21% (70 years) 32% (80 years)
Healy, 2008 (12)	International	28% (59 years)	51% (69 years) 74% (79 years)
Latourelle, 2008 (32)	UK	10% (50 years)	67% (90 years)
Hulihan, 2008 (33)	Tunisia	20% (<50 years)	80% (70 years)
Troiano, 2010 (34)	Algeria	1% (50 years)	7% (80 years)
Goldwurm, 2011 (35)	Italy	12% (60 years)	23% (70 years) 33% (80 years)
Sierra, 2011 (26)	Spain (Cantabria)	2% (50 years) 12% (60 years)	26% (70 years) 47% (80 years)
Hentati, 2014 (36)	Tunisia	30% (50 years) 61% (60 years)	86% (80 years)
	Norway	3% (50 years) 20% (60 years)	43% (80 years)
Marder, 2015 (37)	Israeal and US Ashkenazi Jewish	-	26% (80 years)
Lee, 2017 (38)	France, Spain US, Germany; Italy; Canada	-	42.5 (80 years)
R1441G			
Ruiz-Martínez, 2010 (39)	Basque Country	12.5% (65 years)	83.4% (80 years)

|Original Table. PD= Parkinson's disease; AJ: Ashkenazi jews; non-AJ: non-Ashkenazi jews

1.2 – Manifest and premotor LRRK2-PD

LRRK2-PD clinically resembles IPD more than any other known autosomal dominant monogenic form. Several studies (12,20,21,70–77) have highlighted these similarities, with some subtle differences. One notable difference is an earlier age of onset in LRRK2-PD,(12,76) with a mean of 58.2 years (SD, 12), compared to IPD. Additionally there is a higher proportion of affected females in some studies of LRRK2-PD (59% vs. 41%).(19,70–76) Family history appears to be more frequent in LRRK2-PD cases,(74) and there is possibly slower progression of the disease compared to IPD.(12,75,76)

Non-motor symptoms occur in LRRK2-PD but, REM sleep behavior disorder (RBD), hyposmia, dysautonomia and neuropsychiatric symptoms are reported to occur less frequently and are milder in patients with G2019S *LRRK2* mutations compared to those with IPD.(54,55,78) However, patients with R1441G mutations exhibit similar frequencies of these non-motor symptoms as patients with IPD.(79,80)

LRRK2-PD patients exhibit the classic motor symptoms of PD, which include bradykinesia, rigidity and resting tremor.(12,20,74–76) These symptoms respond well to dopaminergic treatments and advanced therapies, such as deep brain stimulation.(12) The incidence and severity of motor fluctuations and the occurrence of dyskinesias are similar in both LRRK2-PD and IPD.(12,75) Resting tremor is frequently described as a frequent initial symptom,(73) particularly in R1441G carriers.(71,79) As the disease progresses, tremor (G2019S: 89%; R1441G: 76%; $p = 0.046$) and the development of gait instability (G2019S: 70%; R1441G: 48%; $p = 0.023$) are more frequent among G2019S carriers.(76) Limb dystonia has been reported in some patients, although not in all series.(19,28,78)

Like IPD, LRRK2-PD has a premotor phase that predates the onset of classical motor symptoms by years or even decades, during which pathological changes develop.(53–55,81–84) This prodromal phase of LRRK2-PD is not well understood. However, studies involving asymptomatic relatives of patients with LRRK2-PD G2019S and R1441G mutations, indicate that *LRRK2* non-manifesting carriers (NMC) exhibit higher scores than non-carriers on both motor and non-motor symptoms, including hyposmia, anxiety, depression, dysautonomia and RBD. (53–55,81–84) These symptoms seem to be more prevalent in older subjects, and hyposmia, in particular, more frequent among R1441G-NMC.(80)

Understanding the natural history and diagnosing patients in the premotor phase of LRRK2-PD is particularly important in the context of clinical trials with disease-modifying drugs. Interventions at this stage could, if effective, delay or prevent the development of manifest disease. Such early diagnosis and intervention are critical for improving patient outcomes and enhancing the quality of life for those at risk of developing LRRK2-PD. Moreover, early identification of individuals in the premotor phase can aid in the design of clinical trials by providing a clearer understanding of the disease's progression and the effects of potential treatments.

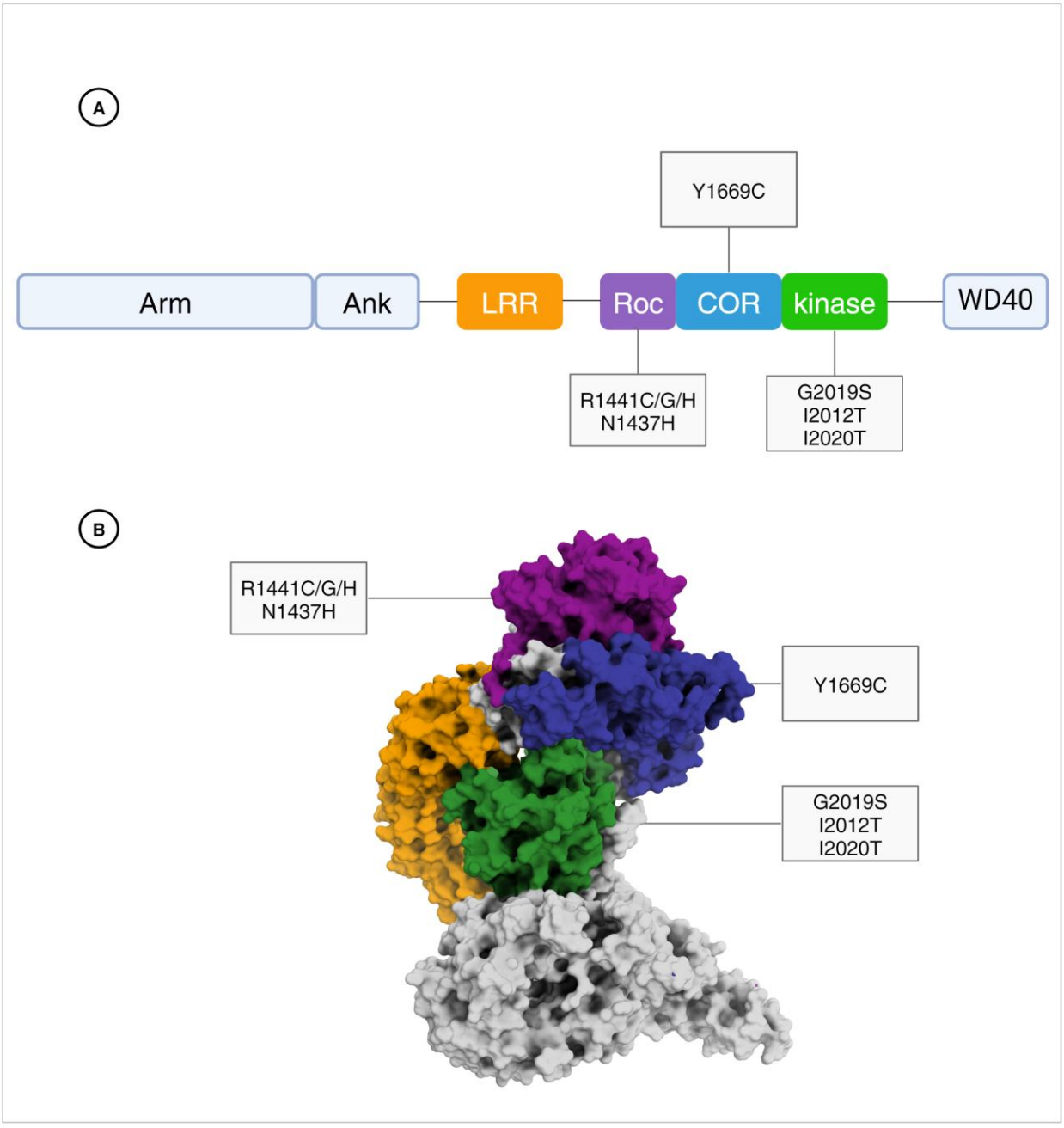
In conclusion, while LRRK2-PD shares many clinical features with IPD, it also presents features that can aid in its identification and management. Continued research into the premotor phase and the development of reliable biomarkers is essential for advancing the treatment and prevention of LRRK2-PD. Understanding these subtle differences and the progression of the disease will be key in tailoring therapeutic approaches and improving patient care in the future.

1.3 – LRRK2-PD pathophysiology

LRRK2 is a large protein consisting of 2527 amino acids, which contains multiple functional domains and exhibits its catalytic activity as a homodimer through the serine–threonine kinase and the Roc-COR GTPase.(85) (**Figure 2**) It is expressed in various tissues, with elevated levels observed in the lungs, kidneys, brain, and particularly within peripheral blood mononuclear cells, such as neutrophils and monocytes.(86) In the cell, LRRK2 dimers localize around vesicle membranes, such as endosomes, lysosomes or autophagosomes,(87) where they exhibit increased kinase activity.(88) Although the precise biological function of LRRK2 within the cell remains unknown, it has been implicated in vesicle trafficking and intermembrane transport,(89) protein degradation,(90) microtubule and cytoskeletal dynamics(91,92) and the biology of cellular organelles such as mitochondria,(93) the trans-Golgi network and lysosomes.

All pathogenic mutations of *LRRK2* are located in its Roc-COR-kinase catalytic core (**Figure 2**) and are associated with a toxic gain-of-function of the protein kinase.(42–44)

Figure 2 –
Schematic structure of LRRK2 protein



|Original Figure.

Legend to Figure 2:

A. This figure ilustrates a linear model of the LRRK2 protein domains and pinpoints the locations of the pathogenic mutations linked to LRRK2-associated Parkinson's disease. The structure is depicted from left to right, beginning with the Armadillo (Arm) repeats, followed by Ankyrin (Ank) repeats, Leucine-Rich Repeat (LRR), Ras of Complex Proteins (Roc), C-terminal of Roc (COR), Kinase, and WD40 domains.

Roc domain shown in purple, is a GTPase domain that regulates the activity of LRRK2. COR domain shown in blue, is important for the stabilization of the Roc domain. Kinase domain shown in yellow is responsible for the phosphorylation activity of LRRK2. Pathogenic mutations in *LRRK2* associated with Parkinson's disease are indicated in grey boxes and all of them are limited to these three critical domains, where they disrupt the normal function of LRRK2, contributing to the pathogenesis of Parkinson's disease.

B. Cryo-EM structure of LRRK2 monomer. The same colour-coding of domains is used as in figure A. Location of the pathogenic Parkinson's disease mutations. (Courtesy of Miquel Angel Galmés Ordinas).

To date, only two direct substrates of LRRK2 have been identified. The first is the autophosphorylation of the LRRK2 protein itself at residue Ser1292 of the Roc domain,(94) which regulates its kinase activity and its interaction with other proteins, such as the 14-3-3 proteins. In one study, decreased blood levels of phospho-Ser935-LRRK2 have been observed in LRRK2-PD patients compared to controls.(95,96) The second substrate of LRRK2 is the RAB family of proteins, small proteins that are key in the generation, organization and transport of vesicles through the cytoskeleton.(51,97–100) Evidence from animal models and cultured cells of LRRK2-PD patients indicates that excessive phosphorylation of RAB proteins by mutated forms of LRRK2 contributes to the pathogenesis of PD.(98) In humans, increased phospho-RAB10 levels have been observed in LRRK2-R1441G PD patients but not in carriers of the G2019S mutation.(99) This phosphorylation is reversible by LRRK2 kinase inhibitors, providing the only known *bona fide* LRRK2 substrate in human cells to date. (99)

Kinase inhibition in LRRK2-PD

The evidence that pathogenic mutations in the *LRRK2* gene increase kinase activity, along with the identification of substrate phosphorylation as a pathogenic outcome, has accelerated the development of LRRK2 kinase inhibitors as potential treatments for LRRK2-PD. Over the past decade, different molecules inhibiting LRRK2 kinase at different sites, have been reported.(101) To date, at least three of these compounds - DNL-201, DNL-151, NEU-723 - have advanced to clinical trials for IPD and LRRK2-PD.(102) Results from these studies, conducted with both healthy volunteers and PD patients, indicate that orally administered kinase inhibitors can effectively inhibit LRRK2 and are well-tolerated at pharmacologically relevant doses.(102) These encouraging data support the continued exploration of kinase inhibitors as potential therapies for LRRK2-PD and IPD.

LRRK2 mutations alter peripheral innate immunity and microglial activity

There is increasing evidence of immune dysregulation in LRRK2-PD. Studies on cell and animal models of mutant *LRRK2* exposed to bacterial infections show an aberrant inflammatory response compared to the wild-type form of the gene.(103) Additionally epidemiological studies support this link between immunity and LRRK2. Regular use of non-steroidal anti-inflammatory drugs (ibuprofen, aspirin) has been associated with a reduced risk of developing PD in a study involving 259 patients with LRRK2-PD and 318 LRRK2-NMC.(104) Also, *LRRK2* mutations have been linked to diseases caused by immune system dysfunction such as inflammatory bowel diseases(105,106) or chronic infectious diseases such as leprosy or tuberculosis.(107,108) Studies on patients show that in both IPD and LRRK2-PD there is an increase in inflammatory markers compared to healthy controls.(56) In a percentage of asymptomatic carriers of this mutation, an increase in pro-inflammatory cytokines in blood and CSF has been detected,(109) suggesting a role for inflammation even in presymptomatic stages of the disease.

Microglia plays a relevant role of innate immunity in the brain, due to its central role in the neuroinflammatory response, protein degradation, and synthesis of neurotrophic factors.(110,111) Microglia are highly represented in the substantia nigra pars compacta and the nucleus striatum of the brain. Neuropathological studies demonstrate the presence of reactive microglia in the brains of PD patients.(111,112) It is believed that in PD, activation of microglia initially exerts a protective function, through the synthesis of neurotrophic factors stimulating the clearance of α-syn, while in a chronic situation the production of inflammatory cytokines may have deleterious effects on neuronal function even stimulating α-syn aggregation and propagation.(113) In turn, extracellular α-syn stimulates microglia to adopt a pro-inflammatory phenotype. Mutations in *LRRK2* may alter vesicle formation and degradation of pathological extracellular α-syn species by microglial cells, disrupting the balance between activated and quiescent microglia, and thereby stimulating gliosis and chronification of the neurodegenerative process.(113,114) (**Figure 3**) Microglia activation induced by *LRRK2* mutations suggests that increased neuroinflammation is likely to trigger heightened neurodegeneration. One hypothesis is that *LRRK2* mutations disrupt essential cellular pathways, such as lipid vesicle trafficking, thereby modifying the interaction between neurons and non-neuronal cells. This disruption may, in turn, trigger inflammatory responses

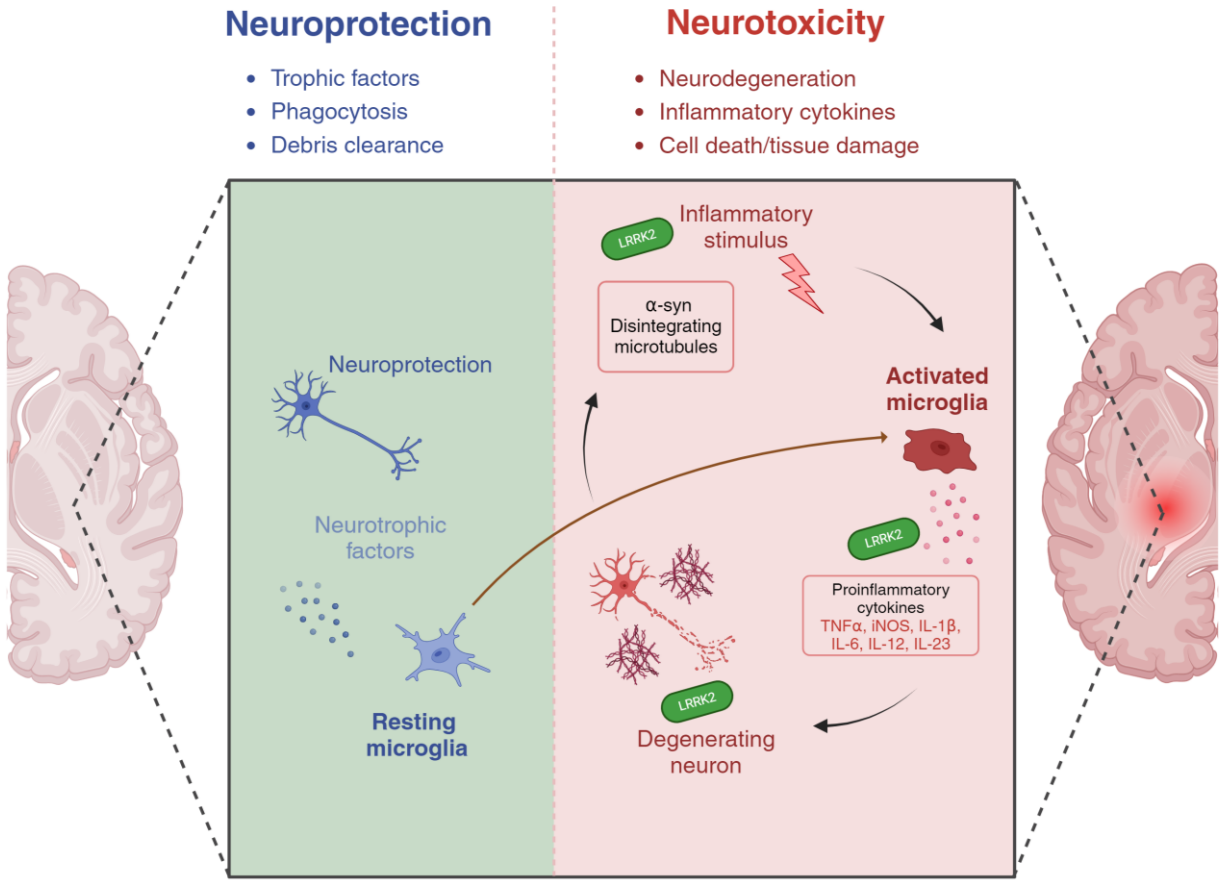
that contribute to the chronic progression of the neurodegenerative process. Since neuroinflammatory processes evolve with aging, this could explain the age-dependent penetrance observed in *LRRK2* mutations.(115)

Studying microglial activation in vivo is possible using functional brain imaging such as positron emitted tomography (PET), a non-invasive imaging technique that allows visualization of abnormal protein deposition or other molecular targets.

Both the innate and adaptative immune system are implicated in the pathogenesis of IPD.(114) Post-mortem analyses of IPD patients' brains have revealed reactive microglia in the substantia nigra pars compacta and striatum. Elevated levels of cytokines, such as TNF, IL-1 or IL-6, secreted by activated microglia have been detected in both post-mortem tissue and cerebrospinal fluid (CSF) of PD patients. (114) PET studies on IPD patients, using ligands like [11C](R)-PK11195, which binds to activated microglia, have reported an increased in binding in patients compared to controls. (114) Increased activation of microglia in the substantia nigra has been demonstrated in subjects in premotor phases of IPD, such as patients with RBD.(116)

Figure 3 –

Possible role of microglia and LRRK2 in the pathogenesis of LRRK2-PD



|Original Figure.

Legend to Figure 3:

Microglia exhibit diverse phenotypes and could play a critical role in Parkinson's disease pathogenesis. In response to inflammatory stimuli, such as peripheral immune cell infiltration or extracellular α-synuclein, microglia shift from a neuroprotective "resting" state to an "activated" ameboid state. Activated microglia secrete pro-inflammatory cytokines found in the substantia nigra, striatum, and cerebrospinal fluid of Parkinson's disease patients, promoting a sustained pro-inflammatory environment that leads to neurodegeneration.

LRRK2 regulates phagocytosis, cell signaling, and autophagy in cells through Rab GTPases. Pathogenic *LRRK2* mutations enhance LRRK2 expression in immune cells peripherally and cause lysosomal defects in glial cells within the central nervous system, leading to impaired phagocytosis and debris accumulation. In neurons, *LRRK2* mutations disrupt mitochondrial function, increase oxidative stress, perturb calcium homeostasis, and result in cell death.

1.4 – LRRK2-PD has a pleomorphic neuropathology

In IPD, the progressive dysfunction and degeneration of neurons are associated with the presence of Lewy type pathology. This pathology is characterized by the abnormal accumulation of α-syn aggregates within cell bodies and neurites of neurons throughout the central nervous system.(117) These aggregates are a hallmark feature of the disease and are widely recognized as a major contributor to neuronal loss and the associated motor and non-motor symptoms of PD. According to Braak staging hypothesis, α-syn aggregation initially begins in the dorsal motor nuclei of the glossopharyngeal and vagal nerve, as well as the anterior olfactory nucleus. It subsequently progresses in an upward trajectory through the brainstem, followed by the involvement of the anteromedial temporal mesocortex, and eventually affects the neocortical areas. This progression correlates with the the onset and worsening of clinical symptoms, as the pathological changes ascend through the brainstem and into cortical brain regions.(118)

In LRRK2-PD, heterogeneous neuropathology is observed. In some instances, there is evidence of nigral degeneration occurring in the absence of overt protein aggregation. In other cases, a diverse range of proteins may accumulate including α-syn, tau, amyloid or TAR DNA-binding protein 43 (TDP-43).(41) This variation underscores the complex amd multifaceted nature of LRRK2-PD pathology. Interestingly different pathologies have been reported among carriers of the same mutations, as illustrated in **Table 3 and Figure 4**, and in rare instances, even among members of the same family.(119)

Lewy pathology has been identified in approximately 60% of confirmed and reported cases of LRRK2-PD to date.(120–129) The majority of these cases involve carriers of the G2019S mutation. In addition to Lewy pathology, 3-repeat tau co-pathology is also frequently observed. (**Table 3**; **Figure 4**).

A retrospective clinico-pathological analysis of subjects with the *LRRK2* G2019S mutation showed an association between Lewy type synucleinopathy and certain non-motor symptoms, such as cognitive impairment, dementia and anxiety.(127) However, despite these observations, there is still insufficient data to definitively correlate the various histopathological features associated with LRRK2-PD with distinct clinical phenotypes. This highlights the need for further research to unravel the complex relationships between genetic mutations, pathological findings, and clinical manifestations in LRRK2-PD.

Table 3 | Neuropathology of LRRK2-PD with G2019S and R1441G mutations.

Study author, year	Clinical Phenotype (n)	n of cases with LB	n of cases with Tau inclusions	n of cases with other pathologies
***LRRK2* p.G2019S (n=50)**				
Rajput, 2006 (120)	PD (1)	0	1 PSP like (4R) / Nft + AD like (3R+4R)	1 Aβ + AGD
Ross, 2006 (121)	PD (8), AD (1)	8	3 Nft	6 Aβ
Gaig, 2007 (62) *Gaig, 2008* (63)	PD (1) PD (1)	0 1	1 0	1 Ubq (MB) 0
Giasson, 2006 (122) *Henderson, 2019* (123)	PD (3) PD (9)	2 5	3 Nft 9 Nft, NP, NT	3 Aβ plaques n.d
Dächsel, 2007 (124)	FTD (1)	0	0	1 Ubq (MB)
Silveira-Moriyama, 2008 (125)	PD (4)	4	4	n.d
Poulopoulos, 2012 (126)	PD (3)	3	1 Nft 2 rare or very rare Nft	n.d
Kalia, 2015 (127)	PD (3)	2	0	1 Aβ
Vilas, 2018 (64)	PD (1) PSP (1)	1 0	2 Nft, coiled bodies, tufted astrocytes	0
Bukhari, 2022 (128)	PD (2)	2	n.d	n.d
Agin-Liebes, 2023 (129)	PD (11)	8	11	7 Aβ 8 LATE
TOTAL	PD 47 AD 1 FTD 1 PSP 1	36 (76.6%) 0 0 0	36 (72%)	18 (36%) Aβ 2 (5.1%) Ubq 1 (2.5%) AGD 11 (22%) LATE
***LRRK2* p.R1441G (n=9)**				
Martí-Massó, 2009 (130)	PD (1)	0	1	1 Aβ
Kalia, 2015 (127)	PD (1)	0	0	0
Vilas, 2019 (65)	PD (1)	0	Nft, NT	0
Vinagre-Aragón, 2021 (79)	PD (5)	0	n.d	n.d
Bukhari, 2022 (128)	PD (1)	1	n.d	n.d
TOTAL	PD 9	1 (11%)	1 (11%)	1 (11%)

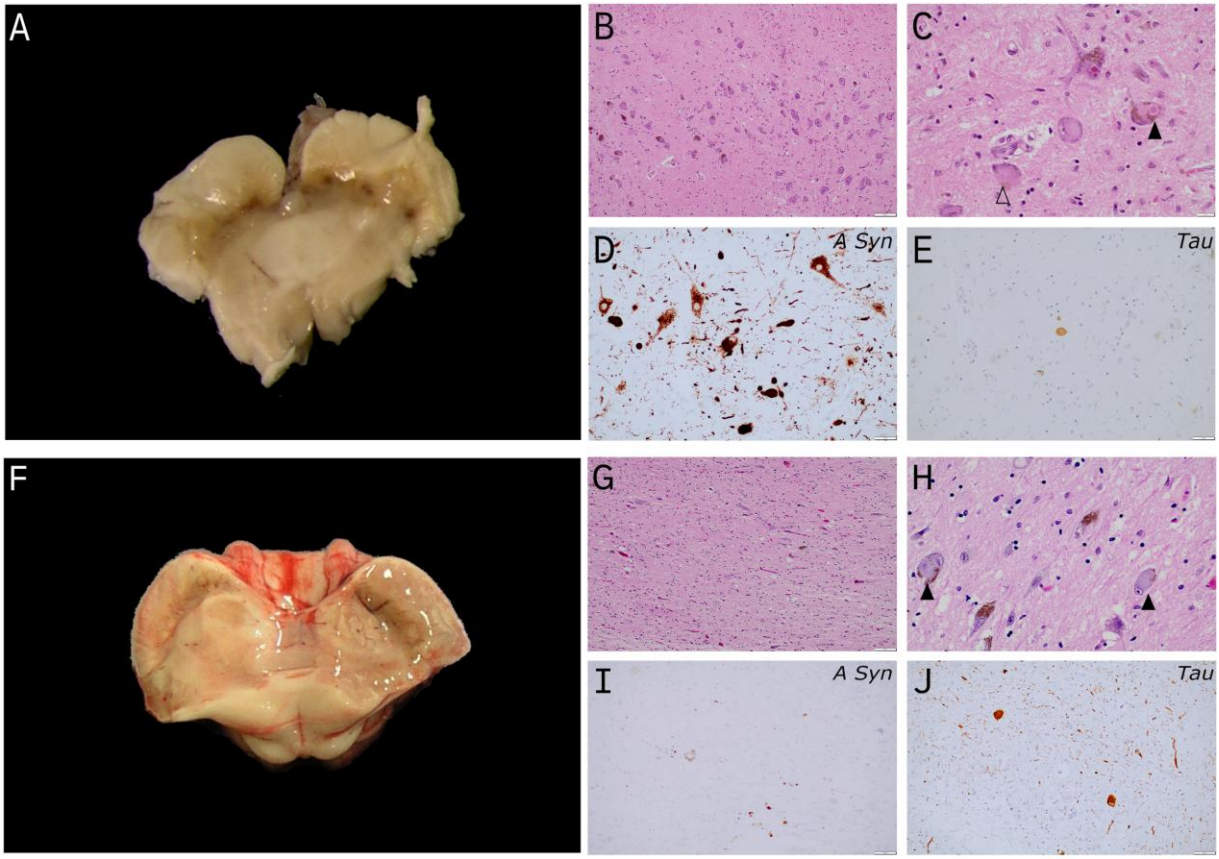
|Original Table. PD= Parkinson's disease; Aβ =amyloid-beta Ubq= ubiquitin; MB= Marinesco bodies: ubiquitin-immunoreactive neuronal intranuclear inclusions; n.d= not detailed; Nft feurofibrillary tangles; NP = neuritic plaques; NT = neuropil threads; PSP= Progressive supranuclear palsy; AGD= argyrophilic grain disease; FTLD = frontotemporal lobar degeneration, LATE= limbic predominant age-related TDP-43 encephalopathy neuropathological change.

Figure 4 –

Variable neuropathological findings in LRRK2-PD patients with different mutations

A-E a case with the R1441G mutation with Lewy body pathology and **F-J** a case with the G2019S mutation with 4 repeat tauopathy compatible with progressive supranuclear palsy

Images extracted from Banc de Teixits Neurològics de l'Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer cases.



|Original Figure.

Legend to Figure 4:

A: Midbrain section of a case with R1441G mutation, shows severe depigmentation of the substantia nigra. **B-C**: Hematoxilin-Eosin (H&E) stain of substantia nigra shows slight neuronal loss and moderate gliosis. There is marked loss of cytoplasmic neuromelanin in many of the neurons of the substantia nigra; Lewy body (black arrowhead) and pale bodies (white arrowhead) are detected **D**: α-Synuclein (*A Syn*) immunohistochemistry highlights the presence of abundant Lewy bodies and neurites in substantia nigra. **E**: Hyperphosphorilated tau staining (*Tau*), shows occasional tangles with pretangles and neurites in substantia nigra.

F: Midbrain section of a case with G2019S mutation, shows marked depigmentation of the substantia nigra. **G**-**H**: H&E stain of substantia nigra shows marked loss of pigmented neurons; globose neurofibrillary tangles can be observed in remaining neurons (black arrowheads). **I**: *A Syn* immunohistochemistry of substantia nigra does not show pathological inclusions; free neuromelanin and hemosiderin deposits can be observed. **J**: AT8 immunohistochemistry of substantia nigra reveals neurofibrillary tangles and pretangles as well as neuropil threads.

Magnifications: B and G 100x; D, E, I and J 200x; C and H 400x.

LRRK2 mutations have been shown in preclinical studies to play a role in the pathological aggregation of α-syn, contributing to the formation of Lewy bodies.(131,132) Additional research in cell and animal models suggests that *LRRK2* mutations enhance the cell-to-cell spread of α-syn, thereby promoting PD progression.(132) Phosphorylation of α-syn at specific residues (Ser 87, Ser 129 and Tyr125) promotes its aggregation leading to the generation of the pathological species (fibrils and fibers) found in Lewy bodies and Lewy neurites.(133) One initially proposed hypotheses linking *LRRK2* mutations to PD development suggested that the heightened kinase activity of the LRRK2 protein played a role in the increased phosphorylation of α-syn, contributing to its enhanced aggregation and toxicity.(9) However, there is currently no demonstrated direct interaction between the LRRK2 protein and α-syn. Evidence indicates common functional pathways between the two proteins, such as their interaction with RAB proteins or 14-3-3 proteins.(50,98)

Our understanding of the factors that lead to the development of distinct pathologies in LRRK-PD and their impact on the clinical characteristics of patients remains limited. Further research is needed to elucidate these mechanisms and their implications for disease progression and treatment.

1.5 – α-Syn based biomarkers in LRRK2-PD

According to the definition proposed by the NIH Biomarker working group,(134) biomarkers are objective characteristics that indicate a normal biological process, a pathological process or a response to a therapeutic intervention. Potential biomarkers have been identified for LRRK2-PD in both the premotor and overt phases of disease, including clinical biomarkers,(52–55,83,84,135,136) imaging,(53,60,61,82,135,137) and molecular biomarkers assessed in blood,(57,138) urine(137,139–142) or CSF.(58,59,143) (**Table 4**)

Similar to approaches in IPD and considering that a majority of G2019S LRRK2-PD subjects exhibit typical Lewy-type pathology, a primary strategy for developing biomarkers for LRRK2-PD has centered on α-syn. Standard immunohistochemical techniques for detecting α-syn in CSF have been employed in LRRK2-PD, revealing no differences compared to control subjects. (58,59)

In 2016, a significant advancement in PD biomarker research was achieved with the demonstration that misfolded α-syn aggregates could be reliably detected in CSF and brain of IPD patients, using a seeding amplification assay (SAA) - a technology originally employed for prionic disease diagnosis.(144) This breakthrough transformed the field of PD biomarkers by significantly enhancing diagnostic accuracy for synucleinopathies. Development of the α-syn SAA represents an important step towards the biological diagnosis of PD, making it possible to detect the disease before clinical changes are observed.

SAA leverage the unique properties of pathologically aggregated α-syn in PD, which enable even small quantities of the protein present in a sample to initiate the amplification of recombinant monomeric α-syn through different methodologies. These methodologies include real-time quaking-induced conversion (RT-QuIC), and protein misfolding cyclic amplification. (**Figure 5**) During the amplification process, pathological α-syn seeds convert normal α-syn into a misfolded state, creating a cascade that can be measured. This amplification can be quantified through fluorescence measurements, which provide a sensitive readout of the presence of misfolded α-syn.

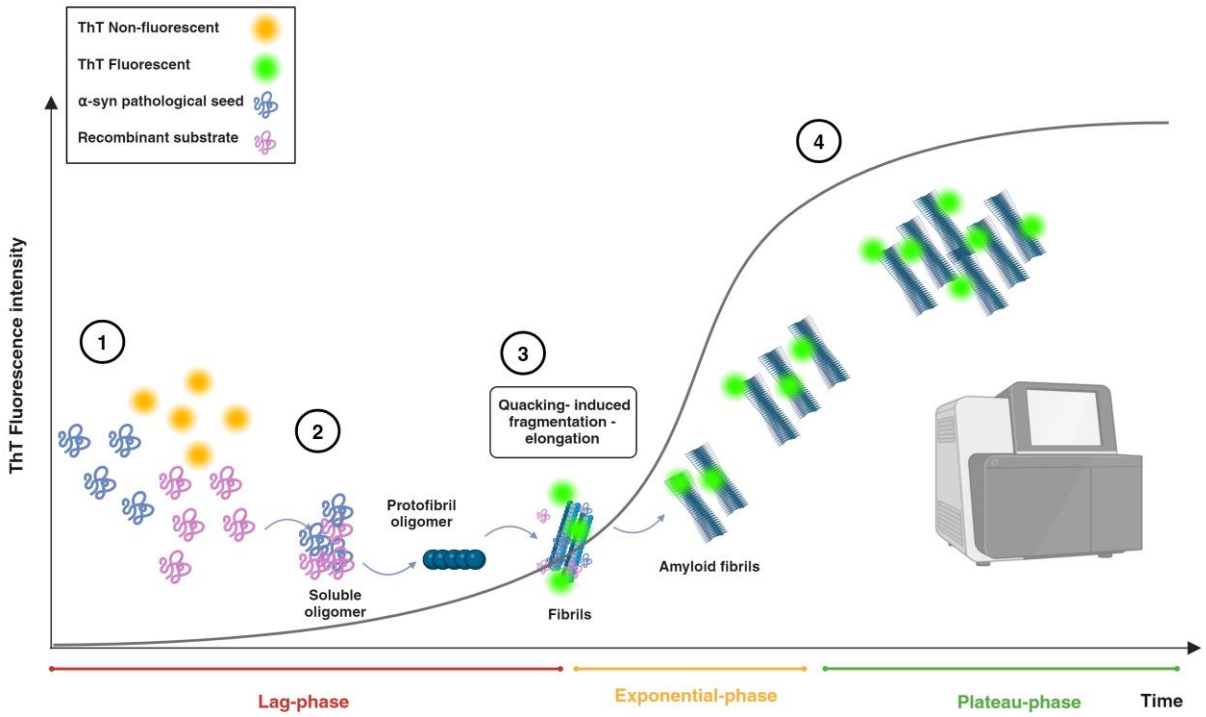
Table 4 | Candidate biomarkers for G2019S and R1441G LRRK2 Parkinson's disease

	Finding	Diagnostic potential		Disease Progression	Comments
		Premotor LRRK2-PD	**Manifest LRRK2-PD**		
Olfaction (53,136,145)	Decreased UPSIT	No	Less frequent than IPD	NA	
Blood (57,138)	Differencial microRNAs	NA	Yes	NA	More studies needed
	Plasma α-syn levels	NA	Yes Higher than IPD	NA	More studies needed
Urine (137,139–142)	Elevated ratio of p-Ser-1292 LRRK2/ total LRRK2	Yes	Yes	Possible	More studies needed
	Elevated BMP	Yes	Yes	No	
CSF (58,59,143)	Levels α-syn $A\beta_{1-42}$, T-tau, P-tau_{181}	No	No α-syn lower than IPD	No	
	Mitochondrial DNA	No	Yes Higher than IPD	No	More studies needed
MRI (38,60,145–148)	Volumetry changes	Yes	Uncertain	NA	Inconsistent findings among studies
	fMRI: altered connectivity at resting state and motor and cognitive tasks	Yes	Yes	NA	Different mehodologies between studies. More studies needed
	NM-MRI:	No	Yes NM-SN loss similar to IPD NM-LC less frequent than IPD	NA	More studies needed
DAT-SPECT (53,137,145,149, 150)	Reduced DAT uptake	Yes	Yes Greater asymmetrical striatal DAT uptake	Possible	More studies needed to determine its utility as progression marker
Transcranial Sonography (53,135,145,147)	SN hyperechogenicity	Yes	Yes Similar to IPD	NA	In *LRRK2*-NMC SN hyperechogenicity is related to other surrogate biomarkers (DAT-SPECT or RBD)

|Original Table. UPSIT: University of Pennsylvania Smell Identification Test; α-syn : α-synuclein; BMP: bis(monacylglycerol) phosphate; CSF: cerebrospinal fluid; $A\beta_{1-42}$: Amyloid-beta 1-42; T-tau: Total tau; P-tau_{181}: Phospho-tau-181; MRI: Magnetic ressonance imaging; fMRI: functional MRI; NM-MRI: neuromelanin sensitive MRI; NM-SN : neuromelanin-substantia nigra; NM-LC : neuromelanin-locus coeruleus; DAT-SPECT: dopamine transporter single-positron emission tomography. SN: substantia nigra. *LRRK2*-NMC : *LRRK2* non-manifesting carriers

Figure 5 –

Schematic representation of α-Syn RT-QuiC assay



|Original Figure.

Legend to Figure 5:

Schematic diagram of the potential seeding conversion mechanism of α-syn in RT-QuIC assay. 1) The RT-QuIC reaction buffer consists of human recombinant full-length α-syn, thioflavin T (ThT), and phosphate buffer. This buffer is seeded with biological tissue containing pathological α-syn seeds (e.g., cerebrospinal fluid). 2) The pathological seeds initiate the aggregation of monomeric α-syn. This aggregation leads to the formation of oligomers, which elongate into fibrils, causing an increase in ThT fluorescence. 3) Quaking events fragment the longer fibrils into shorter, more reactive oligomers, which further act as seeds for the conversion of monomeric α-syn. 4) The characteristic kinetic curve of a positive RT-QuiC reaction observed is depicted.

Mounting evidence demonstrates that α-syn SAA can distinguish IPD patients from healthy controls or those with tauopathies with sensitivities and specificities exceeding 90% in brain tissue and CSF.(144,151–153) (**Table 5**). This high level of accuracy, previously unachieved in the field of PD biomarkers, speaks to the robustness of the assay and its potential utility in clinical diagnosis. Beyond CSF and brain tissue, these techniques have also achieved good accuracy when applied to more accessible tissues, including skin,(154–156) olfactory mucosa obtained with nasal swabs,(157) saliva,(158,159) and blood.(160) (**Table 6**). The possibility to use such samples significantly broadens the potential for early and accurate diagnosis of PD.

Interestingly, SAA have proved effective in detecting α-syn aggregates in individuals with suspected prodromal PD, such as those with RBD,(161,162) and hyposmia. The detection of pathological α-syn aggregates in these prodromal conditions supports the potential of this biomarker for identifying synucelinopathies in the earliest disease stages.

Given that LRRK2-PD is characterized by variable pathology and lacks distinct clinical or imaging features to differentiate cases with specific underlying pathologies, techniques that can reliably identify cases with synucleinopathy in vivo, such as α-syn SAAs, could have a significant impact. A positive result from an α-syn SAA would allow for the inclusion of such cases in disease modification trials targeting α-syn and the exclusion of those with a negative SAA result. This targeted approach could enhance the efficacy of clinical trials by focusing on patients most likely to benefit from the intervention.

Moreover, the SAA results would enable researchers to study differences in clinical phenotype, disease progression, and the development of motor and non-motor complications linked to the underlying pathology. Understanding these differences is essential for counsenling to patients, and developing personalized treatment plans. Additionally, this knowledge opens a new field of research focused on the detection of abnormal synuclein aggregation in asymptomatic *LRRK2* mutations carriers. Combined with other biomarkers, α-syn SAA results would facilitate the design of prevention strategies to delay or prevent the development of manifest parkinsonism.

Table 5 | Studies of α-syn seeding amplification assay accuracy in cerebrospinal fluid in idiopathic Parkinson's disease

Study	Autopsy	Disease	N	N Control	Sensitivity	Specificity
Fairfoul,2016 (144)	Yes	Pure DLB	12	20	92%	100%
		DLB with AD	17	20	65%	100%
		AD with ILBD	13	20	15%	100%
		PD	2	20	100%	100%
	No	PD	20	15	95%	100%
		IRBD	3	15	100%	100%
Shahnawaz,2017 (151)	No	PD	76	65	88%	94%
		DLB	10	65	100%	94%
Manne, 2020 (154)	No	PD	15	11	94%	100%
Groveman, 2018 (163)	Yes	PD	12	31	92%	100%
		DLB	17	32	94%	100%
Bongianni, 2019 (164)	Yes	DLB	7	49	100%	96%
		mixed LBD*	20	51	90%	96%
	No	DLB	26	10	65%	100%
Kang, 2019 (152)	No	PD	105	79	96%	82%
Rossi, 2020 (153)	Yes	DLB	14	101	100%	98%
		mixed LBD†	7	102	86%	98%
	No	DLB	34	166	97%	94%
		PD	71	167	94%	94%
		IRBD	18	168	100%	94%
		PAF	28	169	93%	94%

|Original Table. DLB: dementia with Lewy bodies; AD: Alzheimer's disease; ILBD: incidental Lewy body disease; PD: Parkinson's disease; IRBD: idiopathic REM sleep behaviour disorder *Mixed LBD = DLB and AD mixed pathology (*n* = 15); DLB with tau (*n* = 2) and sporadic Creutzfeldt–Jakob disease with incidental LB pathology (*n* = 3); † Mixed LBD: sporadic Creutzfeldt–Jakob disease with incidental LB pathology (*n* = 5), 1 primary CNS lymphoma with incidental LB pathology, 1 metabolic encephalopathy with incidental LB pathology.

Table 6 | Studies of α-syn seeding amplification assay accuracy in peripheral tissues in idiopathic Parkinson's disease

Study	Tissue	Autopsy	Disease	N	N Control	Sensitivity	Specificity
Manne, 2020 (165)	SMG	Yes	PD	13	16	100%	94%
			ILBD	3	16	100%	94%
Luan, 2022 (159)	Saliva	No	PD	75	18	76%	94.4%
Vivacqua, 2023 (158)	Saliva	No	PD	37	23	83.8%	82.6%
Wang, 2020 (156)	Abdominal skin	Yes	PD	47	73	94%	93%
			DLB	7	73	100%	93%
	Biopsy skin	No	PD	20	21	95%	100%
Manne, 2020 (166)	Frozen skin	Yes	PD	25	25	96%	96%
	FFPE skin		PD	12	12	75%	83%
Mammana, 2021 (155)	Skin	Yes	PD/DLB	2	40	100%	98%
			ILBD	7	40	86%	98%
Okuzumi, 2023 (160)	Blood (serum)	No	PD	221	128	95%	92.1%
			DLB	10	128	90%	92.1%
			IRBD	9	128	44%	92.1%
		Yes	PD	4	3	100%	100%
Schaeffe, 2024 (167)	Blood (plasma)	No	PD	80	20	98.8%	100%

|Original Table. PD: Parkinson's disease; ILBD: incidental Lewy body disease; DLB: dementia with Lewy bodies; IRBD: idiopathic REM sleep behaviour disorder.

In conclusion, the development and application of α-syn SAA represent a significant advancement in the diagnosis and management of PD. These assays offer a high degree of sensitivity and specificity, can be applied to a variety of tissue samples, and have the potential to detect disease in its earliest stages. As research continues to evolve, the integration of SAAs with other biomarkers will likely enhance our ability to diagnose, treat, and ultimately prevent LRRK2-PD, providing hope for those affected by this debilitating condition.

Hypothesis:

- Single nucleotide polymorphisms across various genes modify the penetrance of G2019S LRRK2-Parkinson's disease.

- Increased activation of microglia, leading to neuroinflammation, plays a role in the early, premotor, stages of LRRK2-Parkinson's disease .

- The detection of α-synuclein aggregates in cerebrospinal fluid through seeding amplification assays identifies *LRRK2* mutation carriers with an underlying synucleinopathy.

Objectives:

1. Assess the impact of single nucleotide polymorphisms in the *SNCA* and *DNM3* genes on the penetrance of LRRK2-Parkinson's disease.

2. Study central nervous system microglial activation in pre-clinical stages of LRRK2-Parkinson's disease.

3. Determine the presence of α-synuclein aggregates in the cerebrospinal fluid of *LRRK2* mutation carriers using a seeding amplification assay.

4. Perform α-synuclein seeding amplification assay in postmortem brain and cerebrospinal fluid of LRRK2-Parkinson's disease cases, to assess the test's accuracy in identifyingLewy body pathology.

Material, Methods, and Results:

Article 1 –

α-Synuclein (*SNCA*) but not dynamin 3 (*DNM3*) influences age at onset of leucine-rich repeat kinase 2 (*LRRK2*) Parkinson's disease in Spain.

Garrido A*, Fernández-Santiago R*, Infante J, González-Aramburu I, Sierra M, Fernández M, Valldeoriola F, Muñoz E, Compta Y, Martí MJ, Ríos J, Tolosa E, Ezquerra M; Barcelona LRRK2 Study Group†. Mov Disord. 2018 Apr;33(4):637-641.

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α-synuclein (*SNCA*) but not dynamin 3 (*DNM3*) Influences Age at Onset of leucine-rich repeat kinase 2 (LRRK2) Parkinson's Disease in Spain

Rubén Fernández-Santiago, PhD,[1,2*] Alicia Garrido, MD,[3] Jon Infante, MD, PhD,[2,4] Isabel González-Aramburu, MD,[2,4] María Sierra, MD, PhD,[2,4] Manel Fernández,[1] Francesc Valldeoriola, MD, PhD,[1,2,3] Esteban Muñoz, MD, PhD,[1,2,3] Yaroslau Compta, MD, PhD,[1,2,3] María-José Martí, MD, PhD,[1,2,3] José Ríos, MSc,[5,6] Eduardo Tolosa, MD, PhD,[1,2,3] Mario Ezquerra, PhD,[1,2*] and the Barcelona LRRK2 Study Group[†]

[1]Laboratory of Parkinson Disease and Other Neurodegenerative Movement Disorders, Clinical and Experimental Research, Department of Neurology, Hospital Clínic of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain [2]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas, Madrid, Spain [3]Movement Disorders Unit, Department of Neurology, Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain [4]Movement Disorders Unit, Department of Neurology, Hospital Universitario Marqués de Valdecilla, Universidad de Cantabria, Santander, Spain [5]Medical Statistics Core Facility, Institut d'Investigacions Biomèdiques August Pi i Sunyer and Hospital Clinic, Barcelona, Spain [6]Biostatistics Unit, Faculty of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain

ABSTRACT

Objectives: A recent study showed that Arab-Berbers GG homozygous at rs2421947(C/G) in the dynamin 3 gene (*DNM3*) had 12.5 years earlier age at onset of leucine-rich repeat kinase 2 (*LRRK2*)-associated Parkinson's disease (PD) (L2PD). We explored whether this variant modulates the L2PD age at onset in Spain.

Methods: We genotyped rs2421947 in 329 participants (210 L2PD patients, 119 L2PD nonmanifesting p.G2019S carriers), and marker rs356219 (A/G) in the α-synuclein gene (*SNCA*).

Results: By Kaplan Meier and Cox regression analyses, we did not find an association of the *DNM3* polymorphism with L2PD age at onset. However, we found an association of the *SNCA* marker with up to an 11 years difference in the L2PD median age at onset (58 years for GG carriers vs 69 years for AA).

Conclusion: Our results indicate that *SNCA* rs356219 but not dynamin 3 *DNM3* rs2421947 modifies the penetrance of the mutation G2019S in the Spanish population by influencing the L2PD age at onset. These findings suggest that different genetic modifiers may influence the L2PD age at onset in different populations.

Key Words: Parkinson's disease (PD); leucine-rich repeat kinase (LRRK2); G2019S mutation; age at onset (AAO)

Pathogenic mutations in leucine-rich repeat kinase 2 (*LRRK2*) including p.G2019S are a frequent cause of Parkinson's disease (PD). LRRK2-associated PD (L2PD) resembles common sporadic PD (sPD),[1-3] and mutation G2019S was identified in autosomal-dominant familial (4%) but also in sPD (1%) cases.[1] Yet the G2019S penetrance is incomplete[1,4-7] and increases progressively with age, suggesting additional modifier genes and/or environmental factors that are

***Corresponding authors**: Drs. R. Fernández-Santiago and M. Ezquerra, Laboratory of Neurodegenerative Diseases, CELLEX building, Faculty of Medicine (UB), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, University of Barcelona, Casanova 143, Floor 3B, 08036, Barcelona; E-mail: ruben.fernandez.santiago@gmail.com; E-mail: ezquerra@clinic.ub.es

[†]See appendix.

Rubén Fernández-Santiago and Alicia Garrido contributed equally to this work. Eduardo Tolosa and Mario Ezquerra are cosenior authors. Members of the Barcelona LRRK2 Study Group are listed in the Acknowledgments.

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unknown until now. A study in Tunisian Arab-Berber L2PD patients showed that GG homozygous at the single nucleotide polymorphism (SNP) rs2421947(C/G) had 12.5 years earlier age at onset (AAO) [8] than CC carriers. This association was held significant in a meta-population analysis including Algeria, France, Norway, and the United States.[8] The SNP rs2421947 maps to dynamin 3 (*DNM3*), a Guanosine-5′-triphosphate (GTP)-binding protein associated with microtubules which is involved in vesicle-trafficking and endocytosis. Because different genetic modifiers potentially modulate the penetrance of pathogenic mutations in different PD populations,[9] here we investigated the association of *DNM3* rs2421947 with L2PD AAO in the Spanish population. Our sample comprised 329 participants from Barcelona and Santander, of which 210 were L2PD patients carrying G2019S (affected carriers), and 119 L2PD nonmanifesting G2019S carriers (L2NMC; nonaffected carriers). We also genotyped SNP rs356219(A/G) located in the 3' untranslated region (UTR) of α-synuclein (*SNCA*), which has been previously suggested to modulate PD AAO in the Spanish[10] and other European populations.[11]

Methods

Participants

Local ethics committees at the Hospital Clínic de Barcelona (Barcelona, Spain) and Hospital Marqués de Valdecilla (Santander, Spain) approved the study. The participants gave written informed consent. Blood samples from L2PD patients and L2NMC were obtained at the Movement Disorders Unit of the Hospital Clínic de Barcelona and collaborating centers (The Barcelona LRRK2 Study Group), and also at the Hospital Marqués de Valdecilla of Santander in the Atlantic North area of Spain. The study encompassed 210 G2019S L2PD patients (146 from Barcelona and 64 from Santander) and 119 G2019S L2NMC (56 from Barcelona and 63 from Santander) (Supplementary Table 1). The entire sample of 329 participants included 127 (39%) unrelated participants and 202 (61%) genetically related participants who belonged to a total of 62 pedigrees (Supplementary Table 2). All participants were white Europeans of Spanish descent. Of the current sample of 329 G2019S carriers, a subset of 44 were previously genotyped for *SNCA* rs356219,[10] whereas 285 were newly recruited.

Clinical Evaluation

The patients had a clinical diagnosis of definite PD according to the UKPDS criteria,[12] except that a positive family history was not considered an exclusion criterion, or a neuropathological diagnosis of definitive PD according to proposed criteria.[13] Similar to the previous study,[8] AAO of motor symptoms was self-reported by patients.

Genotyping

We used Taqman SNP assays-on-demand on a StepOnePlus Real-time PCR System to genotype *DNM3* rs2421947 (#C-15804853-10), *SNCA* rs356219 (#C-1020193-10), and *LRRK2* G2019S (#C-63498123-10) (Thermo Fisher Scientific, Waltham, MA, USA). Call rates were 96% for *DNM3* and 97% for *SNCA*. Allelic frequencies were in Hardy-Weinberg equilibrium for *DNM3* ($P = .18$) and *SNCA* ($P = 1$) SNPs.

Cox Regression Analyses

We performed a Kaplan-Meier analysis of the cumulative incidence of PD onset using AAO as continuous time variable, censoring L2PD participants (PD affected) at documented AAO and L2NMC individuals (nonaffected) at the age at last examination, and providing a median estimation of AAO and 95% confidence intervals (CI) from this analysis. We used Cox regression to estimate disease onset risk by means of hazard ratio (HR) and 95% CI using AAO as time of follow-up and last observation of nondiagnosis of PD as a censure value for L2NMC. We tested both markers under a codominant inheritance model to evaluate specific effects of each genotype. For HR estimation, we used two Cox frailty analyses - crude estimation and gender-adjusted estimation-both with random intercepts to account for correlation of observations within families ("family" considered a random analysis factor). For the sensitivity analysis, we applied the same models using a marginal approach to account for the influence of random effects of the factor "family" in frailty Cox models ("family" considered a fixed study design factor). The analyses were made using the entire sample and stratifying by center. Type I error was fixed for 2-sided $P < .025$ Bonferroni correction for 2 analyses (*DNM3* and *SNCA*). We performed Kaplan-Meier analyses using SPSS version 20 (IBM, Armonk, NY, USA) and Cox analyses using R version 2.15.3 (package survival version 2.37-7).

Results

The Kaplan-Meier analysis of *DNM3* rs2421947 (Fig. 1A) showed a median follow-up time to PD onset of 67 years for CC carriers, 64 for GC, and 64 for GG (Table 1), with overlapping 95% CI (Fig. 1C). Frailty (family) Cox regression analysis adjusted by gender under the codominant inheritance model revealed no statistically significant effect of *DNM3* genotypes on PD onset risk (CC vs GC, $P = .94$; CC vs GG, $P = .84$; Table 1). In the same conditions as noted previously, the Kaplan-Meier analysis of *SNCA* rs356219 (Fig. 1B) showed a median cumulative

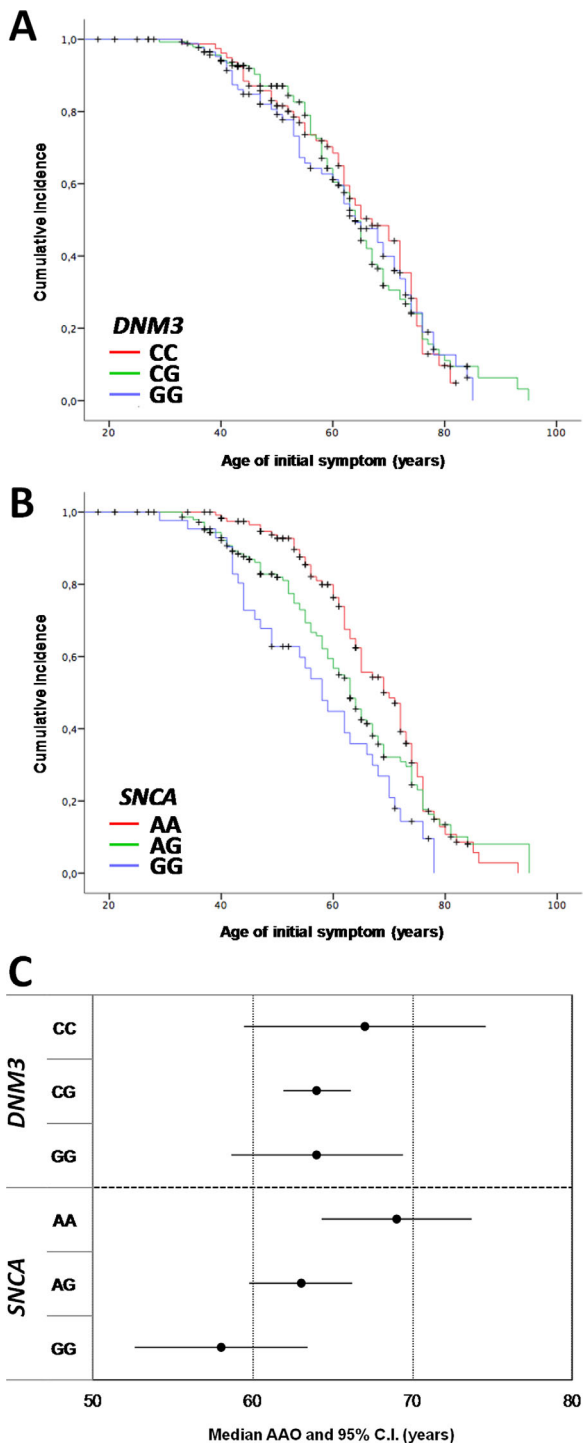

FIG. 1. Age-associated cumulative incidence of PD onset in leucine-rich repeat kinase p.G2019S carriers from Spain (PD affected and nonaffected censored at age at last examination; n = 329). Kaplan-Meier curve of cumulative incidence of PD onset stratified by *DNM3* rs2421947 genotypes (**A**) or by *SNCA* rs356165 genotypes (**B**). Median age-associated cumulative incidence of PD onset (dots) and CI (lines; **C**). AAO, age at onset; CI, confidence interval; *DNM3*, dynamin 3; *SNCA*, α-synuclein.

incidence of disease onset of 69 years for AA carriers, 63 for AG, and 58 for GG (11 years earlier) (Table 1), with distinct not-overlapping 95% CI (Fig. 1C). Frailty (family) Cox regression revealed that these AAO differences were statistically significant for GG versus AA at a Bonferroni-adjusted level of statistical significance of $P < .025$ (AA vs AG, $P = .06$; AA vs GG, $P < .001$; Table 1). Stratifying by center, we found similar HR estimations for *DNM3* and *SNCA* polymorphisms, but the *SNCA* marker did not reach statistical significance because of the reduction in sample size and statistical power (Table 1). Alternatively, akin to frailty models, we obtained largely the same association results with L2PD AAO for *SNCA*, but not for *DNM3* markers using marginal models. In addition, as complementary analysis, we performed classical linear regression considering only L2PD patients with documented AAO (Supplementary Table 3). In line with Cox regression analyses, linear regression again revealed no association of *DNM3* rs2421947, but of *SNCA* rs356219 with AAO.

Collectively, using different methods we found an association of genetic variability in the *SNCA* gene, namely rs356219, but not of *DNM3* rs2421947 with L2PD AAO in the Spanish population, which was in the order of up to 11 years AAO difference for *SNCA*.

Discussion

This is the first study investigating the association of *DNM3* rs2421947 with L2PD AAO in the Spanish population, which was first reported in Arab-Berbers.[8] Our study also aimed at extending the previously described link of *SNCA* rs356219 with L2PD AAO using a larger sample of G2019S carriers from Barcelona and from other regions in Spain (Cantabria). In our sample, we were unable to detect an association of *DNM3* rs2421947 with AAO. However, genotyping the same participants we observed a significant association of *SNCA* rs356219 with L2PD AAO in which carriers of the GG genotype developed disease 11 years earlier when compared with AA, thus confirming previous observations.[10]

The finding that *DNM3* rs2421947 does not seem to influence AAO of L2PD in Spaniards could be attributed to several factors. First, although our sample size of G2019S carriers (n = 329) was comparable to the Arab-Berber (n = 367) or the replication (n = 263) cohorts in the previous study, it could still be limited to detect subtle associations in *DNM3*. Yet using the same sample we found association of *SNCA* rs356219 with L2PD AAO. Second, the association of *DNM3* with AAO was previously confirmed in a meta-population comprising L2PD participants from Tunisia (n = 367), Algeria (n = 46), France (n = 65), Norway (n = 64), and the United States (n = 88) (HR [95% CI] = 1.61 [1.15-2.27]).[8] Still, the subpopulations from France and Norway showed no or limited association (HR = 0.71 and 1.17, respectively). Our results are consistent with these two other European populations and suggest that potential genetic heterogeneity in the meta-population might limit detection in our sample. Indeed, genetic heterogeneity in genome-wide

TABLE 1. Distribution of genotypic frequencies in *DNM3* and *SNCA* polymorphisms in PD affected and nonaffected LRRK2 p.G2019S mutation carriers, median PD AAO estimates (in years) obtained from Kaplan-Meier analysis of PD cumulative incidence (age at last examination in nonaffected), and association analysis with AAO by Cox regression analysis using frailty models, under a codominant inheritance model, adjusting by family and gender, using the entire sample or segregating by center

Genotypes	Frequency	Median AAO, years (95% CI)	HR (95% CI)	*P* value
DNM3 rs2421947(C/G)				
All (N = 329)				
CC	0.26	67 (59.4-74.6)	1	
GC	0.46	64 (61.9-66.1)	0.99 (0.69-1.41)	.94
GG	0.28	64 (58.6-69.4)	1.03 (0.69-1.53)	.84
Barcelona (N = 202)				
CC	0.22	62 (59.2-64.8)	1	
GC	0.46	60 (56.8-63.2)	0.91 (0.58-1.43)	.7
GG	0.31	60 (52.3-67.7)	0.95 (0.58-1.54)	.82
Santander (N = 127)				
CC	0.31	72 (64.7-79.3)	1	
GC	0.46	69 (62.7-75.3)	1.13 (0.61-2.08)	.7
GG	0.31	73 (70.7-75.3)	1.07 (0.53-2.20)	.85
SNCA rs356219(A/G)				
All (N = 329)				
AA	0.39	69 (64.3-73.7)	1	
AG	0.47	63 (59.8-66.2)	1.35 (0.99-1.86)	.06
GG	0.14	58 (52.6-63.4)	2.07 (1.36-3.14)	<.001
Barcelona (N = 202)				
AA	0.32	63 (60.5-65.5)	1	
AG	0.49	60 (56.6-63.4)	1.18 (0.80-1.73)	.38
GG	0.19	58 (51.4-64.6)	1.57 (0.98-2.54)	.06
Santander (N = 127)				
AA	0.49	73 (71.1-74.9)	1	
AG	0.45	67 (62.0-72.0)	1.63 (0.97-2.75)	.06
GG	0.06	55 (35.9-74.1)	1.64 (0.56-4.79)	.36

Bonferroni-adjusted level of statistical significance of $P < .025$. AAO, age at onset; CI, confidence interval; *DNM3*, dynamin 3; HR, hazard ratio of each genotype compared to reference genotype; LRRK2, leucine-rich repeat kinase 2; N, number of participants; *SNCA*, α-synuclein.

association studies in other neurodegenerative diseases such as amyotrophic lateral sclerosis has been previously related to nonreplication in independent samples.[14-16] Third, it is plausible that the *DNM3* association with AAO could be population specific and that its effect may be restricted to or more prominent in Arab-Berbers. For instance, genetic variability in *SNCA* such as rs356165 which was previously associated with PD AAO in several cohorts from Spain[10,17] has been shown not to influence AAO in Arab-Berbers,[18] thus illustrating that different genetic or environmental modifiers could influence L2PD AAO in distinct populations.[9] Fourth, it is important to highlight that genetic modifiers may constitute only one factor, but lifestyle, environmental hazards, or comorbidities are other factors that could also be involved in determining AAO. Finally, AAO was self-reported by L2PD patients in our study and in the previous study which could represent a potential source of bias.

Previously we genotyped the *SNCA* polymorphism rs356219 in L2PD patients from Barcelona carrying the G2019S mutation and reported a nearly 7-years earlier mean AAO for the GG genotype.[10] Here we validated these findings in a larger sample of newly recruited G2019S mutation carriers from Barcelona and Santander (n = 285). Another study in Spain has also reported an association of *SNCA* rs356219 with increased disease risk and differential AAO of sPD.[17] Moreover, in other European populations such as Germans, this polymorphism has also been shown to influence AAO of sPD.[11] In these studies, the G allele or the GG genotype were associated with earlier AAO of sPD. The *SNCA* rs356219 marker is located within the gene 3' UTR and has been associated with gene expression changes of the *SNCA* transcript in blood, cerebellum, and substantia nigra of PD patients.[19,20] These studies have suggested that this polymorphism or other functional SNP in linkage disequilibrium could influence L2PD AAO by determining *SNCA* expression levels. Similarly, we cannot exclude that other *SNCA* polymorphisms in linkage disequilibrium with rs356219 could be responsible for the association with L2PD AAO, and thus elucidating the true functional variant or variants involved in AAO requires further studies. Yet in summary, our study has limitations, including a limited sample size compared to sPD studies, the AAO data self-reported by patients, and a limited number of genotyped candidate SNPs.

Our results indicate that *SNCA* rs356219 or another functional variant in linkage disequilibrium, but not *DNM3* rs2421947, could represent a modifier of G2019S penetrance and AAO of L2PD. This study suggests that distinct genetic modifiers may influence L2PD AAO in different populations. Further studies are warranted to identify the specific genetic modulators of G2019S expressivity in different worldwide populations. ■

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The Barcelona LRRK2 Study Group: Parkinson's Disease and Movement Disorders Unit, Neurology Service, Institut de Neurociències, Hospital Clínic de Barcelona, University of Barcelona, Barcelona (Eduard Tolosa, María-José Martí, Yaroslau Compta, Francesc Valldeoriola, and Esteban Muñoz); Neurology Service, Hospital Universitari Germans Trias i Pujol, Badalona (Lourdes Ispierto, Dolores Vilas, and Ramiro Álvarez); Neurology Service, Hospital Universitari Vall D'Hebron, Barcelona (Oriol De Fàbregues, and Jorge Hernández-Vara); Neurology Service, Hospital Del Mar, Barcelona (Víctor Puente); Neurology Service, Hospital Universitari de Bellvitge, Barcelona (Matilde Calopa, Serge Jauma, and Jaume Campdelacreu); Parkinson's Group, Clínica Teknon, Barcelona (Angels Bayés); Neurology Service, Hospital de Sant Joan d'Espí - Moises Brogi, Sant Joan d'Espí (Asunción Ávila, and Nuria Caballol); Neurology Service, Hospital Universitari Mutua de Terrasa, Barcelona (Miquel Aguilar, and Pau Pastor); Hospital Mateu Orfila, Maó, Menorca (Pilar Casquero).

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Appendix

The Barcelona LRRK2 Study Group: Parkinson's Disease and Movement Disorders Unit, Neurology Service, Institut de Neurociències, Hospital Clínic de Barcelona, University of Barcelona, Barcelona (Eduard Tolosa, María-José Martí, Yaroslau Compta, Francesc Valldeoriola, and Esteban Muñoz); Neurology Service, Hospital Universitari Germans Trias i Pujol, Badalona (Lourdes Ispierto, Dolores Vilas, and Ramiro Álvarez); Neurology Service, Hospital Universitari Vall D'Hebron, Barcelona (Oriol De Fàbregues, and Jorge Hernández-Vara); Neurology Service, Hospital Del Mar, Barcelona (Víctor Puente); Neurology Service, Hospital Universitari de Bellvitge, Barcelona (Matilde Calopa, Serge Jauma, and Jaume Campdelacreu); Parkinson's Group, Clínica Teknon, Barcelona (Angels Bayés); Neurology Service, Hospital de Sant Joan d'Espí - Moises Brogi, Sant Joan d'Espí (Asunción Ávila, and Nuria Caballol); Neurology Service, Hospital Universitari Mutua de Terrasa, Barcelona (Miquel Aguilar, and Pau Pastor); Hospital Mateu Orfila, Maó, Menorca (Pilar Casquero).

Supporting Data

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

Article 2 –

Imaging dopamine function and microglia in asymptomatic *LRRK2* mutation carriers.

Garrido A*, Gersel Stokholm M*, Tolosa E, Serradell M, Iranzo A, Østergaard K, Borghammer P, Møller A, Parbo P, Stær K, Brooks DJ, Martí MJ, Pavese N.

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ORIGINAL COMMUNICATION

Imaging dopamine function and microglia in asymptomatic LRRK2 mutation carriers

Morten Gersel Stokholm[1] · Alicia Garrido[2,3] · Eduardo Tolosa[2,3,4] · Mónica Serradell[3] · Alex Iranzo[3,4] · Karen Østergaard[5] · Per Borghammer[1] · Arne Møller[1] · Peter Parbo[1] · Kristian Stær[1] · David J. Brooks[1,6] · Maria José Martí[3,4] · Nicola Pavese[1,6]

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Abstract

Neuroinflammation (microglial activation) and subclinical nigrostriatal dysfunction have been reported in subjects at risk of Parkinsonism. Eight non-manifesting carriers (NMCs) of LRRK2 G2019S mutation had ^{11}C-PK11195 and ^{18}F-DOPA PET to assess microglial activation and striatal dopamine system integrity, respectively. Comparisons were made with healthy controls. Five LRRK2-NMCs had subclinical reductions of putaminal ^{18}F-DOPA uptake. Three of them had significantly raised nigral ^{11}C-PK11195 binding bilaterally. These findings indicate that nigrostriatal dysfunction and neuroinflammation occur in LRRK2-NMCs. Studies in larger cohorts with appropriate follow-up are needed to elucidate the significance of neuroinflammation in the premotor phase of LRRK2-PD.

Keywords Genetics · Parkinson's disease · Clinical neurology · PET · Movement disorders

Mutations in the leucine-rich repeat kinase 2 gene (LRRK2) are known to cause inherited Parkinson's disease (PD). LRRK2-associated PD (LRRK2-PD) presents with a low penetrant autosomal-dominant inheritance pattern with a cumulative risk of developing PD ranging from 26 to 80% at the age of 80 years [1, 2]. Patients with the G2019S mutation, the most common mutation in LRRK2-PD, are clinically indistinguishable from idiopathic PD and most cases present similar pathological findings with degeneration of dopaminergic neurons in the substantia nigra and occurrence of Lewy-type α-synuclein pathology [3]. Studying non-manifesting carriers (NMCs) of the LRRK2 G2019S mutation provides an opportunity to identify pathophysiological processes occurring in the premotor phase of genetic PD. This is essential to identify biomarkers and therapeutic targets to halt disease progression.

Microglia, the major resident immune cells of the central nervous system, monitors the brain milieu in their resting physiologic state, but when activated by injury, they can express both neuroprotective and cytotoxic phenotypes. It is hypothesized that a pro-inflammatory phenotype, leading to neuronal dysfunction, may drive neurodegeneration [4]. Microglial activation has been observed in idiopathic PD [5], but its role in the pathophysiology of genetic PD is unknown. A recent study using positron emission tomography (PET) imaging demonstrated increased microglial activation in the substantia nigra along with putaminal dopaminergic dysfunction in patients with idiopathic rapid eye movement sleep behavior disorder [6], a condition that may progress over time to a neurodegenerative alpha-synucleinopathy [7].

Morten Gersel Stokholm and Alicia Garrido contributed equally to the manuscript.

✉ Nicola Pavese
npavese@cfin.au.dk

[1] Department of Nuclear Medicine and PET Centre, Aarhus University Hospital, Noerrebrogade 44, bldg. 10G, 8000 Aarhus C, Denmark

[2] Movement Disorders Unit, Neurology Service, Hospital Clínic de Barcelona, Barcelona, Spain

[3] Centro de Investigación Biomédica en Red Sobre Enfermedades Neurodegenerativas (CIBERNED), Hospital Clínic, IDIBAPS, Universitat de Barcelona, Barcelona, Spain

[4] Department of Neurology, Hospital Clínic de Barcelona, Barcelona, Spain

[5] Department of Neurology, Aarhus University Hospital, Aarhus C, Denmark

[6] Division of Neuroscience, Newcastle University, Newcastle upon Tyne, England

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The present study aimed to investigate whether raised levels of microglial activation and alterations of the nigrostriatal dopaminergic function occur as well in LRRK2-NMCs.

Methods

Eight LRRK2-NMCs were recruited from Hospital Clínic de Barcelona between September 2016 and May 2018. Inclusion of LRRK2-NMC was based on the absence of Parkinsonism and availability to perform the study. All NMC had a 60.5-min ^{11}C-(R)-PK11195 PET scan (ECAT HRRT; CTI/Siemens, Knoxville, TN, USA) to assess levels of microglial activation (expressing 18-kDa translocator protein) and a 94.5-min ^{18}F-DOPA PET scan to assess the integrity of the dopaminergic system. Each subject had a T1-weighted MRI (3 T MAGNETOM Skyra, Siemens Healthcare, Germany) performed for co-registration of PET images. PET findings were compared with those of 29 healthy controls (^{11}C-PK11195 PET $n=20$, ^{18}F-DOPA PET $n=9$). Control PET scans were acquired for two former projects using the exact same scanner and scan protocols [6, 8]. All participants were examined to exclude Parkinsonism. Assessments with the MDS-UPDRS part III, and Mini-Mental State Examination were also performed. All the assessments of this study were performed at the Department of Nuclear Medicine and PET Centre, Aarhus University Hospital.

Volumes of interest (VOI) sampled included the substantia nigra (^{11}C-PK11195 PET only) plus putamen and caudate nucleus for both PET tracers. Image analysis was performed as previously described [6]. Briefly, parametric images of ^{11}C-PK11195 binding potentials (BP_{ND}) were generated using a simplified reference tissue model with an individual tissue non-specific input function extracted with a supervised cluster-analysis approach. Similarity between the ^{11}C-PK11195 reference input function extracted from LRRK2-NMCs and controls was confirmed with a repeated measurement analysis (χ^2 test, $p>0.05$). In those VOI's where ^{11}C-PK11195 binding is lower than that of the selected reference tissue voxels, this model computes a negative BP_{ND}. ^{18}F-DOPA influx constant (Ki) images were generated using the Patlak graphical approach with the occipital lobe as the non-specific uptake reference region.

Group differences in ^{11}C-PK11195 BP_{nd} and ^{18}F-DOPA Ki were interrogated with unpaired Student's t test ($p<0.05$), normal distribution of data was checked with normal probability plots and D'Agostino–Pearson normality test. For descriptive purposes, individually *raised* regional ^{11}C-PK11195 binding and *reduced* regional ^{18}F-DOPA uptake was defined as a statistically significant deviation from the controls mean value (left and right side averaged) of two or more standard deviations (z score ≥ 2 and z score ≤ -2). Statistical analysis and graphical presentations were performed in Stata IC 14.2 (StataCorp LP, TX, USA) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

Results

Eight LRRK2-NMCs, 7 men, with a mean UPDRS part III score of 2.6 (SD;1.6) and a mean age of 55.8 years (range 39.8–73.4), were compared to 20 ^{11}C-PK11195 controls (12 men, mean age 66.8 years, range 58–80) and 9 ^{18}F-DOPA controls (9 men, mean age 64.6 years, range 59.9–69.5). The left–right averaged putaminal ^{18}F-DOPA uptake was significantly ($p=0.006$) reduced by −9.2% (range −16.9% to 4.7%) in LRRK2-NMCs compared to controls, while caudate ^{18}F-DOPA uptake was similar ($p=0.468$). No group differences were observed in VOI analysis with ^{11}C-PK11195 PET (substantia nigra $p=0.075$, putamen $p=0.678$, caudate nucleus $p=0.695$).

Five out of eight LRRK2-NMCs had *reduced* ^{18}F-DOPA uptake in putamen (z score ≤ -2), four unilateral and one bilateral (Table 1; Fig. 1). Three out of eight LRRK2-NMC had bilaterally *raised* levels of ^{11}C-PK11195 binding in the substantia nigra (z score ≥ 2), coincidental with unilaterally *reduced* putaminal ^{18}F-DOPA uptake (z score ≤ -2) in two and bilaterally reduced in one (Table 1; Fig. 1). One subject had unilateral *raised* putaminal ^{11}C-PK11195 binding without ipsilateral *reduced* putaminal ^{18}F-DOPA uptake (Table 1).

Discussion

This PET imaging study performed in LRRK2-NMCs found *reduced* putaminal ^{18}F-DOPA uptake in five out of eight LRRK2-NMCs as previously reported in subjects without manifest PD carrying different subtypes of LRRK2 mutations [9]. Three carriers in our cohort had bilaterally raised substantia nigra ^{11}C-PK11195 binding, indicating microglial activation, together with reductions in putaminal ^{18}F-DOPA uptake (unilaterally *reduced* in two subjects and bilaterally in one). To our knowledge, this is the first report of activated microglia in LRRK2-NMC.

While not in all cases, a reduced putaminal ^{18}F-DOPA uptake without increased ipsilateral nigral ^{11}C-PK11195 binding was observed (subject 4 and 5; Fig. 1), suggesting that in LRRK2-NMC, dysfunction of putaminal dopaminergic axonal terminals may antedate microglial activation in nigral cell bodies, presumed to be related with neurodegeneration [4]. A similar pattern (normal range substantia nigra ^{11}C-PK11195 binding and reduced ipsilateral putaminal ^{18}F-DOPA uptake) has been reported previously in occasional subjects with prodromal PD [6] and manifest idiopathic PD patients [5]. The significance of microglial

Table 1 ^{11}C-PK11195 BP_{nd} and ^{18}F-DOPA Ki in controls and non-manifesting LRRK2 carriers

	^{11}C-PK11195 BP_{nd}		^{18}F-DOPA Ki	
	Controls ($n=20$)	LRRK2-NMCs ($n=8$)	Controls ($n=9$)	LRRK2-NMCs ($n=8$)
Group analysis				
Substantia nigra	−0.006 (0.09)	0.08 (0.16)	–	–
Putamen	0.08 (0.11)	0.06 (0.13)	0.0131 (0.00069)	0.0120* (0.00087)
Caudate	−0.15 (0.12)	−0.17 (0.13)	0.0113 (0.00085)	0.0120 (0.0017)

	Reduced putamen ^{18}F-DOPA Ki	*Raised* substantia nigra ^{11}C-PK11195 BP_{nd}	*Raised* putamen ^{11}C-PK11195 BP_{nd}	UPDRS-III	MMSE
Individual analysis					
Carrier 1				0	28
Carrier 2				5	30
Carrier 3	○	●		4	30
Carrier 4	○			3	30
Carrier 5	○			2	29
Carrier 6				3	30
Carrier 7	●	●		3	29
Carrier 8	○	●	○	1	26

Values from both left and right side are averaged in the group analysis. Values are mean and standard deviation in brackets. *$p<0.05$ in group analysis

Unilateral (○) or bilateral (●) *reduced* putaminal ^{18}F-DOPA Ki value (z score ≤ -2) and/or *raised* substantia nigra ^{11}C-PK11195 BP_{ND} (z score ≥ 2) in individual carriers

NMCs non-manifesting carriers, *UPDRS-III* Unified Parkinson's Disease Rating Scale—part three, *MMSE* Mini-Mental State Examination

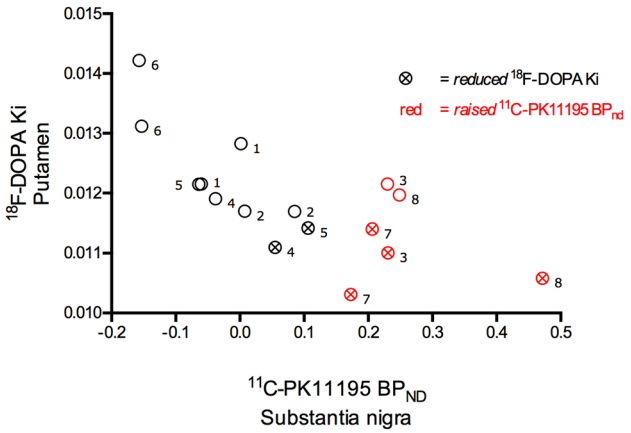

Fig. 1 ^{18}F-DOPA uptake in putamen and ipsilateral ^{11}C-PK11195 binding in substantia nigra in non-manifesting LRRK2 G2019S carriers. Values from left and right side in eight non-manifesting LRRK2 G2019S carriers are depicted. ⊗ indicates significantly reduced ^{18}F-DOPA Ki values, which are two or more standard deviations below the average mean of controls (z score ≤ -2). Red colour indicates significantly raised ^{11}C-PK11195 BP_{ND} two or more standard deviations above the average mean of controls (z score ≥ 2). Observations from each individual carrier are marked with numbers from one to eight, which corresponds to their number in Table 1, individual analysis

activation in the pathophysiology of PD is yet to be established. The findings in the current study, together with those found in a previous one examining prodromal PD [6] support the concept that similar alterations occur in the premotor phase in both genetic and idiopathic forms of the disease. Microglial cells are able to express either a neuroprotective or a cytotoxic phenotype [4]. Which of these is occurring in the examined LRRK2-NMC can only be determined with PET tracers binding specifically to each microglia phenotype; however, this kind of in vivo tracer is currently not available.

There are limitations to this study to be considered. First, the small number of cases examined may preclude the generalization of the results. Second, the LRRK2-NMC included in this study are younger than the controls which may account for a selection bias. Considering the age-related and low and penetrance of LRRK2 G20109S mutation [1, 2], it is possible that we have studied subjects who will never develop PD or at a very early phase of the disease when biochemical and pathological events have not yet started [10]. However, the alteration of ^{18}F-DOPA PET in five LRRK2-NMC indicates that in most of LRRK-NMC in this cohort, pathological changes are already occurring. Given that age is the greatest risk factor for developing LRRK2-PD, we investigated whether PET findings were more likely to occur in the eldest LRRK2-NMC. We found that age did not correlate with PET alterations in the studied cohort, suggesting that other factors may influence ^{18}F-DOPA dysfunction and microglial activation (individual data including age, not shown to avoid subject identification). Third, as previously

reported in animal models [11], levels of microglial activation in PD may be influenced by the presence of pathological α-synuclein inclusions. In LRRK2 G2019S PD, a pleiotropic neuropathology has been reported, including subjects without Lewy-type α-synuclein pathology [3, 12]. This pathological variability may possibly influence the heterogeneity of the results. Finally, ^{11}C-PK11195 may not only bind to the 18-kDa translocator protein located on mitochondria inside the activated microglial cells but also on astrocytes; however, observations show that this only accounts for minor degree of ^{11}C-PK11195 signal [13].

This is the first study performed in LRRK2-NMCs showing dopamine dysfunction in five out of eight subjects and concomitant nigral microglial activation in three of them. These results suggest that PET imaging with ^{18}F-DOPA and ^{11}C-PK11195 could help identify NMCs with ongoing nigrostriatal pathology and show that at least in some cases, neuroinflammation could play a role in the pathophysiology of early phases of LRRK2-PD. The contribution of the observed in vivo microglial response and its clinical relevance at individual level still needs to be elucidated. Prospective studies using similar PET tracers in a larger study population with appropriate clinical follow-up may clarify these issues.

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Author contributions Study design: NP, ET, MGS, and AG. Data acquisition: MGS, AG, MS, PP, KS, MJM, and NP. All authors contributed to data analyses and the writing of the manuscript. We confirm that all authors have contributed to this work, and have read and approved the manuscript.

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Compliance with ethical standards

Conflicts of interest ET reports grants from the MJFox Foundation and the Instituto de Salud Carlos III. KØ reports grants from The Danish Parkinson Association, The Danish Council for Independent Research, and Lundbeck Foundation, during the conduct of the study and personal fees from Medtronic Inc., UCB, Fertin Pharma, and AbbVie outside the submitted work. DJB reports grants from The Danish Council for Independent Research, Lundbeck Foundation, The Danish Parkinson Association, European Union FP7 programme, and Alzheimer Research UK and personal fees from GE Healthcare and Plexxikon outside the submitted work. NP reports grants from The Danish Council for Independent Research during the conduct of the study. The other authors declare no competing interests.

Ethical approval The local Ethics Committee at both centres, Aarhus and Barcelona, approved the study and all participants gave written informed consent according to the Declaration of Helsinki before study enrollment.

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Article 3 –

α-Synuclein RT-QuIC in cerebrospinal fluid of LRRK2-linked Parkinson's disease.

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RESEARCH ARTICLE

α-synuclein RT-QuIC in cerebrospinal fluid of LRRK2-linked Parkinson's disease

Alicia Garrido[1,2], Graham Fairfoul[3], Eduardo S. Tolosa[2,4], Maria José Martí[1,2,4], Alison Green[3], & on behalf of the Barcelona LRRK2 Study Group[†]

[1]Parkinson's Disease and Movement Disorders Unit, Institut Clínic de Neurociències, Hospital Clinic de Barcelona, Barcelona, Spain
[2]Centre for Networked Biomedical Research on Neurodegenerative Diseases (CIBERNED), Madrid, Spain
[3]The National CJD Research & Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh EH4 2XU, United Kingdom
[4]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona (UB), Barcelona, Spain

Correspondence
Eduardo Tolosa, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona (UB), C/Villarroel 170, Barcelona 08036, Spain. Tel: 3493-227-5785; Fax: 3493-227-5783; E-mail: etolosa@clinic.ub.es

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[†]Barcelona LRRK2 Study group members are listed in the Acknowledgments.

Abstract

Background: Leucine-rich kinase 2 (LRRK2)-linked Parkinson's disease (PD) is clinically indistinguishable from idiopathic PD (IPD). A pleiotropic neuropathology has been recognized but the majority of studies in LRRK2 p.G2019S patients reveal Lewy-type synucleinopathy as its principal histological substrate. To date no in vivo biomarkers of synucleinopathy have been found in LRRK2 mutation carriers. **Objectives**: We used real-time quaking-induced conversion (RT-QuIC) technique to assess the presence of alpha-synuclein (a-syn) aggregates in cerebrospinal fluid (CSF) of LRRK2 p.G2019S carriers. **Methods**: CSF samples of 51 subjects were analyzed: 15 LRRK2 p.G2019S PD, 10 IPD, 16 LRRK2 p.G2019S nonmanifesting carriers (NMC) and 10 healthy controls. The presence of parkinsonism and prodromal symptoms was assessed in all study subjects. **Results**: Forty percent ($n = 6$) LRRK2-PD, and 18.8% ($n = 3$) LRRK2-NMC had a positive a-syn RT-QuIC response. RT-QuIC detected IPD with 90% sensitivity and 80% specificity. No clinical differences were detected between LRRK2-PD patients with positive and negative RT-QuIC. A positive RT-QuIC result in LRRK2-NMC occurred in a higher proportion of subjects meeting the Movement Disorder Society research criteria for prodromal PD. **Interpretation**: RT-QuIC detects a-syn aggregation in CSF in a significant number of patients with LRRK2-PD, but less frequently than in IPD. A small percentage of LRRK2-NMC tested also positive. If appropriately validated in long-term studies with large number of mutation carriers, and hopefully, postmortem or in vivo confirmation of histopathology, RT-QuIC could contribute to the selection of candidates to receive disease modifying drugs, in particular treatments targeting a-syn deposition.

Introduction

Pathogenic mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are one of the most frequent causes of inherited Parkinson's disease (PD).[1,2] Among the different mutations in this gene, p.G2019S is the most frequently found in the population worldwide accounting for 1% of sporadic and 4% of familial PD patients.[2] LRRK2-linked PD (LRRK2-PD) presents with an autosomal-dominant inheritance pattern with incomplete penetrance. The cumulative risk of developing PD in LRRK2 p.G2019S cases is age dependant and varies widely depending on the studies, from 25% to 42.5% at age 80 years[3,4] and currently is not possible to identify which subjects will develop the disease. The clinical picture of LRRK2-PD is largely indistinguishable from that of idiopathic PD (IPD).[2,5] Aggregated alpha-synuclein (a-syn) in the form of Lewy-type pathology[6] in the nervous tissue, similar to that encountered in IPD, has been reported to occur in a majority of LRRK2-PD p.G2019S cases, but LRRK2-PD can have a pleiotropic neuropathological substrate.[6–11] Kalia and colleagues have suggested that the occurrence

of nonmotor symptoms such as cognitive impairment/dementia, and anxiety is related to an underlying Lewy-type synucleinopathy (LTS) in LRRK2-PD p.G2019S cases,[9] however, it is currently not possible to reliably identify these cases in vivo.

Seeking for evidence of abnormal a-syn aggregation in different body tissues and fluids of patients with suspected synucleinopathy has been one of the main strategies in PD biomarkers research during the last years.[12–15] Recently, an assay to detect a-syn oligomers in cerebrospinal fluid (CSF), the real-time quaking-induced conversion (RT-QuIC) test, has proven to identify PD patients with high sensitivity and specificity.[16] This technique, previously used as a diagnostic tool in diseases caused by prion proteins, is based on the capacity of misfolded a-syn to induce the aggregation of monomeric species of a-syn.[17,18] Fairfoul and colleagues demonstrated in CSF of pathologically confirmed patients with Lewy body disease a positivity of the test in 92% of cases, whereas none of the controls were positive. A validation cohort of clinically diagnosed PD subjects resulted in a sensitivity of the test of 95% and a specificity of 100%. Shahnawaz and colleagues have equally demonstrated high sensitivity and specificity of a similar assay, based on aggregated a-syn amplification in the CSF, for the detection of PD subjects.[19] In the study by Fairfoul et al. three subjects with idiopathic rapid eye movement sleep behavior disorder (RBD), considered a prodromal stage of PD[20] also presented abnormal aggregation of a-syn in CSF, suggesting that this test could be used as a marker of premotor PD.

The objective of our study was to assess the presence of a-syn aggregation in CSF of LRRK2 mutation carriers using RT-QuIC. Detection of such aggregates could be indicative of the presence of an ongoing synucleinopathy. Patients with manifest LRRK2-PD and a positive test could eventually be candidates to receive disease modifying drugs, in particular treatments directed against a-syn. In LRRK2 nonmanifesting carriers (NMC) the identification of a-syn in CSF may possible identify subjects who will develop the motor syndrome of PD.

Methods

Patient groups and clinical assessment

This study was carried out in the Neurology Service at the Hospital Clinic of Barcelona, in collaboration with the National CJD Research & Surveillance Unit in Western General Hospital, University of Edinburgh. The local ethics committee approved all clinical protocols and written informed consent was obtained from all the participants prior to enrollment.

We analyzed 51 CSF samples from LRRK2-PD ($n = 15$) and LRRK2-NMC ($n = 16$) carrying the p.G2019S mutation, IPD ($n = 10$) and HC ($n = 10$). IPD and LRRK2-PD subjects were identified and recruited in the Movement Disorders Unit from the Hospital Clínic of Barcelona and collaborating centres of the Barcelona LRRK2 study group. LRRK2-NMC were identified among the relatives of LRRK2-PD subjects. All LRRK2 mutation carriers ($n = 31$) of our cohort belong to 21 different families. None of the 15 LRRK2-PD cases are related. Among the 16 LRRK2-NMC, eight subjects are relative to six LRRK-PD, and eight subjects are not and belong to six different families. Genetic analysis was performed as previously described.[21] DNA was extracted from peripheral blood following standard procedures. Genotyping of the G2019S mutation was performed using the predesigned TaqMan assay C-63498123-10 SNP rs34637584 and the R1441G/C/H mutation using a commercial TaqMan assay. Genotyping was performed on a StepOnePlus Real-time PCR System (Life Tech. Inc.). CSF samples of 10 HC subjects were obtained for comparative purposes among subjects undergoing a surgical procedure in our institution that required an epidural anesthesia.

All the participants of this study were comprehensively assessed for demographic and clinical characteristics. Patients with IPD and LRRK2-PD were diagnosed clinically according to UK PD Society Brain Bank criteria[22] and recruited between May 2012 and October 2017. At the time of inclusion to the study, none of the LRRK2-NMC and none of the healthy controls presented signs of parkinsonism. The severity of parkinsonism was assessed with the Movement Disorders Society (MDS) sponsored revision of the Unified Parkinson's Disease Rating Scale part III (MDS-UPDRS-III),[23] the Hoehn and Yahr (H&Y) stage,[24] and the Schwab and England scale (S&E)[25] Nonmotor symptoms were assessed using the MDS-UPDRS part I and specific validated questionnaires for each symptom. For evaluation of depression the Geriatric Depression Scale-15[26] or the Beck Depression Inventory-II[27] were used; dysautonomic symptoms were evaluated with the Scale for Autonomic Function (SCOPA-AUT)[28]; olfaction was assessed using the Spanish version of the University of Pennsylvania Smell Test (UPSIT).[29] The presence of RBD was based on the RBD screening questionnaire[30] and the Epworth Sleepiness Scale[31] was used to evaluate somnolence.

All LRRK2-NMC consented to undergo a dopamine transporter (DaT) SPECT (^{123}I-ioflupane) in less than 30 days from the lumbar puncture. SPECT images were acquired in a dual-headed gamma camera (E-Cam, Siemens), 64 images/head, with a matrix of 64 × 64. SPECT images were classified visually by the local nuclear medicine team of the hospital nuclear medicine physicians as

normal when a symmetric intense tracer uptake in striatum both caudate nucleus and putamen was observed. If a unilateral or bilateral reduction was observed in the striatal tracer uptake, the DAT-SPECT was considered as abnormal.[32] We also calculated the likelihood ratio and posttest probability for conversion to manifest PD for each LRRK2-NMC, based on the MDS research criteria for prodromal PD as described elsewhere.[33,34] We included the available risk markers (age, sex, genetic status, and habits) and prodromal markers (subtle motor and nonmotor symptoms with the previously published cutoffs and DaT SPECT results) for each subject. LRRK2 carriers have been followed up prospectively and 12 of 16 NMC have undergone additional clinical evaluations during 2018.

CSF collection and handling

CSF collection protocol has been described elsewhere.[35] Briefly, all the participants underwent the lumbar puncture after 8-hour fast. Lumbar puncture was performed using 20 or 22G needle, after local anesthesia. CSF was collected into siliconized polypropilene tubes. 15–20 mL of CSF were collected at room temperature, centrifuged at 2000*g* for 10 min, then transferred to 1.5 mL precooled siliconized polypropilene aliquot tubes and immediately freezed on dry ice. The frozen aliquots were stored at −80°C, until March 2018, when thawed, aliquoted into coded 0.5–1 mL siliconized polypropilene tubes, refrozen and transferred on dry ice from Barcelona to the University of Edinburgh (Scotland, UK) for the study described here.

Real-time quaking-induced aggregation for alpha-synuclein

The CSF samples were analyzed and reported without prior knowledge of the clinical diagnosis and genetic status of the donor. The RT-QuIC reaction buffer (RB) was composed of 100 mmol/L phosphate buffer (pH 8.2), 10 μmol/L Thioflavin T (ThT) and 0.1 mg/mL human recombinant full-length (1–140aa) a-syn (Stratech, Cambridge, UK). Each well of a black 96-well plate with a clear bottom (Nalgene Nunc International, Fisher Scientific Ltd, UK) contained 98, 90, or 85 μL RB (depending on volume of seed added) and 37 $\pm$ 3 mg of 0.5 mm zirconium/silica beads (Thistle Scientific Ltd, Glasgow, UK). Reactions were seeded with 2 μL of working strength brain homogenate, 15 μL of undiluted CSF to a final reaction volume of 100 μL. The plates were sealed with a plate sealer film (Fisher Scientific Ltd, UK) and incubated in a BMG OPTIMA FluoSTAR plate reader at 30°C for 120 h with intermittent shaking cycles: double orbital with 1 min shake (200 rpm), 14 min rest. ThT fluorescence measurements (450 nm excitation and 480 nm emission) were taken every 15 min. Each sample was run in duplicate. A positive response was defined as a relative fluorescence unit (rfu) value of >2SD above the mean of the negative controls at 120 h in both of the CSF duplicates. If only one of two CSF sample replicates gave a positive response, the RT-QuIC analysis of the CSF samples was repeated in quadruplicate. A positive response in two or more of the replicates was considered positive. If only one of the replicates was positive, the RT-QuIC was considered to be negative. The final fluorescence value was the mean fluorescence value taken at 120 h. The maximal fluorescence value was the highest mean fluorescent value seen during the a-syn RT-QuIC analytical run of 120 h. A measure of the lag-phase was taken to be the time it took to get to 50% aggregation as measured by a rfu value of 44,000.

Statistical analysis

Qualitative variables were described by absolute and relative frequencies (%) and analyzed by the Fisher exact test. Quantitative variables were described by median and interquartile range (25th and 75th percentiles) and analyzed using the Kruskal–Wallis test for overall comparisons and the Mann–Whitney *U* test for pairwise comparisons.

Results

Compared to IPD, LRRK2-PD subjects were younger (55 [48–64] years vs. 63.5 [57.5–70] years; $P = 0.007$), age at onset of motor symptoms was earlier (49 [44–57] years vs. 57.5 [50–62]; $P = 0.03$) and disease duration was shorter (5 [3–6] vs. 8 [5.5–8]; $P = 0.01$). There were no differences in the motor features, nonmotor symptoms or disease severity between LRRK2-PD and IPD. The LRRK2-NMC group was younger than the control group (51.5 [38.5–64] vs. 68.5 [59.5–72.2]; $P = 0.01$).

After decoding the CSF samples, the results showed that nine of 10 subjects in the IPD group and two of 10 in the control group were positive for RT-QuIC reaction, obtaining a sensitivity of the test of a 90%, and a specificity of 80%. Six of 15 LRRK2-PD subjects had positive results resulting in a sensitivity of 40% and three of 16 LRRK2-NMC (18.8%) also tested positive (Table 1). Of the six LRRK2 patients that were positive, three had lag-phases and final fluorescence values that were indistinguishable from the majority of the IPD group with the remaining three patients having lag-phases that were longer and final fluorescence value that were less than the majority of the IPD group (Fig. 1). None of the LRRK2

mutation carriers with a positive RT-QuIC result were related. Two of the three LRRK2-NMC with a positive RT-QuIC had LRRK2-PD relatives with a negative RT-QuIC result.

The medical records of the IPD subject with a negative result were subsequently reviewed. This subject's motor symptoms started at the age of 50 years, with asymmetric rest tremor and bradykinesia. However, he has atypical features like poor levodopa response, absence of motor fluctuations or levodopa-induced dyskinesias, and development of prominent lateralized trunkal dystonia (Pisa syndrome). He has bilaterally reduced striatal DaT binding and a normal brain magnetic resonance. One of the two control subjects with positive RT-QuIC reaction has developed an action tremor of the hands and was diagnosed of having an action tremor of unknown cause 2 years after his participation in the study, and presents now with cognitive complains. The rest of control subjects have not developed symptoms or signs suggestive of a neurodegenerative disease.

No statistically significant differences were observed on MDS-UPDRS part I, II, and III scores, H&Y stage, S&E and UPSIT scores between LRRK2-PD with positive or negative RT-QuIC tests (Table 2) and LRRK2-NMC with positive and negative RT-QuIC result (Table 3). Two LRRK2-NMC, both with a negative RT-QuIC result, reported to have vivid dreams and mobilization while sleeping, but no video-polisomnography was performed. Five of the 16 LRRK2-NMC had abnormal UPSIT scores after adjusting for age and gender; of these two had positive and three negative RT-QuIC results. Two of three subjects with positive RT-QuIC result (66.7%) had an abnormal DaT-SPECT, whereas only two of 13 (15.4%) who were negative had altered striatal DaT binding (Table 3). Three NMC, all with an altered DaT SPECT met MDS criteria for probable prodromal PD (>80% of threshold); two had positive a-syn RT-QuIC result.

Twelve of 16 LRRK2-NMC, including the three positive RT-QuiC cases, were clinically re-evaluated during 2018, after a mean of 36.2 months (s = 21.6 months) of follow-up, and none of them has motor symptoms suggestive of clinical PD. Only one LRRK2-NMC has mild bradykinesia on the evaluation after 31 months. This subject has negative RT-QuIC, but an abnormal nigrostriatal function on the DaT-SPECT at the time of CSF collection, and meets the criteria for probable prodromal PD.

Table 1. Prevalence of CSF positive a-syn RT-QuIC test, lag-phase and final fluorescence values in IPD, LRRK2-PD, LRRK2-NMC, and healthy controls.

Subject group (n)	Positive RT-QuIC [% (n)]	Lag-phase in positive RT-QuIC (hours)[2]	Final fluorescence in positive RT-QuIC (rfu[1])[2]
IPD (10)	90 (9)	132 ± 20	60955 ± 7685
LRRK2-PD (15)	40 (6)	151 ± 33	50402 ± 13862
LRRK2-NMC (16)	18.8 (3)	125 ± 51	41228 ± 21852
HC (10)	20 (2)	120 ± 40	49016 ± 17578

CSF, cerebrospinal fluid. IPD, idiopathic Parkinson's disease. LRRK2-PD, Leucine-rich repeat kinase 2 Parkinson's disease; LRRK2-NMC, Leucine-rich repeat kinase 2 nonmanifesting carriers; HC, Healthy controls.

[1]rfu, relative fluorescence unit.

[2]Mean ± SD.

Discussion

Our study confirms that a-syn aggregation in CSF using RTQuIC identifies IPD with high specificity (90%) and sensitivity (80%). Positive a-syn aggregation in the CSF occurred in a significant number of patients with LRRK2-PD p.G2019S but less frequently than in patients with IPD.

Based on our results in IPD and previously published data referred above[16] it seems safe to presume that our LRRK2-PD subjects with a positive RTQuIC result have an underlying LTS. The percentage of positive subjects among the LRRK2-PD group is lower than in IPD and lower than expected since a majority of LRRK2 p.G2019S cases are thought to have LTS.[6] This lower percentage of positive RT-QuIC in the LRRK2 cases could reflect the reported neuropathological variability in LRRK2-PD p.G2019S. RT-QuIC negative cases may not have LTS but an alternative neuropathological substrate. Nonspecific degeneration of the substantia nigra without abnormal protein deposits has been described in LRRK2-PD p.G2019S and still in other cases ubiquitin or tau inclusions constitute the only abnormality.[36–39] Studies with larger cohorts of prospective brains of LRRK2 mutation carriers have not been performed and the current assumption that the majority of LRRK2 p.G2019S have a synucleinopathy is based on limited case reports and screens from LB specimens in brain banks,[8] being possibly confounded by a selection bias.

It is also possible that CSF a-syn seeds in LRRK2-PD patients differ from those with IPD in their potency to induce fibril formation and this could be altering the RT-QuIC result. In our LRRK2-PD cases with a positive RT-QuIC, three subjects had longer lag-time phases and lesser final fluorescence values compared to IPD (Fig. 1) supporting this hypothesis. Mamais et al.[40] have shown distinct biochemical properties of aggregated a-syn in LRRK2-PD p.G2019S compared to IPD, detecting a lower percentage of highly insoluble a-syn species

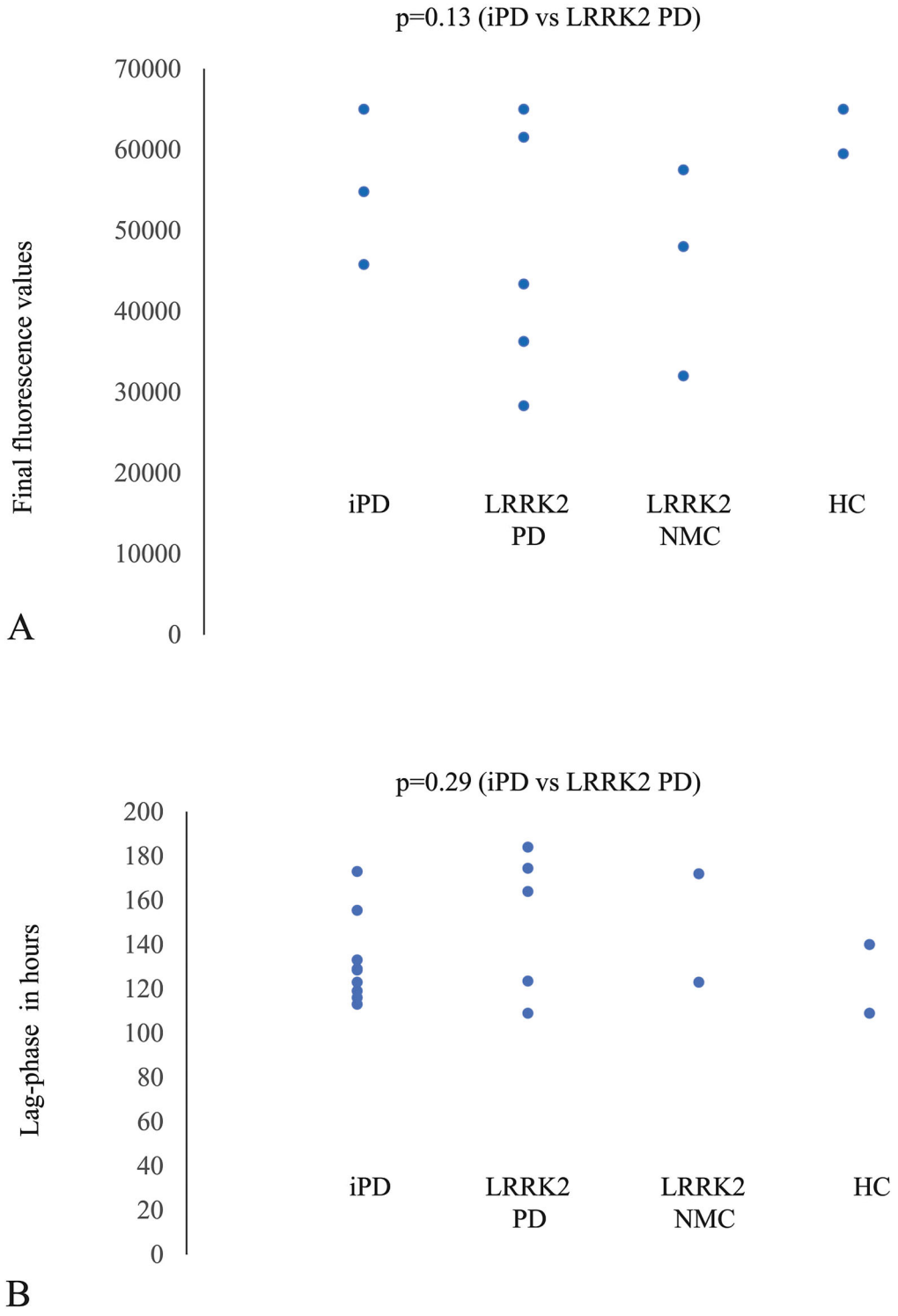

Figure 1. Final fluorescence values (A) and Lag-phase (B) of positive a-syn RT-QuIC subjects in IPD, LRRK2-PD, LRRK2-NMC and healthy controls (HC). All but two of the IPD patients with positive RT-QuIC had final fluorescence values of 65,000, with the remaining two patients having values of 54,801 and 45,792, respectively. The seven IPD that had final fluorescent values of 65,000 are represented by a single point on Figure 1A. Among the LRRK2-PD patients with positive RT-QuIC two patients had a final fluorescence value of 65000 and appear as a single point on Figure 1A. One LRRK2-PD patient and one LRRK2-NMC with positive RT-QuIC had unmeasurable lag-phases as the final fluorescence values were less than 44,000 in both duplicates and are not represented in Figure 1B.

Table 2. Nonmotor symptoms of LRRK2 Parkinson's disease patients.

	Positive RT-QuIC						Negative RT-QuIC								
Sex	M	M	M	M	M	M	F	F	M	F	F	F	F	M	F
Age	51	38	51	59	50	64	45	44	55	68	68	57	48	64	60
Cognitive impairment[1]	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0
Depression[1]	2	0	1	0	3	1	4	0	0	0	0	0	0	1	1
Urinary problems[1]	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0
Constipation problems[1]	2	0	1	0	0	1	2	0	0	0	1	0	1	0	3
REM sleep behavior disorder[2]	No	No	No	No	No	Yes	No	No	No	No	No	No	Yes	No	No
Hyposmia[3]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes

MDS, International Parkinson's disease and Movement Disorders Society.

[1]International Parkinson's disease and Movement Disorders Society Unified Parkinson's Disease Rating Scale part I scores: 0 = normal; 1 = slight, 2 = mild, 3 = moderate, 4 = severe (Goetz et al. 2008).

[2]Based on the REM sleep behavior questionnaire (Stiasny-Kolster et al. 2007).

[3]Based on the University of Pennsylvania Smell Test normative values (Doty et al. 1995).

Table 3. Clinical characteristics and MDS prodromal criteria likelihood ratio of LRRK2 nonmanifesting carriers.

	Positive RT-QuIC			Negative RT-QuIC												
Sex	M	F	M	M	M	M	F	M	M	F	M	M	F	F	M	M
Age	64	47	52	40	64	38	61	27	71	60	28	37	43	51	68	72
Cognitive impairment[1]	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Depression[1]	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Bladder symptoms[1]	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Constipation[1]	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
RBD[2]	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	No
Hyposmia[3]	Yes	No	Yes	No	No	No	No	Yes	No	Yes	No	No	No	No	Yes	No
Abnormal DaT-SPECT	Yes	No	Yes	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No
Meets criteria for prodromal PD*	Yes	No	Yes	No	No	No	No	No	Yes	No	No	No	No	No	No	No
LR for probable prodromal PD PD[†]	947.6	2.9	1076.8	127.3	2.3	2.9	1.3	29.5	289.3	16.2	0.74	0.74	9.2	5.42	174.7	5.2

RBD, REM sleep behavior disorder; MDS, International Parkinson's disease and Movement Disorders Society; PD, Parkinson's disease; LR, likelihood ratio.

[1]International Parkinson's disease and Movement Disorders Society Unified Parkinson's Disease Rating Scale part I scores: 0 = normal; 1 = slight, 2 = mild, 3 = moderate, 4 = severe (Goetz et al. 2008).

[2]Based on the REM sleep behavior questionnaire (Stiasny-Kolster et al. 2007).

[3]Based on the University of Pennsylvania Smell Test normative values (Doty et al. 1995).

*The subject meets the probability threshold of >80% for probable prodromal PD based on the MDS research criteria for prodromal PD (Berg et al. 2015, Mirelman et al. 2018).

[†]Total likelihood ratio based on the MDS Research Criteria for prodromal PD (Berg et al. 2015, Mirelman et al. 2018). Missing values were not imputed (LR = 1).

in LRRK2-PD brain homogenates. Diversity in the structure and self-aggregation potency of a-syn could contribute to variability in RT-QuIC quantification and even account for a negative result in some cases. Another possible explanation for a negative test in the presence of LTS could be that the negative LRRK2-PD cases presented less a-syn burden, insufficient to induce significant aggregation. So far, though, the studied LRRK2-PD brains with LTS follow the staging scheme of Braak,[41] and in studies comparing LRRK2-PD to IPD histopathology no differences in Lewy body and neurite distribution have been observed.[40]

In LRRK2-NMC RT-QuIC CSF positivity occurred in three of the 15 (18.8%) subjects suggesting an ongoing synucleinopathy in these cases. Two LRRK2-NMC with a positive result had a relative with manifest LRRK-PD who had a negative RT-QuIC result. Variable neuropathological substrates, even in the same kindreds as has been well documented to occur in cases with other pathogenic LRRK2 mutations,[42] could explain this finding. A decline in DaT-SPECT (^{123}I-ioflupane) binding has been previously suggested to be a useful tool to predict phenoconversion in LRRK2-NMC.[43] In our study two of three (66.7%) LRRK2-NMC with a positive RTQuIC had an abnormal DaT-SPECT result, whereas this occurred only in two of 13 (15.3%) of the negative ones. The greater frequency of altered DaT-SPECT uptake among the RTQuIC-positive cases is in line with the interpretation that these asymptomatic carriers are possibly those that will eventually evolve into manifest PD. The higher proportion of probable prodromal PD based on the MDS prodromal criteria[33,34] in those subjects with a positive RTQuIC reaction, also supports this hypothesis.

We found a positive RT-QuIC test in two healthy controls. One of these individuals has developed an action tremor of the hands since the time of CSF acquisition and may be harboring a neurodegenerative disorder. The presence of phosphorylated a-syn aggregates in the nervous system is not exceptional in living individuals without neurological diseases.[44–46] These cases may not represent false-positive cases but rather possibly be cases of incidental Lewy body disease. Postmortem studies in the brain show LTS in about 15–20% of elder population without parkinsonism or dementia during life.[22,47,48]

A major caveat of our study is the lack of neuropathological studies to establish a correlation between RT-QuIC result and histological diagnosis. An additional limitation of our study is the small number of subjects studied, precluding generalization of the results. Still the good sensitivity and specificity found in the IPD group, practically identical to those encountered by others using similar techniques[16,19] supports the validity of our data. Further RT-QuIC studies with greater number of LRRK2 mutation carriers, sufficient longitudinal follow-up to assess phenoconversion in the asymptomatic carriers and, eventually, postmortem or in vivo[49] studies to define the type of pathological lesions occurring in these subjects will be needed to validate our observation.

In conclusion, our results show that RT-QuIC methodology detects a-syn aggregation in CSF in a significant number of patients with LRRK2-PD p.G2019S but less frequently than in patient with IPD. In our cohort of LRRK2-NMC, almost 20% of subjects were positive for RT-QuIC suggesting that a positive test in LRRK2-NMC may possibly identify those at major risk to eventually develop parkinsonism. The high sensitivity and specificity of the results in IPD patients in this and previous studies support the notion that the presence of a-syn aggregation in CSF detected by RT-QuIC or similar a-syn amplification methods, if appropriately validated, could contribute to the selection of candidates to receive disease modifying drugs, particularly treatments targeting a-syn deposition.

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Authors Contributions

E.Tolosa designed the study; A.Garrido, and A.Green contributed to the design of the study. G Fairfoul performed the experiments, A Green analyzed the RT-QuIC data. A.Garrido, E. Tolosa, MJ. Marti and A. Green interpreted the results. A. Garrido and E. Tolosa and A. Green wrote the manuscript. All authors reviewed and commented on the manuscript.

Conflicts of Interest

Alicia Garrido has no competing interests.
Alison Green has no competing interests.
Graham Fairfoul has no competing interests.
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Article 4 –

Brain and Cerebrospinal Fluid α-synuclein Real-Time Quaking-Induced Conversion Identifies Lewy Body Pathology in LRRK2-PD.

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Brain and Cerebrospinal Fluid α-Synuclein Real-Time Quaking-Induced Conversion Identifies Lewy Body Pathology in LRRK2-PD

Alicia Garrido, MD,[1,2,3] Graham Fairfoul, M.Sc,[4] Eduardo Tolosa, MD, PhD,[2,3*] Maria J. Marti, MD, PhD,[1,2,3] Mario Ezquerra, PhD,[2] and Alison J E Green, PhD[4]

[1]*Parkinson's Disease and Movement Disorders Unit, Institut Clínic de Neurociències, Hospital Clinic de Barcelona, Barcelona, Spain* [2]*Laboratory of Parkinson Disease and Other Neurodegenerative Movement Disorders: Clinical and Experimental Research, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, Barcelona, Spain* [3]*Centre for Networked Biomedical Research on Neurodegenerative Diseases (CIBERNED), Barcelona, Spain* [4]*The National CJD Research & Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh, United Kingdom*

ABSTRACT: Background: The neuropathology of Parkinson's disease (PD) associated with leucine-rich repeat kinase 2 (LRRK2) mutations (LRRK2-PD) is heterogeneous and varies with the type of mutation. There are only a few studies evaluating seeding aggregation assays to detect α-synuclein (α-syn) in patients with LRRK2-PD.

Objective: We aimed to investigate whether α-syn real-time quaking induced conversion (RT-QuIC) is a sensitive biomarker of synucleinopathy in LRRK2-PD.

Methods: We studied α-syn RT-QuIC in brain tissue and postmortem ventricular cerebrospinal fluid (CSF) of LRRK2-PD cases with and without Lewy-type pathology.

Results: The accuracy of α-syn RT-QuIC in substantia nigra and CSF samples of patients with LRRK2-PD was 100%. The test also obtained 100% sensitivity to detect misfolded α-syn in substantia nigra of cases with idiopathic PD and was negative in the substantia nigra of all the control brains without Lewy-type pathology.

Conclusions: Substantia nigra and ventricular CSF RT-QuIC discriminates with high sensitivity and specificity LRRK2 cases with Lewy-type pathology from those without it. RT-QuIC assay could be of particular interest in the selection of cases for clinical trials in this genetic form of PD. © 2022 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; LRRK2; α-synuclein; RT-QuIC; Lewy body

***Correspondence to:** Dr. Eduardo Tolosa, Institut d'Investigacions Biomèdiques August Pi i Sunyer, University of Barcelona, C/Villarroel 170, Barcelona 08036, Spain; E-mail: etolosa@clinic.cat

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Introduction

Mutations in leucine-rich repeat kinase 2 (LRRK2) cause late-onset parkinsonism, which is clinically indistinguishable from idiopathic Parkinson's disease (iPD).[1-6] A pleiomorphic pathology has been reported since the first descriptions of LRRK2-PD,[7-10] and although many patients with PD with G2019S LRRK2 mutations exhibit α-synuclein (α-syn)-positive Lewy-type pathology (LTP),[10,11] nearly half of the remaining cases are Lewy body negative.[10,11] In patients carrying non-G2019S variants, LTP is even rarer, and nigral degeneration without abnormal protein deposition or associated to tau, Aβ, ubiquitin, TDP-43, or α-syn glial-cytoplasmic deposits is found.[8,10-19]

Seeding amplification assays (SAAs) such as real-time quaking-induced conversion (RT-QuIC) have shown in cerebrospinal fluid (CSF) and other tissues sensitivity and specificity values greater than 85% to identify a synucleinopathy.[21-24]

We aimed to investigate in a series of LRRK2-PD autopsy cases whether α-syn RT-QuIC can accurately separate LTP from non-LTP cases in postmortem brain tissue and CSF.

Subjects and Methods

Brain and postmortem ventricular CSF samples were obtained from brain bank cases of six subjects diagnosed

during life as LRRK2-PD, seven cases with histological diagnosis of synucleinopathy (six with premortem diagnosis of iPD and one case without known parkinsonism during life), and five cases without LTP (with a clinical diagnosis of frontotemporal dementia in one patient, vascular dementia in one, and three donors without a known neurodegenerative disease), matched for age and sex, at the Neurological Tissue Bank of the Biobank-Hospital Clinic–Institut d'Investigacions Biomèdiques August Pi i Sunyer (BTN-IDIBAPS brain bank).

Five patients with LRRK2-PD harbored the G2019S variant and one the R1441G variant. Genetic studies were performed, as previously described,[24] either by extracting DNA from in vivo collected peripheral blood or from brain tissue. Genotyping of the G2019S variant was performed on a StepOnePlus Real-Time PCR System (Life Technologies) using the commercial predesigned TaqMan assay C-63498123-10 single-nucleotide polymorphism rs34637584, and a TaqMan assay on demand (Life Technologies) was used for identifying the R1441G variant[25] with subsequent DNA sequencing. The histological findings of five of the LRRK2-PD cases used for this study have been published.[26,27] The local ethics committee of Hospital Clinic Barcelona approved the neuropathological and the study protocols, and donors or next of kin provided informed consent for brain donation.

Collection of Samples

Brain and ventricular CSF extraction and neuropathological examination were performed following the standardized procedures of the BTN-IDIBAPS brain bank.[28]

At the time of brain extraction, ventricular CSF was collected in 14 instances (3 LRRK2-PD, 5 iPD, and 6 non-PD control subjects) in a polypropylene tube using a 10-cc syringe and kept at 4°C until the processing. CSF samples were centrifuged for 10 minutes, at 4000*g* at 4°C in less than 2 hours from the collection, and gently mixed to avoid gradient effects. After centrifugation, CSF was immediately frozen at −80°C. Brain and CSF samples were stored in the BTN-IDIBAPS brain bank at −80°C until the time of this study.

Neuropathological Description

Three cases with LRRK2-PD, all carrying the G2019S variant, exhibited LTP with moderate nigral loss and α-syn inclusions predominantly in the midbrain and extending to the temporal neocortex (Braak stages 4–5). The other three LRRK2-PD cases (two harboring the G2019S and one the R1441G variant) showed a moderate neuronal loss in substantia nigra (SN) and locus coeruleus, without LTP. No protein inclusions were detected in the R1441G case; neuronal and glial 4R tau inclusions were present in one G2019S case, in which the pathological diagnosis of progressive supranuclear palsy was established; and tau inclusions characteristic of Alzheimer's disease (AD) were present in the other case. The six cases with a clinical diagnosis of iPD exhibited the classical LTP Braak stages 5 and 6 with variable degree of AD copathology. One case without known neurodegenerative disease during life had Lewy-type synucleinopathy with olfactory bulb and brainstem involvement (Braak stage 1) and AD-type pathology Braak stage 2.

The neuropathological diagnosis of the remaining LTP-negative cases was of AD at different stages (Braak stages 2–5) in three cases and primary age-related tauopathy in two cases, with some degree of small vessel disease in all of the cases.

α-Syn RT-QuIC in Brain Homogenates and CSF Samples

Frozen brain sections from the SN (n = 17) and anterior cingulate (AC) (n = 18) and CSF (n = 14) samples were transferred on dry ice to the National CJD Research & Surveillance Unit in Western General Hospital, University of Edinburgh, for analysis in January 2021 and stored at −80°C on arrival.

Samples were analyzed without prior knowledge of the donor's pathological diagnosis or genetic status. α-syn RT-QuIC was performed following a previously described methodology.[21] Each sample was run in duplicate. A positive response was defined as a relative fluorescence unit value of >2 standard deviations greater than the mean of the negative controls at 120 hours in both the BHs and CSF duplicates. If only one of two sample replicates responded positively, the RT-QuIC analysis was repeated in quadruplicate. A positive response in two or more of the replicates was considered positive. If only one of the replicates was positive, the RT-QuIC was considered to be negative.

Statistical Analysis

All analyses were done with RStudio. Qualitative variables are presented by absolute and relative frequencies (%) and analyzed by Fisher exact test. Quantitative variables are presented by the median and interquartile range (25th and 75th percentiles) and analyzed using the Kruskal–Wallis test. Sensitivity and specificity, with 95% confidence limits, for α-syn-RT-QuIC were calculated in all groups. A P value <0.05 was considered significant.

Results

The median age of LRRK2-PD subjects at death was 75.5 (70–83) years. Half of the LRRK2-PD patients were female. There were no significant demographic differences between LRRK2-PD, iPD, and control cases. The time between the demise and brain and CSF

collection ranged from 3 to 18.5 hours and did not differ significantly between groups.

All LRRK2 cases with neuropathologically confirmed LTP showed positive SN and AC α-syn RT-QuIC, whereas in LRRK2-PD cases without abnormal α-syn aggregates on the immunohistochemical analysis, SN α-syn RT-QUIC was negative (Table 1, Fig. 1). In one LRRK2-PD patient without LTP, in which SN was not available, the α-syn RT-QuIC of the AC was negative. One of the LRRK2-PD subjects without Lewy body disease had a positive curve in the AC.

All non-LRRK2 PD cases with LTP had a positive reaction of the α-syn RT-QuIC assay in the SN and AC (Table 1). Controls brains without LTP proved negative in α-syn RT-QuIC SN in all cases. Three control cases (LTP negative) had a positive test in the AC. Overall, SN α-syn RT-QuIC had a 100% sensitivity and 100% specificity to detect an underlying LTP synucleinopathy. RT-QuIC in the AC, however, was positive in cases with a negative result in the SN (in one LRRK2 brain and three controls).

Data on ventricular CSF were available in 14 subjects: 3 LRRK2-PD, 6 brains with LTP, and 5 control subjects. α-syn RT-QuIC results in CSF showed good agreement with nigral α-syn RT-QuIC results and the presence or absence of LTP on neuropathological examination (Table 1, Fig. 1). Only one iPD case had a negative CSF α-syn RT-QuIC reaction. Overall, CSF α-syn RT-QuIC had a 88.9% sensitivity and 100% specificity to detect underlying LTP.

Discussion

We report in this article the results of an α-syn seeding assay in postmortem brain and CSF from LRRK2-PD. RT-QuIC detected α-syn aggregation in postmortem SN and CSF of LTP-proven PD cases, either carrying a LRRK2 mutation or idiopathic, with high sensitivity and specificity. A similar high accuracy of α-syn RT-QuIC has been reported earlier in CSF of non-LRRK2 PD subjects with neuropathologically confirmed diagnosis.[22,30-32]

In a previous study, performed in 31 G2019S LRRK2 carriers, including patients with manifest PD and unaffected subjects, we found that α-syn RT-QuIC in lumbar CSF was positive in 40% of the LRRK2-PD cases and in 18% of unaffected carriers.[33] We hypothesized that the smaller percentage of positive cases among LRRK2-PD compared with the iPD cases was related to the known neuropathological variability of LRRK2-PD, with the positive cases identifying those with an underlying synucleinopahty. The lack of autopsy confirmation rendered the conclusions tentative. The results of this study showing that α-syn RT-QuIC can identify the LRRK2-PD cases with pathology-proven LTP with 100% sensitivity and 100% specificity in both SN and CSF support our initial hypothesis.

TABLE 1 *α-Synuclein real-time quaking induced conversion diagnostic value in postmortem brain tissue and ventricular CSF*

Diagnostic category	n	SN pos	Sensitivity (%)	Specificity (%)	AC pos	Sensitivity (%)	Specificity (%)	CSF pos	Sensitivity (%)	Specificity (%)
LRRK2-PD LTP^{+}	3	3/3	100	–	3/3	100	–	2/2	100	–
LTP^{+} controls[a]	7	7/7	100	–	7/7	100	–	5/6	83	–
LRRK2-PD LTP^{-}	3	0/2	–	100	1/3	–	67	0/1	–	100
LTP^{-} controls[b]	5	0/5	–	100	3/5	–	60	0/5	–	100

[a]Six idiopathic Parkinson's disease cases with classical LTP (Braak stages 5–6) and one case with LTP from a patient without parkinsonism (Braak stage 1).
[b]Frontotemporal dementia (n = 1), vascular dementia (n = 1), and control subjects without neurodegenerative disease (n = 3).
Abbreviations: CSF, cerebrospinal fluid; SN, substantia nigra; pos, positive; AC, anterior cingulate; LRRK2-PD, Parkinson's disease associated with leucine-rich repeat kinase 2 mutations; LTP, Lewy-type pathology.

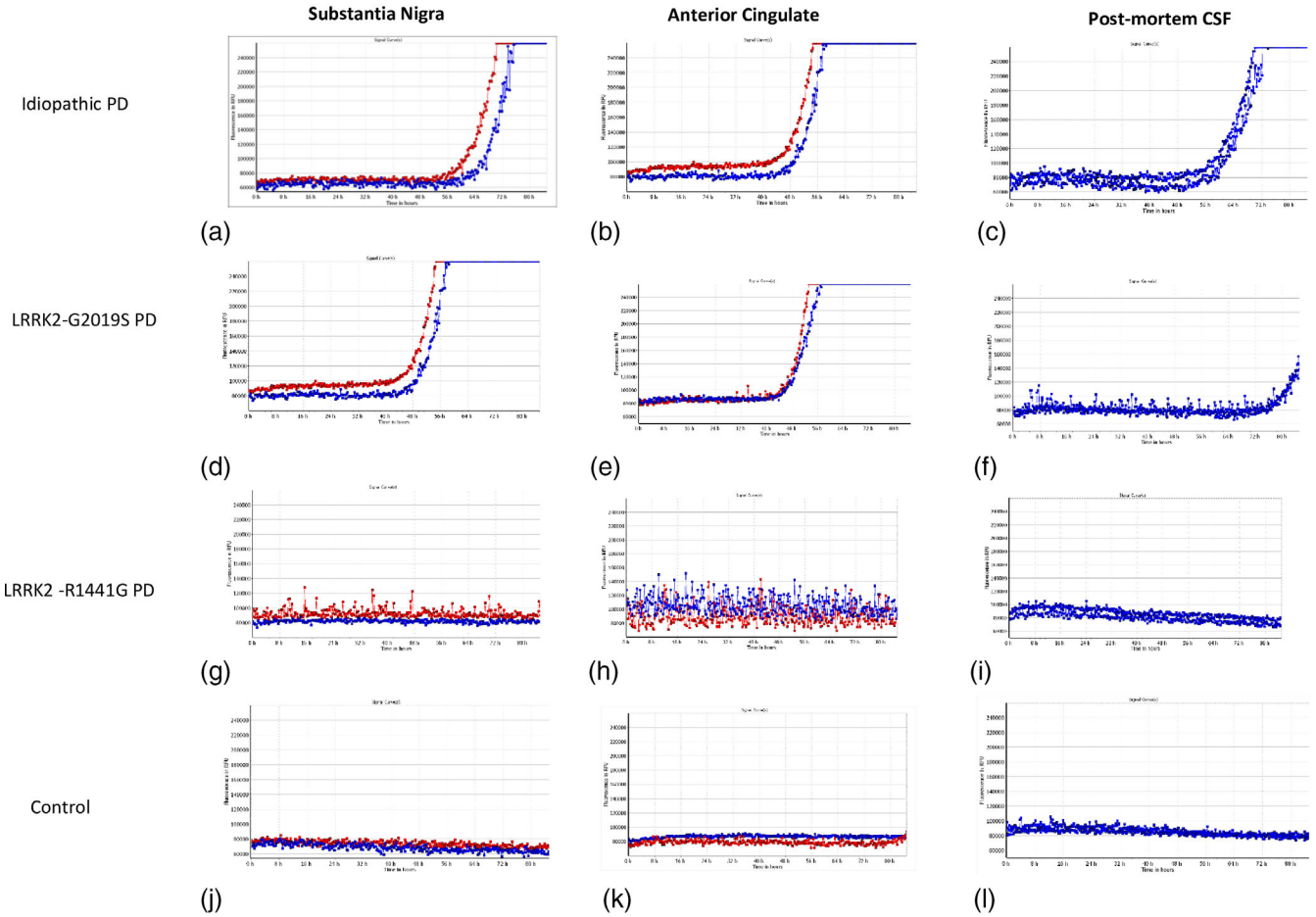

FIG. 1. α-Synuclein real-time quaking-induced conversion reaction curves of substantia nigra (**A**, **D**, **G**, **J**), anterior cingulate (**B**, **E**, **H**, **K**), and postmortem cerebrospinal fluid (**C**, **F**, **I**, **L**) from a patient with idiopathic Parkinson's disease (PD) (**A–C**), one with leucine-rich repeat kinase 2 (LRRK2)-G2019S PD (**D–F**), one with LRRK2-R1441G PD (**G–I**), and a control subject (**J–L**). The two lines shown in each figure represent replicates from the same subject. [Color figure can be viewed at wileyonlinelibrary.com]

In this study, α-syn RT-QuIC in the SN gave the highest positive and negative predictive values, in line with the known universal presence of LTP in this structure in case of LTP-associated PD. In the AC, RT-QuIC, unexpectedly, had a low specificity, being positive in some of the controls. The significance of these findings is unclear. The AC is considered a brain region very frequently involved with LTP in PD,[34,35] and we suspect that the presence of α-Syn aggregates in this brain region of the LTP-positive brains could be the reason that so many PD-LTP-positive cases were AC positive for RT-QuIC. Unexpected RT-QuIC results have been reported in CSF studies where 10% to 25% of healthy control subjects were found to be positive, and this has been attributed to an underlying subclinical synucleinopathy.[24,33] In brain tissue, a recent study[36] has demonstrated positive RT-QuIC results in brain regions without LTP, suggesting that α-Syn aggregates could remain undetected by immunohistochemical methods and still generate a positive α-Syn RT-QuIC curve. SAAs in different brain regions, with and without detectable LTP by immunochemistry, are needed to try to understand these results.

Results in ventricular CSF achieved a high level of correlation with those in the SN. Only one CSF sample of an iPD case had a negative α-syn RT-QuIC reaction. Postmortem CSF collected at the time of autopsy may provide results different from those in lumbar CSF obtained in patients in the clinic,[36] because CSF constituents' origin may vary according to the site of collection (eg, choroid plexus and brain vs. subarachnoid structures, meninges, and dorsal roots).[37] Whether lumbar CSF allows for a similar detection of α-syn aggregates as ventricular CSF has not been determined. The results in ventricular postmortem CSF reported in this article, though, mimic those in patients with and without underlying synucleinopathies,[21,22] suggesting that ventricular postmortem CSF behaves similar to in vivo lumbar CSF in regard to α-syn seeding assays.

In summary, in this α-syn aggregation study, in postmortem brain and CSF from neuropathologically documented cases, α-syn RT-QuIC correctly identified as

positive the LRRK2-PD cases with LTP, whereas it was negative in those without LTP. RT-QuIC was also positive in all control PD cases with LTP. Our results support the notion that SAA, like RT-QuIC, applied to lumbar CSF may reliably identify those cases with an underlying synucleinopathy and potentially candidates for disease modification therapies targeting α-syn. Despite the small number of cases in our study, the results suggest that α-syn RT-QuIC is a promising tool to evaluate the underlying neuropathology of LRRK2-PD in vivo. Studies comparing SAA results in postmortem brain tissue and lumbar CSF from LRRK2-PD subjects are needed to confirm the usefulness of these assays for the reliable identification of an ongoing central nervous system synucleinopathy. ■

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Somatic *SNCA* Copy Number Variants in Multiple System Atrophy are Related to Pathology and Inclusions

Monica Emili Garcia-Segura,[1] Diego Perez-Rodriguez, PhD,[1] Darren Chambers,[2] Zane Jaunmuktane,[1,3,4] and Christos Proukakis, PhD, FRCP[1*]

*Correspondence to: Prof Christos Proukakis, Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, University College London, Rowland Hill Street, London NW3 2PF, UK; E-mail: c.proukakis@ucl.ac.uk

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[1]*Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, University College London, London, UK* [2]*The Dubowitz Neuromuscular Centre, Division of Neuropathology, UCL Queen Square Institute of Neurology, University College London, London, UK* [3]*Queen Square Brain Bank for Neurological Disorders, UCL Queen Square Institute of Neurology, University College London, London, UK* [4]*Division of Neuropathology, National Hospital for Neurology and Neurosurgery, University College London NHS Foundation Trust, London, UK*

ABSTRACT: Background: Somatic α-synuclein (*SNCA*) copy number variants (CNVs, specifically gains) occur in multiple system atrophy (MSA) and Parkinson's disease brains.

Objective: The aim was to compare somatic *SNCA* CNVs in MSA subtypes (striatonigral degeneration [SND] and olivopontocerebellar atrophy [OPCA]) and correlate with inclusions.

Methods: We combined fluorescent in situ hybridization with immunofluorescence for α-synuclein and in some cases oligodendrocyte marker tubulin polymerization promoting protein (TPPP).

Results: We analyzed one to three brain regions from 24 MSA cases (13 SND, 11 OPCA). In a region preferentially affected in one subtype (putamen in SND, cerebellum in OPCA), mosaicism was higher in that subtype, and cells with CNVs were 4.2 times more likely to have inclusions. In the substantia nigra, non-pigmented cells with CNVs and TPPP were about six times more likely to have inclusions.

Conclusions: The correlation between *SNCA* CNVs and pathology (at a regional level) and inclusions (at a single-cell level) suggests a role for somatic *SNCA* CNVs in MSA pathogenesis. © 2022 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: snca; alpha-synuclein; mosaicism; multiple system atrophy; somatic mutation

Background

Multiple system atrophy (MSA) can be classified pathologically into striatonigral degeneration (SND), olivopontocerebellar atrophy (OPCA), and mixed.[1] α-Synuclein, encoded by *SNCA*, forms aggregates, and glial cytoplasmic inclusions (GCIs) in oligodendrocytes are likely central to pathogenesis.[2] Rare inherited *SNCA* mutations lead to synucleinopathies, generally classified as Parkinson's disease (PD), often with dementia. These are most often copy number variants (CNVs), specifically gains, which increase mRNA,[3] and disease severity depends on gene dosage.[4]

Synthesis of results and discussion

In recent years, there has been a rapid expansion of knowledge on the epidemiology and clinical manifestations of LRRK2-PD, as well as advancements in understanding the LRRK2 protein pathways and their role in the pathogenesis of PD. However, several critical questions remain unanswered in the field such as the factors affecting the penetrance of *LRRK2* pathogenic mutations, the mechanisms by which these mutations result into neurodegeneration, and the absence of predictive biomarkers for disease onset and progression.

SNCA rs356219 but not dynamin 3 DNM3 rs2421947 modifies the penetrance of the G2019S mutation of LRRK2 in the Spanish population

In this study involving 329 LRRK2-PD patients and LRRK2-NMC from Spain, we aimed to evaluate the impact of polymorphisms in the *SNCA* (SNP rs356219) and *DNM3* (SNP rs2421947) genes on LRRK2-PD penetrance.

Our analysis revealed a significant effect of the *SNCA* rs356219-G allele on the age at onset of LRRK2-PD. Specifically, carriers of the rs356219-G allele exhibited a significant reduction in the age at onset compared to rs356219-A allele carriers ($p < 0.001$). This finding aligns with previous studies conducted in Spanish and other populations.(68,168) In the study by Botta-Orfila et al. (68), conducted in Spanish population, the risk allele lowered the age at onset by 9 years compared to the homozygous AA allele carriers (55.37±1.54 vs 64.19±2.8; $p = 0.006$). In an earlier study by Cardo et al.(168), the difference between the two alleles in age at onset was even shorter (57.6±12.5 vs 60.10±12.8; $p = 0.003$). Contrary to a previous study,(66) our investigation did not find a significant association between *DNM3* rs2421947 and penetrance of LRRK2-PD.

The observed association of risk *SNCA* polymorphisms with an earlier age at onset underscores the significant role of additional genetic factors in influencing disease manifestation beyond the *LRRK2* mutation. *SNCA* mutations, including both multiplications and missense mutations, lead to an aggressive phenotype of PD characterized by juvenile or early onset and the presence of α-syn aggregation in neurons in the form of Lewy body

pathology.(2,169) Common risk *SNCA* variants might exert an influence on gene regulation, resulting in elevated intracellular levels of α-syn. According to this hypothesis an increased transcription of *SNCA* risk alleles might contribute to a higher risk and earlier onset of LRRK2-PD, aligning with the pathogenic effect of increased gene dosage observed in patients with genomic multiplications.(169) Nevertheless, studies investigating whether the risk SNPs in *SNCA* correlate with the clinical features of LRRK2-PD or its pathological substrate are still lacking. Analysis of data from the participants in the Parkinson's Progression Markers Initiative (PPMI) including IPD and controls found that another SNP in the *SNCA* gene (rs356182) was associated with elevated CSF levels of α-syn.(170) Notably this association was predominantly driven by the control group. The advancement in development of disease biomarkers holds significant promise as it could enable the correlation between the genomic profile and the molecular and clinical features of LRRK2-PD. Additionally, it could provide a means to identify LRRK2-NMC in whom symptoms have not yet manifested, but the neurodegenerative process has initiated, shifting the assessment of LRRK2 penetrance from clinical symptoms to biomarkers.

The non-significant association of *DNM3* rs2421947 in our cohort, different to the opposite results observed in an analysis performed in Algerian, European and north-American cohorts(66), could be attributed to several factors. First, although our sample size of G2019S carriers (n=329) was comparable to the Arab-Berber (n=367) or the replication (n=263) cohorts in the previous study, it may still be insufficient to detect subtle associations in *DNM3*, and larger cohorts may be necessary to achieve sufficient statistical power. Nonetheless using the same sample we found association of *SNCA* rs356219 with L2PD AAO. Second it might suggest potential variations in genetic factors across different populations with diverse ethnic background, and highlights the complexity of gene-gene interactions modifying LRRK2-PD penetrance. Indeed, ethnicity plays a significant role in penetrance due to shared genetic and environmental factors within specific populations. In a meta-analysis (40) the stratification of *LRRK2* G2019S patients according to their ethnic background showed higher penetrance in Israeli Ashkenazi Jews (>90% penetrance at 80 years of age) compared to a population of Ashkenazi Jews from New York (24% penetrance at 80 years of age). This difference reflects the additional influence of environmental factors.

It is important to consider that studies examining penetrance may have methodological limitations and ascertainment bias. Firstly, there are no prospective

longitudinal studies designed to determine the penetrance of LRRK2-PD in the general population, and the available data is a statistical estimation based on selected populations of patients with PD, guided by the age at which the phenotype (i.e. the cardinal motor symptoms of PD) is identified. In the absence of biomarkers, the diagnosis of the disease is subjected to clinical diagnostic criteria and limited by the disease's broad expressivity and the physician's expertise. Although the reported age at onset has been well correlated with the age at diagnosis,(171) there may be also a recall bias. Secondly, most studies on unaffected carriers encompass subjects from specifically chosen family cohorts exhibiting autosomal dominant inheritance of PD. This involves multi-incident families likely predisposed to manifest the disease due to other shared genetic or environmental factors.

Furthermore, investigations focusing on individual loci yield insights into only a minor portion of the overall risk. Among the candidate polymorphisms identified through GWAS studies, including *SNCA*, *DNM-3*, TMEM175/G-cyclin-associated kinase *(GAK)*, brain-derived neurotrophic factor (*BDNF*), *MAPT*, bone marrow stromal antigen 1 (*BSTM1*), *RAB-29*, and vesicle-associated membrane protein 4 (*VAMP4*), only variants in *SNCA* and *MAPT* have been consistently replicated across different LRRK2-PD cohorts.(172) To enhance statistical power, it is crucial to expand the scope of studies to encompass larger and more diverse populations. This approach allows for a comprehensive evaluation of the cumulative impact of multiple risk polymorphisms at the individual level. A recent proposal introduces a cumulative genetic risk index, or Polygenic Risk Score (PRS), including 89 PD risk loci identified through GWAS studies. (172) This scoring system was evaluated in a population of 800 *LRRK2* G2019S mutation carriers (439 individuals with PD and 394 asymptomatic cases). The results reveal that individuals in the highest decile of PRS face a six-fold higher risk of developing PD compared to those in the lowest decile, and this heightened risk is associated with a lower age of onset.(172) Moreover, in asymptomatic *LRRK2* G2019S carriers, a higher PRS has been associated with an increased risk of developing PD (OR, 1.34; 95% CI [1.09, 1.64]; p = 0.005). These findings underscore the importance of considering a broader genetic landscape and the cumulative effects of multiple risk loci in understanding the risk of PD.

Increased neuroinflammation and putaminal denervation is observed in early stages of LRRK2-PD

In this study we examined the presence of activated microglia and the integrity of the nigrostriatal pathway in asymptomatic carriers of the *LRRK2* G2019S mutation compared to a control group of healthy individuals. We employed PET imaging using the first-generation tracer [^{11}C](R)-PK11195 and the [^{18}F]-DOPA tracer to assess the occurrence of both phenomena. We postulated that nigroestriatal degeneration would precede the onset of microglial activation.

The key findings revealed a decreased left-right putaminal [^{18}F]-DOPA uptake in the LRRK2-NMC group (− 9.2%, range − 16.9% to 4.7%; p = 0.468), without differences in the [^{11}C]-(R)-PK11195 uptake between groups. Notably, individual-level analysis indicated that 5 out of 8 LRRK2-NMC exhibited unilateral or bilateral reduced putaminal [^{18}F]-DOPA uptake, with three of them showing a bilateral increase in [^{11}C]-(R)-PK11195 uptake in the substantia nigra.

These results confirm the presence of neuroinflammation in the presymptomatic phase of LRRK2-PD, similar to the alterations observed in subjects with idiopathic RBD, a prodromal phase of IPD.(116) This finding supports a role of inflammation in LRRK2-PD pathogenesis. Our study is unique in conducting an in-vivo evaluation of microglial activity in LRRK2-NMC using PET tracers. The [^{11}C]-(R)-PK11195 tracer binds to the protein translocator receptor, which is present in activated microglia. However, the use of this tracer has limitations. The expressivity of the receptor is subject to the polymorphisms of its gene and is also present in other peripheral cells that may have infiltrated the central nervous system. Additionally, activated microglia can express both a compensatory, neuroprotective phenotype, and a cytotoxic deleterious state, and the significance of microglial activation in LRRK2-PD is yet to be established. Further studies using more specific microglial PET tracers and conducted in larger cohorts are necessary to validate our findings. Nevertheless, growing evidence from human cell and animal models suggests that *LRRK2* mutations have a pathogenic effect on microglial state, leading to altered functions such as decreased phagocytosis, altered cytokine production, and increased oxidative stress.(173)

Few studies have explored imaging changes in LRRK2-NMC using alternative PET tracers. A previous study on unaffected carriers of various *LRRK2* mutations using PET scan

with [^{18}F]-DOPA, and [^{11}Cd]-threo-methylphenidate, a marker of the membrane dopamine transporter, revealed reduced putaminal uptake compared to controls, similar to the findings of our study.(174)

In our study, two LRRK2-NMC individuals exhibited diminished putaminal [^{18}F]-DOPA uptake without concurrent increased ipsilateral nigral [^{11}C]-(R)-PK11195 binding, suggesting that striatal axon degeneration in the putamen may precede microglial activation in nigral neurons. Similar observations have been reported in subjects with RBD.(116) These results highlight the potential of PET tracers to assess the changes occurring in LRRK2-PD and their temporal sequence.

There are several limitations to this study that should be considered. First, the small size may limit the ability to generalize the findings. Second, the LRRK2-NMC group was younger than the control group, potentially inducing selection bias. Given the age-related low penetrance of *LRRK2* G2019S mutation, it is possible that some may never develop PD, or are in a early stage where biochemical and pathological changes may have yet not manifested. However, the observed alterations in [^{18}F]-DOPA PET scans in five LRRK2-NMC participants suggest that pathological changes are already present in most of this cohort. As age is a significant risk factor for LRRK2-PD, we examined whether PET findings correlated with age but found no such relationship, indicating other factors might influence [^{18}F]-DOPA dysfunction and microglial activation. Additionally, microglial activation levels in PD may be affected by the presence of pathological α-syn inclusions. In *LRRK2* G2019S PD, the variability in neuropathology, including cases without Lewy body type pathology, could contribute to the heterogeneity of the results. Lastly, while [^{11}C]-(R)-PK11195 primarily binds to the 18-kDa translocator protein on activated microglia, it may also bind to astrocytes, confounding our interpretation, though this likely accounts for only a minor part of the [^{11}C]-(R)-PK11195 signal.

α-Syn amplification assays in CSF and brain tissue identify neuronal synuclein disease

We report two different studies assessing α-syn SAA in LRRK2-PD. One, investigated the presence of α-syn aggregated in CSF collected in vivo from *LRRK2* mutation carriers. The second study aimed at correlating the results of α-syn SAA in CSF with those of post-mortem brain tissue from LRRK2-PD patients.

While α-syn RT-QuIC on CSF samples has been consistently positive in more than 95% of IPD patients,(144,151–153) our initial study in CSF collected in vivo from LRRK2-PD cases, revealed a positive result of α-syn RT-QuIC in only 40% of samples. Subsequent studies confirmed this lower sensitivity in LRRK2-PD compared to IPD. (**Table 7**) Brockmann and colleagues found that 78% of LRRK2-PD patients (n=7) exhibited a positive RT-QuIC result compared to 97% in the IPD group.(175) In a recent study performed on samples from the PPMI, which included 123 LRRK2-PD patients the test demonstrated a sensitivity of 67,5% in the LRRK2-PD group, compared to 87.7% in the IPD group.(176)

Table 7 | Comparison of α-syn RT-QuIC Sensitivity in cerebospinal fluid of *LRRK2* mutation carriers.

Study	Cohort	Number of subjects	Sensitivity in *LRRK2* mutation carriers (%)	Sensitivity in IPD (%)
Garrido et al. 2019	LRRK2-PD LRRK2-NMC	15 16	40% 18.8%	90%
Brockmann et al. 2021(175)	LRRK2-PD LRRK2-NMC	9 3	78% 33%	97%
Siderowf et al. 2023(176)	LRRK2-PD LRRK2-NMC	123 159	67.5% 14%	87.7%

|Original Table. IPD: idiopathic Parkinson's disease; LRRK2-PD: LRRK2 associated Parkinson's disease; LRRK2-NMC: LRRK2 non-manifesting carriers.

Neuropathological pleomorphism is characteristic of LRRK2-PD, with fewer cases displaying Lewy pathology compared to IPD. We hypothesized that the lower frequency of positive α-syn RT-QuIC in CSF samples of LRRK2-PD cases (40%) compared to the high sensitivity (90%) and specificity (80%) of the test in IPD cases in the same study, reflects the absence of synuclein-type pathology in the RT-QuIC negative LRRK2-PD cases. However, the absence of autopsy confirmation rendered these findings inconclusive.

Support for this hypothesis came from the second study conducted on postmortem tissue, including brain homogenates from the substantia nigra and anterior cingulate and ventricular CSF. This study revealed that all cases with synucleinopathy confirmed by immunohistopathology - the gold-standard technique for detecting pathological α-syn - had a positive α-syn RT-QuIC test in both substantia nigra and anterior cingulate. In contrast, cases without Lewy-type pathology in the substantia nigra showed negative RT-QuIC results. Interestingly, the RT-QuIC assay in the anterior cingulate exhibited lower specificity, yielding positive results in some control cases, the reason for which remains unclear. Similar unexpected positive RT-QuIC results have been observed in CSF studies, up to 20% of healthy controls tested positive (**Table 5**), possibly indicating subclinical synucleinopathy. Moreover, a study found positive RT-QuIC results in brain regions of Alzheimer's disease patients, even though no Lewy bodies were detected in their neuropathological evaluation,(177) suggesting that α-syn aggregates may evade detection by immunohistochemistry while still generating a positive RT-QuIC signal.

Further research using seeding assays in various brain regions, both with and without immunohistochemically detectable Lewy-type pathology, is necessary to clarify these findings. Additionally, autopsy data from the study by Siderowf and colleagues, which included a LRRK2-PD case carrying the G2019S mutation, revealed negative RT-QuIC result, and the hystopathological examination showed nigral cell loss and depigmentation without Lewy bodies or neurites, aligning with our results. (176)

The results from ventricular CSF showed strong correlation with those from the substantia nigra, with only one CSF sample from an idiopathic PD case testing negative for α-syn RT-QuIC. It is important to note that postmortem CSF, collected during autopsy, may produce different results compared to lumbar CSF obtained from living patients, as CSF composition may vary based on the collection site (e.g., choroid plexus and brain versus

subarachnoid structures, meninges, and dorsal roots). However, the findings from ventricular postmortem CSF in this study mirror those observed in patients with and without underlying synucleinopathies, suggesting that ventricular postmortem CSF behaves similarly to in vivo lumbar CSF in α-syn seeding assays.

Limited data are available on the clinical features distinguishing LRRK2-PD cases with underlying synucleinopathy from those with a different neuropathological substrate.(127) Our investigation revealed no statistically significant differences in motor and non-motor symptoms severity or disability between LRRK-PD cases with a positive or negative RT-QuIC results. The only variable that showed a significant difference was male sex, more prevalent among positive RT-QuIC cases (p = 0.007; data unpublished). This observation, coupled with an older age, was corroborated by findings in two subsequent studies(175,176) In the study involving PPMI samples, a higher test sensitivity was observed in LRRK2-PD individuals with hyposmia (89.9%; CI 82.7–97.0) compared to those with normal olfaction (34.7%; 21.4–48.0).(176)

18% of asymptomatic *LRRK2* G2019S carriers tested positive for α-syn RT-QuIC in our study. (**Table 7**) This suggests that a positive SAA test is a surrogate marker of ongoing synucleinopathy before the motor manifestation of LRRK-PD occurs. Other studies have reported the high performance of α-syn RT-QuIC in detecting IPD in premotor stages, including individuals with RBD and hyposmia(144,162) In our study, LRRK2-NMC with a positive RT-QuIC reaction exhibited more prodromal symptoms and a higher likelihood of meeting the International MDS criteria for prodromal PD.(178) Notably, the oldest LRRK2-NMC with a positive result of our cohort developed motor symptoms of PD three years after the CSF was collected, underscoring the potential predictive value of α-syn RT-QuIC. While we recognize the need for further validation in larger cohorts with ongoing longitudinal follow-up and postmortem data to support these findings, our report is the first to suggest that α-syn SAA could be a promising biomarker for detecting an ongoing synucleinopathy in early, premotor, stages of LRRK2-PD.

The understanding of the biological underpinnings of PD is continually advancing as research uncovers new information about its complex mechanisms. In recent years, significant progress has been made in identifying the neuropathological features of PD and developing methods to detect α-syn aggregate using SAA. These advancements have led to an effort aimed at biologically defining PD. In mid-2023, two prominent research groups undertook the ambitious task of establishing the first comprehensive biological definition of PD.(179,180) This new definitions integrate the detection of α-syn aggregates via SAA with evidence of PD-related neurodegeneration. The scientific evidence supporting these is definitions underscores the significance of α-syn SAA positivity in conjunction with neurodegenerative changes observed in dopaminergic neurons.

Importantly, insights from neuropathology have revealed that the presence of Lewy pathology, traditionally considered a hallmark of PD, is not necessarily required for a clinical diagnosis of the disease. This is particularly evident in cases of LRRK2-PD, where Lewy bodies may be absent despite the presence of other PD-related symptoms and pathology. This finding has profound implications for our understanding of PD, as it challenges the conventional notion that Lewy pathology is a definitive indicator of the disease. In developing a more precise biological definition of PD, it is crucial to consisder the genetic status of individuals. The findings presented in this thesis highlight the importance of incorporating genetic information into the diagnostic framework for PD.

The studies presented in this thesis (**Table 8**) have attempted to gather new insights into the genetic and pathophysiological basis of LRRK2-PD, along with the identification of potential molecular diagnostic biomarkers. We believe our findings provide additional insights into disease penetrance and the potential role of inflammation in LRRK2-PD. Furthermore we emphasize the potential role of the newly developed α-syn SAA in identifying those cases with intraneuronal synuclein aggregates typical of Lewy type pathology.

Table 8 | Key Findings and Implications of LRRK2-PD studies

Biomarker	**Key finding**	**Implications**
SNCA polymorphysms	Variants in *SNCA* rs356219 change LRRK2-PD penetrance	Promotes individualized diagnostic and prognostic approaches
CSF α-syn SAA Positivity	α-Syn Rt-QuiC is less sensitive in LRRK2-PD compared to IPD	Improves the stratification of patients. Challenges conventional diagnostic criteria
PET Microglial activation	Microglia activation can have a role in early phases of LRRK2 disease	Suggests new therapeutic targets
|Original Table; LRRK2-PD: LRRK2 associated Parkinson's disease; α-syn: α-synuclein; CSF: cerebrospinal fluid; SAA: seeding amplification assay; PET: Positron Emission Tomography		

Future research projects should build on the findings presented in this work to further advance our understanding of LRRK2-PD. First, long-term follow-up studies on individuals with *LRRK2* mutations are essential. These studies should expand the investigations of genetic risk polymorphisms, such as the *SNCA* rs356219-G allele, to develop predictive models of increased penetrance and improved individualized diagnostic and prognostic approaches. Second, to validate the α-syn SAA in larger, more diverse cohorts of LRRK2-PD and LRRK2-NMC with various mutations is crucial to establish the accuracy of this test as a diagnostic tool. To standarize the assay across laboratories and to apply the technique to more accessible tissues in LRRK2 populations could facilitate its use in clinical setting, potentially leading to earlier and more accurate diagnosis of LRRK2-PD. Additionally, to expand research

into the inflammatory mechanisms underlying LRRK2-PD could help identify new therapeutic targets and inform the development of anti-inflammatory treatments for PD.

These results represent a an advance in the field of PD research. By enhancing our understanding of the disease's biological underpinnings and identifying key diagnostic biomarkers, we move closer to discovering effective treatments for LRRK2-PD. This progress brings hope for improved patient outcomes and a future where PD can be more accurately diagnosed and effectively managed.

Conclusions

1. Genetic polymorphisms within the *SNCA* gene contribute to the penetrance of G2019S *LRRK2*-associated Parkinson's disease.

2. Polymorphisms in the *DNM3* gene do not influence age of onset in G2019S *LRRK2*-associated Parkinson's disease within the Spanish population.

3. Activation of central nervous system microglia, indicating neuroinflammatory phenomena, occurs in G2019S *LRRK2* non-manifesting carriers and may be preceded by degeneration of the putaminal axonal terminals.

4. Real-time quacking induced conversion, detects abnormal α-synuclein aggregates in the cerebrospinal fluid of patients with G2019S *LRRK2*-Parkinson's disease, albeit less frequently than in idiopathic Parkinson's disease.

5. The lower frequency of positive α-synuclein real-time quacking induced conversion in cerebrospinal fluid collected in vivo from G2019S *LRRK2*-Parkinson's disease patients, can be attributed to the frequent absence of neuronal synuclein aggregates in this genetic form of Parkinson's disease.

6. A subset of asymptomatic carriers with the G2019S *LRRK2* mutation exhibit abnormal α-synuclein aggregates in cerebrospinal fluid using real-time quacking induced conversion. These individuals present higher clinical scores for probable prodromal Parkinson's disease.

7. α-synuclein real-time quacking induced conversion positivity in postmortem brain and cerebrospinal fluid of G2019S *LRRK2*-Parkinson's disease cases reflects an underlying synucleinopathy.

8. The lower frequency of positive α-synuclein real-time quacking induced conversion in cerebrospinal fluid collected in vivo from G2019S *LRRK2*-Parkinson's disease patients can be attributed to the common absence of neuronal synuclein aggregates in this genetic form of Parkinson's disease.

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