Universidad de Granada

Programa de Doctorado: BIOMEDICINA

Genetic basis of Parkinson's Disease in Southern Spain

Fundamentos genéticos de la Enfermedad de Parkinson en el sur de España

Sara Bandrés Ciga

Granada, 2016

Editor: Universidad de Granada. Tesis Doctorales
Autora: Sara Bandrés Ciga
ISBN: 978-84-9163-056-2
URI: http://hdl.handle.net/10481/44397

Genetic basis of Parkinson's Disease in Southern Spain

Fundamentos genéticos de la Enfermedad de Parkinson en el sur de España

Tesis doctoral presentada por

Sara Bandrés Ciga

para optar al grado de

Doctora con Mención Internacional

Dirigida por los Doctores

Francisco Vives Montero

Raquel Durán Ogalla

Departamento de Fisiología

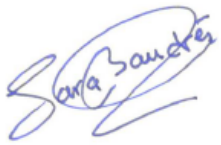
Instituto de Neurociencias

Universidad de Granada

La doctoranda **Sara Bandrés Ciga,** y los directores de tesis **Francisco Vives Montero y Raquel Durán Ogalla**, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo nuestra dirección, y hasta donde nuestro conocimiento alcanza, se han respetado los derechos de otros autores a ser citados, cuando se ha hecho referencia a sus publicaciones.

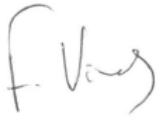
Granada, a 3 de Noviembre de 2016

Doctoranda



Fdo.: Sara Bandrés Ciga.

Directores de la Tesis



Fdo.: Francisco Vives Montero.

Fdo.: Raquel Durán Ogalla

“Our challenges do not define us. Our actions do”

Michael J. Fox

Table of contents

Financiación / Financial sources

Esta Tesis Doctoral es fruto del trabajo de investigación realizado gracias a la financiación nacional recibida por las siguientes fuentes:

- **FPU12-01885.** Beca-Contrato del Programa de Formación de Profesorado Universitario (FPU) (2013-2017) concedida a la doctoranda Sara Bandrés Ciga. Centro: Departamento de Fisiología, Facultad de Medicina e Instituto de Neurociencias Federico-Olóriz.

- **EST13/00569.** Estancia cofinanciada por el Campus de Excelencia Internacional CeiBioTic y la Universidad de Granada, dedicada a la realización de un screening detallado de variantes genéticas patogénicas o de riesgo en genes candidatos asociados a la enfermedad de Parkinson. Centro: Department of Molecular Neuroscience, Institute of Neurology, University College of London, (London, UK). Duración: 3 meses.

- **EST14/0061.** Estancia financiada por el programa FPU, dirigida a la realización del primer estudio de genotipado a gran escala de la enfermedad de Parkinson en España. Centro: Laboratory of Neurogenetics, National Institute on Aging, NIH. (Maryland, USA). Duración: 6 meses.

- Premio Sociedad Andaluza de Neurología al proyecto "Polimorfismos genéticos, biomarcadores plasmáticos y estrés oxidativo en la Enfermedad de Parkinson".

This thesis has been cofinancially supported by the following international sources:

- **Project WT089698/Z/09/Z:** Medical Research Council (London, UK) and Welcome Trust Strategic Award.
- **Project ZO1 AG000949:** Intramural Research Program of the National Institute on Aging, National Institutes of Health (NIH) (part of the Department of Health and Human Services, USA).

Publications

Part of the work of this thesis has been published in the following sources:

A) Peer-reviewed journals:

Bandrés-Ciga, S., Mencacci, N.E., Durán, R., Barrero, F.J., Escamilla-Sevilla, F., Morgan, S., Hehir, J., Vives, F., Hardy, J., Pittman, A.M., 2016. *Analysis of the genetic variability in Parkinson's disease from Southern Spain.* **Neurobiol. Aging.** 37, 210.e1–5.

Bandrés-Ciga, S., Price, T.R., Barrero, F.J., Escamilla-Sevilla, F., Pelegrina, J., Arepalli, S., Hernández, D., Gutiérrez, B., Cervilla, J., Rivera, M., Rivera, A., Ding, J.-H., Vives, F., Nalls, M., Singleton, A., Durán, R., 2016*. Genome-wide assessment of Parkinson's disease in a Southern Spanish population.* **Neurobiol. Aging** 45, 213.e3–9.

Bandrés-Ciga, S., Durán, R., Barrero, FJ., Escamilla-Sevilla, F., Pelegrina, J., Medina, JA., Ching-López, A., Cervilla, J., Gutiérrez, B., Vives, F., *Genetic association study of Parkinson's disease related-loci in Southern Spain. (Under review)*

Bandrés, S., Durán, R., Barrero, F., Ramírez, M., Vives, F., 2014. *Proteomic biomarkers in Parkinson's disease.* **Rev. Neurol.** 58, 166–174.

B) Book chapters

Bandrés S, Durán R, Vives F. Biomarcadores en la Enfermedad de Parkinson. Encuentros en Neurociencias (Vol II), Págs (57-80). ISBN: 978-84-96876-87-7. Editorial CIBER, 2013.

Bandrés-Ciga S, Durán R, Barrero FJ, Escamilla-Sevilla F, Pelegrina J, Ramírez M, Vives F. Genética de la Enfermedad de Parkinson: Una actualización. Encuentros en Neurociencias (Vol III), Págs (1-18). ISBN: 978-84-338-5819-1. Editorial Universidad de Granada, 2016.

Other articles published during this thesis:

Mencacci, N.E., R'bibo, L., **Bandres-Ciga, S.,** Carecchio, M., Zorzi, G., Nardocci, N., Garavaglia, B., Batla, A., Bhatia, K.P., Pittman, A.M., Hardy, J., Weissbach, A., Klein, C., Gasser, T., Lohmann, E., Wood, N.W., 2015a. *The CACNA1B R1389H variant is not associated with myoclonus-dystonia in a large European multicentric cohort.* **Hum. Mol. Genet**. 24, 5326–5329.

Mencacci, N.E., Rubio-Agusti, I., Zdebik, A., Asmus, F., Ludtmann, M.H.R., Ryten, M., Plagnol, V., Hauser, A.-K., **Bandres-Ciga, S.,** Bettencourt, C., Forabosco, P., Hughes, D., Soutar, M.M.P., Peall, K., Morris, H.R., Trabzuni, D., Tekman, M., Stanescu, H.C., Kleta, R., Carecchio, M., Zorzi, G., Nardocci, N., Garavaglia, B., Lohmann, E., Weissbach, A., Klein, C., Hardy, J., Pittman, A.M., Foltynie, T., Abramov, A.Y., Gasser, T., Bhatia, K.P., Wood, N.W., 2015b. *A missense mutation in KCTD17 causes autosomal dominant myoclonus-dystonia.* **Am. J. Hum. Genet**. 96, 938–947.

Bandrés Ciga, S., Salvatierra, J., López-Sidro, M., García-Sánchez, A., Durán, R., Vives, F., Raya-Álvarez, E., 2015. *An examination of the mechanisms involved in secondary clinical failure to adalimumab or etanercept in inflammatory arthropathies*. **J Clin Rheumatol** 21, 115–119.

Abbreviations

Aa: Amino acid

AAS: Amino acid substitution

AAO: Age at onset

AD: Alzheimer's disease

ADORA1: Adenosine A1 receptor A_1R gene

ADORA1: Adenosine A1 receptor A_1R protein

ATP13A2: Lysosomal type 5 P-type ATPase gene

ATP13A2: Lysosomal type 5 P-type ATPase

ARJP: Autosomal recessive juvenile parkinsonism

α-syn: α-synuclein

ATP: Adenosine triphosphate

AUC: Area under the curve

BP: Base pair

BST1: bone marrow stromal cell antigen 1 gene

CHR: Chromosome

CMA: Chaperone-mediated macroautophagy

CDCV: Common disease common variant

CDRV: Common disease rare variant

CNS: Central nervous system

CNV: Copy number variant

CI: Confidence Interval

CoQ: Coenzyme Q

dNTPs: Deoxynucleotide triphosphates

ddNTPs: Dideoxynucleotide triphosphates

dATP: Deoxyadenosine triphosphate

dTTP: Deoxythymin triphosphate

dGTP: Deoxyguanin triphosphate

dCTP: Deoxycytosin triphosphate

DNA: Deoxyrribonucleic acid

DNAJC13: DnaJ Heat Shock Protein Family (Hsp40) Member C13 gene

DNAJC6: DnaJ Heat Shock Protein Family (Hsp40) Member C6 gene

DRD: Dopa-responsive dystonia

DJ-1: Protein deglycase DJ-1

EOPD: Early-onset Parkinson's disease

EIF4G1: Eukaryotic Translation Initiation Factor 4 Gamma 1 gene

FBX07: F-box only protein 7 gene

FBX07: F-box only protein 7

FPD: Familial Parkinson's disease

GAK: Cyclin G Associated Kinase gene

DGKQ: Diacylglycerol kinase gene

Gak: Cyclin G Associated Kinase

GCase: Glucocerebrosidase

GWAS: Genome wide association study

GCTA: Genome wide complex trait analysis

GBA: Glucocerebrosidase gene

GBAP: Glucocerebrosidase pseudogen

GCH1: GTP cyclohydrolase 1 gene

GD: Gaucher's disease

GIGYF2: GRB10 Interacting GYF Protein 2 gene

GRS: Genetic risk score

HLA: human leukocyte antigen locus

HLA-DRA: α chain of major histocompatibility complex class II DR gene

HLA-DRB: β chains of major histocompatibility complex class II DR gene

HWE: Hardy-Weinberg equilibrium

IBD: Identity by descent

IL-2: Interleukin-2

IL-6: Interleukin-6

LA: Linkage analysis

LB(s): Lewy Body(ies)

LD: Linkage disequilibrium

LRRK2: Leucine-rich repeat kinase 2 gene

LRRK2: Leucine-rich repeat kinase 2

LOPD: Late-onset Parkinson's disease

MAO-A: Monoamino oxidase A

MAO-B: Monoamino oxidase B

MAPT: Microtubule-protein associated tau gene

MAPT: Microtubule-protein associated tau

MAF: Minor allele frequency

MIR4697: MicroRNA 4697

MLPA: Multiplex ligation probe amplification

MPTP: 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine

MPP+: 1-methyl-4-phenylpyridinium

NGS: Next generation sequencing

NUCKS1: Nuclear Casein Kinase and Cyclin-Dependent Kinase Substrate 1 gene

PARK2: Parkin RBR E3 Ubiquitin Protein Ligase gene

PBS: Phosphate-Buffered Salina

PCR: Polymerase Chain reaction

PCA: Principal component analysis

PCs: Principal components

PINK1: PTEN-induced putative kinase 1 gene

PINK1: PTEN-induced putative kinase 1

PLA2G6: Phospholipase a2 group VI gene

PLA2G6: Phospholipase a2 group VI

PD: Parkinson's disease

QC: Quality control

RAB7L1: RAB7, member RAS oncogene family-like 1 gene

Rab7l1: RAB7, member RAS oncogene family-like 1

ROHs: Runs of homozygosity

ROS: Reactive species of oxygen

RSN: Reactive species of nitrogen

OMI/HTRA2: Serine protease HTRA2 locus

OR: Odds ratio

OS: Oxidative stress

QQ: Quantile- quantile

RAI1: retinoic acid induced 1 gene

SBS: Sequencing by synthesis

SCIP: Screen for Cognitive Impairment in Psychiatry

SNCA: synuclein-alpha gene

SNP: Single nucleotide polymorphism

SNPSpD: single nucleotide polymorphism spectral decomposition

SNV: Single nucleotide variant

SNpc: Substantia nigra pars compacta

SREBF: Sterol regulatory element–binding protein gene

ssDNA: single-stranded DNA

STK39: Serine/Threonine Kinase 39 gene

SYNJ1: Synaptojanin 1 gene

TBE: Tris/Borate/EDTA

TSCA: TruSeq Custom Amplicon Assay

TMEM230: TMEM230 transmembrane protein 230 gene

TMEM230: TMEM230 transmembrane protein 230

TNF-α: Tumor necrosis factor alpha

TSCA: TruSeq Custom Amplicon Assay

UCH-L1: Ubiquitin carboxyl-terminal hydrolase L-1 gene

UPS: Ubiquitin proteasome system

UTRs: Untranslated regions

VPS13C: Vacuolar Protein Sorting 13 Homolog C gene

VPS13C: Vacuolar Protein Sorting 13 Homolog C

VPS35: Vacuolar protein sorting 35 gene

VPS35: Vacuolar protein sorting 35

WES: Whole-exome sequencing

WGS: Whole-genome sequencing

List of Figures

List of Tables

Resumen

En las últimas dos décadas, hemos sido testigos de una revolución en el campo de la genética de la Enfermedad de Parkinson (EP), un trastorno neurodegenerativo que afecta principalmente al movimiento y cuya causa es la muerte de las neuronas dopaminérgicas de la *substantia nigra pars compacta* (SNpc). El descubrimiento de genes asociados a mutaciones deletéreas causantes de la variedad familiar o de variantes genéticas de riesgo asociadas a la forma esporádica, ha proporcionado un mejor conocimiento acerca de los posibles mecanismos moleculares implicados en su patogénesis. Conocer las bases moleculares de esta enfermedad orientará el desarrollo de nuevas terapias farmacológicas que frenen, curen o prevengan la neurodegeneración.

El principal objetivo de la presente Tesis Doctoral es el estudio de la arquitectura genética de la EP familiar y esporádica en el sur de España, evaluando de forma detallada una población que hasta el momento ha sido pobremente estudiada en este contexto.

El primer objetivo de este trabajo se centró en llevar a cabo un *screening mutacional* en genes candidatos previamente asociados a la EP. Para ello, se incluyeron 134 pacientes, de los cuales 97 individuos desarrollaron EP con inicio tardío (EP-ta), 28 con inicio temprano (EP-te) y 9 eran casos familiares (EP-fam). El análisis genético se realizó mediante dos aproximaciones diferentes.

La primera se basó en un estudio de secuenciación de última generación a través de un panel que incluía los siguientes 8 genes: *LRRK2, SNCA, PARK2, PINK1, DJ-1, VPS35, GBA* y *GCH1,* en el grupo EP-te y EP-fam. En segundo lugar, para el grupo EP-ta, se secuenciaron los genes *GBA* (exones 8-11) y *LRRK2* (exones 31 y 41) donde residen la mayoría de las variantes patogénicas conocidas.

Se identificaron mutaciones patogénicas en un total de 30 pacientes (22,4%), destacando los genes *GBA* y *LRRK2* donde fueron particularmente frecuentes con respecto a otras poblaciones. Además, se detectó la deleción en heterocigosis de los exones 3 y 4 en el gen *PARK2* como causa de la EP en un caso familiar de inicio temprano. Uno de los hallazgos más interesantes fue el descubrimiento de la nueva mutación R32S en el gen *VPS35.* Aunque los análisis de simulación *in silico* realizados para predecir su grado de patogenicidad, catalogaron el cambio amino acídico como dañino tanto para la estructura, estabilidad y funcionamiento de la proteína codificada por este gen, se requieren investigaciones adicionales para establecer su papel en la patogénesis de la EP.

El segundo objetivo de la tesis fue el estudio de la variabilidad genética en determinados loci candidatos relacionados con la EP, en un grupo de 113 casos y 374 controles sanos. Concretamente, se analizaron 64 polimorfismos de tipo SNP (*single nucleotide polymorphism*) representativos de regiones específicas del genoma implicadas en la patogénesis de la EP, a través de un array customizado. Nuestros resultados mostraron que las variantes intrónicas de riesgo rs356204 y rs2736990 del gen *SNCA,* estaban asociadas significativamente a la EP en el modelo ajustado.

Los análisis haplotípicos desvelaron una asociación significativa de los haplotipos TTGG y CCAA del gen *GBA* (rs2990245-rs2049805-rs914615-rs206698), y el haplotipo TGGT del gen *SNCA* (rs356204-rs356219-rs2736990-rs356220) con la EP.

El tercer objetivo de esta tesis se centró en realizar el primer estudio genético de asociación a gran escala (GWAS) en enfermos de EP de España. Los GWAS constituyen la principal herramienta metodológica para expandir el espectro de las variantes genéticas de riesgo implicadas en la EP idiopática. Para ello, 240 casos y 192 controles fueron genotipados en el array NeuroX, un chip que contiene alrededor de 240.000 variantes genéticas. Se estudió si existía asociación entre el genotipo de variantes comunes y la EP, así como con la edad de inicio de la sintomatología. Se encontró una leve asociación ($p < 0,05$) entre los siguientes loci candidatos y la EP: *ACMSD/TMEM163, MAPT, STK39, MIR4697 y SREBF/RAI1.* No se detectó ninguna correlación con la edad de inicio.

Se realizó un perfil genético de riesgo incluyendo 30 variantes genéticas asociadas a la EP a través de un coeficiente de riesgo acumulativo. Se demostró que aquellos individuos portadores de un mayor número de alelos de riesgo tenían una predisposición 3,6 veces superior de desarrollar EP con respecto al grupo de individuos en el quintil más bajo del análisis.

Posteriormente, se estudió si existían posibles regiones en homocigosis sobrerrepresentadas en nuestro grupo de casos que pudieran revelar la presencia de variantes recesivas implicadas en la EP.

Se identificaron dos regiones en homocigosis más frecuentes en casos que en controles: una en el gen *HLA-DQB1* (6 casos y 1 control) y otra en el locus *GBA-SYT11* (1 caso).

Se exploró la presencia de variantes raras y de variantes genómicas estructurales. Los resultados no revelaron ninguna variante rara nueva asociada a la EP. Sin embargo, se identificó un largo espectro de variantes genéticas presentes en los casos y ausentes en controles en los genes *PINK1*, *PARK2*, *GIGYF2*, *DNAJC13*, *SYNJ1* y *FBXO7*. Cabe destacar las variantes patogénicas *GBA* N370S y *LRRK2* G2019S encontradas en 8 y 7 casos respectivamente. Finalmente, se identificó una deleción en el exón 6 del gen *PARK2* en un caso.

Los resultados de esta tesis permiten conocer mejor las variantes genéticas presentes en la población estudiada. Los marcadores genéticos descritos pueden ser de utilidad no sólo en el diagnóstico preclínico de la EP sino que en la identificación de individuos en riesgo de desarrollarla.

Abstract

In the last decades, we have witnessed a revolution in the field of genetics of Parkinson's disease (PD), a movement disorder caused by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The discovery of deleterious mutations and genetic risk variants in familial and sporadic PD cases respectively, has increased our knowledge about the possible molecular pathways involved on its pathogenesis. This substantial progress has helped us to better understand such devastating disease, and although the route to PD disease-modifying drugs is still long, it will hopefully be an achievable future goal.

This study aims to elucidate the genetic architecture of familial and sporadic PD in Southern Spain, assessing in detail a population that has so far been poorly studied in this context. Our first aim was to assess the contribution of known candidate genes in a cohort diagnosed with familial or sporadic PD. 134 patients were included in the study of which 97 individuals were diagnosed with late-onset sporadic PD (LOPD), 28 with early-onset sporadic PD (EOPD) and 9 with familial (FPD). Genetic analysis was performed through a next-generation sequencing panel to screen 8 PD-related genes (*LRRK2, SNCA, PARKIN, PINK1, DJ-1, VPS35, GBA,* and *GCH1*) in EOPD and FPD groups and direct Sanger sequencing of *GBA* exons 8-11 and *LRRK2* (exons 31,41) in the LOPD group, where it is thought to be the majority of pathogenic mutations.

We identified pathogenic mutations among 30 patients (22.4%). Mutations in the *GBA* and *LRRK2* genes appeared to be considerably frequent in our population. We also found a heterozygous *PARK2* deletion of exon 3 and 4 as responsible for PD in a familial case. Interestingly, we identified a novel mutation (R32S) in the *VPS35* gene. An *in silico* analysis was performed to predict the possible pathogenicity of this variant. The mutant amino acid was predicted to be damaging for the structure, stability and function of the protein encoded by *VPS35.* However, we cannot assume that this mutation may be causative and further studies should be done to understand its role on the pathogenesis of PD.

The second purpose of this thesis was to study the genetic variability of risk PD related loci, by performing a case-control association study in 113 individuals with PD and 374 healthy controls. We assessed 64 single nucleotide polymorphisms (SNPs) representative from specific regions of the genome previously linked to PD, through a customised array. Our results involved the intronic risk variants rs356204 and rs2736990 of the *SNCA* gene in the pathogenesis of idiopathic PD. Haplotype analyses revealed that the *GBA* haplotypes TTGG and CCAA (rs2990245-rs2049805-rs914615-rs206698), and the *SNCA* haplotype TGGT (rs356204-rs356219-rs2736990-rs356220) were significantly associated to PD.

The third purpose of this thesis was to perform the first genome wide association study (GWAS) of PD in Spain. GWAS constitute the main methodological conception to expand the spectrum of novel putative candidate loci associated to PD.

240 PD cases and 192 controls were genotyped on the NeuroX array, a powerful and reliable tool to screen for approximately 240,000 variants. We estimated genetic variation associated with PD risk and age at onset. We marginally replicated PD association (p-value < 0.05) at the following candidate loci: *ACMSD/TMEM163, MAPT, STK39, MIR4697 and SREBF/RAI1.* No significant correlation was found with age at onset.

We performed a risk profile analysis incorporating 30 SNPs previously associated with PD, by calculating a cumulative genetic risk score. We showed that individuals carrying a higher number of risk alleles where found to be 3.6 times more likely to develop PD than those in the lowest quintile of the analysis.

Subsequently, we studied if there were homozygosity regions overrepresented in our cases which could reveal presence of recessive variants related to PD. We found evidence of runs of homozygosity in two PD-associated regions: one intersecting the *HLA-DQB1* gene (6 cases and 1 control); and another intersecting the *GBA-SYT11* gene (1 case).

We then explored the presence of rare variants and structural genomic variants. We did not reveal any novel rare variant. However, we identified a wide spectrum of genetic variants present in cases and absent in controls among the following genes; *PINK1, PARK2, GIGYF2, DNAJC13, SYNJ1* y *FBXO.* It should be pointed out that the *GBA* N370S and the *LRRK2* G2019S variants were found in 8 and 7 cases respectively. Finally, a deletion was found in the exon 6 of the *PARK2* gene in an EOPD case.

The results of this thesis contribute to better understanding the genetic variants related to PD in the population from Southern Spain. The genetic markers described may be useful not only in the preclinical diagnosis of PD but in identifying individuals at risk of developing it.

I. Introduction

1. PARKINSON'S DISEASE IN THE CONTEXT OF NEURODEGENERATIVE DISEASES

Population ageing is considered a global health success, but also involves new health challenges in the form of age-related conditions including neurodegeneration.

Neurodegenerative diseases are defined as hereditary and sporadic incurable conditions which are characterized by progressive nervous system dysfunction (Forman et al., 2004). They are often associated with atrophy of the affected central or peripheral structures of the nervous system, and some of them share common features such as propensity to form protein aggregates, impairment in intracellular trafficking and dysfunctional protein degradation. They include diseases such as Alzheimer's Disease (AD) and other dementias, Parkinson's Disease, Multiple Sclerosis, Amyotrophic Lateral Sclerosis, Huntington's Disease and Prion Diseases, among others.

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and the first most frequent movement disorder (Gibrat et al., 2009). Such disease is considered as one of the toughest medical and economic challenges facing our society today since there are no available treatments that can stop or reverse the neurodegenerative process. Since it was described as the "shaking palsy" in 1817 by James Parkinson, our understanding of how neurodegeneration begins and progresses is still limited.

PD is and will continue to be a major issue for medicine and public health because of the demographic changes worldwide. Life expectancy is increasing specially in developed countries, due to the advances in the prevention and treatment of diseases.

PD is a multifaceted disease and it is now more often considered as a syndrome. It displays a wide range of clinical symptoms and variations in pathology, as it will be dicussed later on this thesis. Placing all PD patients into the same "umbrella of pure PD" does not seem to be the way to follow, since increasing evidence suggests the existence of different subtypes.

Fortunately, new research opportunities are emerging which will facilitate the development of novel therapeutics for such devastating condition. These combine different scientific approaches such as genetics (the main focus of this thesis), brain imaging, *in vitro* and *in vivo* models of disease, as well as advances in biomarker discovery and comprehensive clinical examinations.

2. EPIDEMIOLOGY

2.1. Prevalence

Prevalence indicates the total number of cases of disease on a given time. In industrialized countries, it is generally estimated that PD affects to about 1-2% in people over 60 years of age and 4% in people over the age of 85 (De Lau et al., 2006).

From an etiological perspective, PD results from different environmental exposition and genetic background that are due to intercultural differences. Some research studies have questioned the role of ethnicity in relation to PD, obtaining conflicting results. It has been reported lower prevalence of PD in South-American and Asian populations than in European populations (reviewed in Wirdefeldt et al., 2011). However, the lower prevalence may result from differences in life expectancy or delay in diagnosis due to limited access to appropriate health services, rather than ethnic differences. Several studies have shown higher prevalence in men than in women (Baldereschi et al., 2000; Van Den Eeden et al., 2003) although other studies found no gender difference (Zhang et al., 2005, De Rijk et al., 1997).

Nowadays, it is estimated that there are about 6 million people affected with PD, and by year 2030 it is expected that the number of individuals will be between 8.7 and 9.3 million people (Bach et al., 2011) as a result of ageing population and an increasing life expectancy.

2.2. Incidence

Compared to prevalence studies, there are relatively few incidence studies of PD. The term incidence refers to the number of new cases of a disease per unit of time, usually 1 year.

Reported incidence rates of PD are about 8–18 per 100,000 persons-year. De Lau et al., have reported that incident rates increase after the age of 50, particularly in men, and increase more rapidly over the age of 75 (De Lau et al., 2004). Apparently, risk for developing PD between the ages of 55 and 85 years is 8.5% for men and 7.7% for women. Between the ages of 75 and 85, the incident rate is 6.8 for men and 4.5 for women per 1,000 people per year. Over the age of 85, it increases to 12.1 and 10.2 for men and women respectively.

The incidence of PD has been reported to be higher in men than in women. A meta-analysis of incidence studies showed a male to female ratio of 1.49 (95% confidence interval (CI) 1.24–1.95) (Wooten et al., 2004). Suggested explanations for the male predisposition include protective effects of estrogens, higher frequency of toxins exposure due to professional issues as well as recessive susceptibility genes on the X chromosome.

Variations in PD incidence across ethnic groups might give clues about etiology, including differential environmental exposures or susceptibility genes. However, comparison among incidence studies of PD has been subjected to differences in methodology, often revealing inconsistent results.

3. CLINICAL ASPECTS OF PARKINSON'S DISEASE

3.1. Motor and non-motor symptoms

PD is characterized by a wide spectrum of symptomatology including the cardinal motor symptoms akinesia/bradykinesia (absence or slowness of movements), muscular rigidity, resting tremor and postural instability.

Resting tremor, is usually the first symptom reported by most of the patients. It mostly occurs in the upper limbs, legs and head. It is absent during voluntary actions, however, some PD patients can also suffer from postural and action tremor, re-emergent tremor and orthostatic tremor. Muscular rigidity can sometimes precede tremor and is defined as a constant increased resistance of a joint to passive movement, resulting in stiffness and a reduced ability to relax limb muscles. When this feature is present with resting tremor, a characteristic type of resistance is observed that has been given the name of "cogwheel-like" rigidity.

PD patients also present problems with executing and programming certain movements. Patients with advanced disease status usually suffer what is known as "freezing phenomena", a situation in which they are completely unable to start walking, resulting in stooped posture (Lees et al., 2009). Both voluntary and involuntary movements are often affected. Therefore, automatic and habitual movements such as swinging the arms while walking, eyes blinking, swallowing saliva or making gestures while talking are partially lost.

Non-motor symptoms have been described as very frequent among PD. Such features, in many cases, reduce quality of life to a greater extent than the cardinal symptoms. Non-motor symptoms include fatigue, anxiety, decreased motivation and apathy, depression, slowness in thinking (bradyphrenia), sleep disorders (especially REM sleep behaviour disorder), autonomic dysfunction such as constipation and hyposmia, and sensory symptoms such as pain (Bostantjopoulou et al., 2013). These symptoms are ocasionally complicated by cognitive decline (dementia) and psychiatric problems (hallucinations).

The motor features of PD are identified relatively late in the pathological process, when approximately 50% of dopaminergic neurons have been lost in the substantia nigra pars compacta (SNpc). However, it should be pointed out that some of the mentioned nonmotor symptoms, such as sleep disorders, loss of smell and depression can precede the onset of motor symptoms by over a decade.

3.2. Diagnosis

To date, an easily applicable and reliable diagnostic test or marker for PD diagnosis is not yet available. Thus, the diagnosis is clinical and based on defined criteria from the UK PD Brain bank **(Figure 1)**. An important limitation is that criteria lead to a diagnosis of probable PD, while a definite diagnosis requires post-mortem confirmation.

A prerequisite for the clinical diagnosis includes bradykinesia with one additional cardinal symptom such as muscular rigidity, resting tremor or postural instability (Hughes et al., 1992).

Moreover, it is crucial to exclude symptoms that might indicate other parkinsonian syndromes that have their own neuropathological changes, and to ascertain at least three supportive criteria for PD, such as unilateral onset of symptoms, persistent asymmetry of clinical symptoms, good response to levodopa treatment and induction of dyskinesias by the dopaminergic treatment.

UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria

Step 1 Diagnosis of Parkinsonian syndrome

- Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive actions)
- And at least one of the following:
  - muscular rigidity
  - 4–6 Hz rest tremor
  - postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction.

Step 2 Exclusion criteria for Parkinson's disease

- History of repeated strokes with stepwise progression of parkinsonian features
- History of repeated head injury
- History of definite encephalitis
- Oculogyric crises
- Neuroleptic treatment at onset of symptoms
- More than one affected relative
- Sustained remission
- Strictly unilateral features after 3 years
- Supranuclear gaze palsy
- Cerebellar signs
- Early severe autonomic involvement
- Early severe dementia with disturbances of memory, language, and praxis
- Babinski sign
- Presence of cerebral tumour or communicating hydrocephalus on CT scan
- Negative response to large doses of levodopa (if malabsorption excluded)
- MPTP exposure

Step 3 Supportive prospective positive criteria for Parkinson's disease

(Three or more required for diagnosis of definite Parkinson's disease)

- Unilateral onset
- Rest tremor present
- Progressive disorder
- Persistent asymmetry affecting side of onset most
- Excellent response (70–100%) to levodopa
- Severe levodopa-induced chorea
- Levodopa response for 5 years or more
- Clinical course of 10 years or more

Figure 1. Clinical diagnosis criteria by the PD Society Brain Bank. (*Reproduced from Hughes et al., 1992).*

3.3. Treatment

There are no disease-modifying drugs available to prevent, cure or stop PD progression. Treatment is just symptomatic and it ameliorates the quality of life of PD patients correcting the motor disturbances. It is believed that even if there would be new treatments, pathology may be too advanced at the point of clinical diagnosis to be efficiently treated by neuroprotective treatments. Thus, identifying individuals at the earliest stages of disease, as it will be discussed later in this thesis, is an important challenge to reach. Predicting PD before the appearance of motor symptoms, would pave the road for clinical trials of novel and efficient neuroprotective therapies.

Among the symptomatic therapies used to treat PD, a precursor to dopamine (levodopa), in combination with a peripheral dopa decarboxylase inhibitors (benserazide or carbidopa), is the most common initial treatment for patients. At the beginning of the treatment, response is usually very good. However, with disease progression, the majority of patients experience shorter duration of response to individual doses (wearing-off symptoms), alternative phases with good and poor response to medication (on-off symptoms) and involuntary movements of the head, body or limbs (dyskinesias).

There are other useful dopaminergic medications to manage these fluctuations. They include monoamine oxidase type B inhibitors (selegiline and rasagiline), catechol-O-methyltransferase inhibitors (tolcapone and entacapone), the NMDA receptor antagonist (amantadine) and dopamine receptor agonists (pramipexole, ropinirole, rotigotine, apomorphin and piribedil) (reviewed in Sveinbjornsdottir S., 2016).

Surgical therapy, usually with deep brain electrical stimulation, is available for a selected proportion of patients when medical therapy fails to control the motor symptoms.

4. NEUROPATHOLOGICAL ASPECTS OF PARKINSON'S DISEASE

PD has distinctive neuropathological brain changes. The pathological hallmark of PD is the progressive loss of dopaminergic neurons mainly in the SNpc and other brain areas, the presence of abnormal proteinaceous spherical bodies called Lewy bodies (LBs) in the surviving neurons (Tolosa et al., 2006) and Lewy neurites spreading out the central nervous system as the disease progresses.

LBs are intracytoplasmatic inclusions particularly rich in aggregated α-synuclein (α-syn), and other numerous proteins including components of the ubiquitin–proteasome system, molecular chaperones and lipids (reviewed in Brundin et al., 2008) **(Figure 2).**

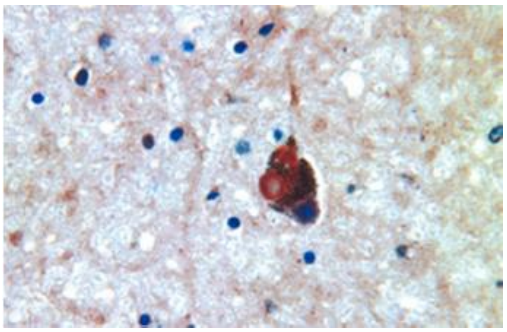


Figure 2. LBs in a degenerating neuron. (*Reproduced from Sveinbjornsdottir S., 2016).*

α-Syn plays a key role in the pathogenesis of PD as it will be discussed later on this thesis. It is physiologically enriched in nerve terminals and althought its functions are not completely elucidated, it is thought to be involved in synaptic vesicle recycling, storage and comparmentalization of neurotransmitters. In PD, natively unfolded α-syn monomers form β-sheet rich oligomers which together lead to protofibrils of heterogenous structure. The protofibrils may give rise to more stable and insoluble amyloid-like fibrils. α-Syn fibrils eventually aggregate and precipitate to form LB **(Figure 3).**

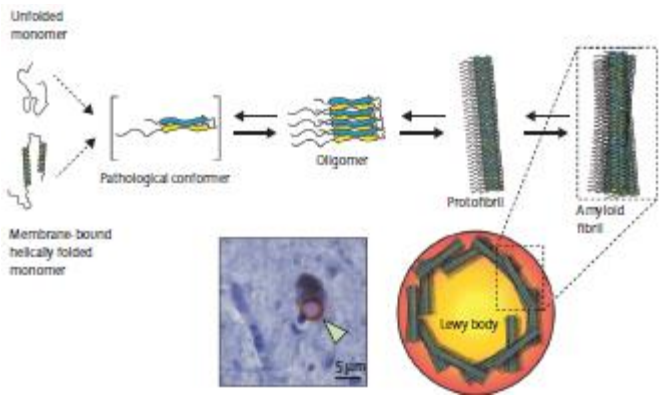


Figure 3. Eschema of α-syn fibrillogenesis. (*Reproduced from Dehay et al., 2015)*

This protein is often posttranslationally modified. The most common posttranslational modification is phosphorylation at residue Ser129 and Ser87 which are observed prominently within LBs (Fujiwara et al., 2002, Okochi et al., 2000). Other posttranslational modifications include nitrosylation (Hodara et al., 2004) and glycosylation.

The native state of α-syn is extensively debated. It can exist in various conformations and oligomeric states in a dynamic equilibrium, modulated by factors that either accelerate or inhibit fibrillation (reviewed in Dehay et al., 2015). The identification and characterisation of the toxic α-syn species remain incomplete. Two hypotheses have been proposed: toxic species could be amyloid-like insoluble fibrils (notably identified in LBs), or soluble prefibrillar intermediates, such as oligomers or protofibrils. Compelling evidence from both *in vitro* and *in vivo* studies has supported the hypothesis that oligomeric species are the toxic species (Fauvet et al., 2012, Winner et al., 2011).

In 2003, Braak et al., proposed that PD progresses in six stages that can be distinguished by the anatomical spread of LB pathology, on the basis of the correlation of neuropathological findings with preclinical and clinical disease. In the pre-symptomatic stages of the disease (stages 1–2), LBs are confined to the medulla oblongata/pontine tegmentum, olfactory bulb/anterior olfactory nucleus and gastrointestinal system. With progression of the disease, SNpc and other areas of the midbrain and forebrain become affected (stages 3–4). It has been suggested that patients develop clinical symptoms of the disease at this stage. In the end stage (stage 5–6), the process affects the neocortex with a wide variety of clinical manifestations.

It has been widely hypothesised that the disease might begin in the gastric autonomic plexus of Meissner and the olfactory nerve endings. Then abnormally folded α-syn could be transferred from one neuron to another along anatomical pathways by a prion-like mechanism within the central nervous system and from the enteric nervous system.

The disease spreading hypothesis has always been a matter of debate. Several compelling studies have shown that intracerebral inoculation of α-syn taken from the SNpc of patients with PD can promote LB pathology in host neurons of animals (Recaesens et al., 2014, Luk et al., 2012). What initiates the misfolding of α-syn and a spreading cascade remains to be established.

5. ETIOLOGY OF PARKINSON'S DISEASE

Although the neuropathology of PD is relatively well understood, the causes that lead to neurodegeneration are quite unknown. PD has a complex etiology which only has started to be decoded in the last decades.

5.1. Genetic factors

Until about 20 years ago, PD was considered the textbook example of a "non-genetic" disorder. The first cross-sectional series of twin studies perfomed in the 1980s, failed to demonstrate significantly higher concordance of PD in monozygotic *versus* dizygotic twins (Ward et al., 1983, Eldridge and Ince, 1984, Duvoisin et al., 1981, Ward et al., 1984). This conclusion was supported by previous epidemiological studies which linked PD to environmental causes such as viral infection or neurotoxins. In 1918, a pandemic influenza virus was strongly associated with postencephalic PD, pointing to viral infection as the major cause of PD.

Moreover, in the 1980s, the observation that drug users exposed to 1-methyl-4-phenyl 1.2,3,6-tetrahydropyridine (MPTP) developed parkinsonian-like features strenghten the notion that PD was a non-genetic disease (Langston and Ballard, 1983). However, molecular genetics changed completely this view thank to the identification of genes linked to familial rare monogenic forms of PD, with autosomal dominant or recessive pattern of inheritance. More recently, an increasing number of risk loci/genes have been seen to predispose to the sporadic form. To date, PD is generally considered a multifactorial disorder that arises from the combination of genes and environmental factors.

The genetic basis of PD is the main issue of this thesis, and it will be addressed with further detail at the end of the introduction.

5.2 Environmental factors

As it has been discussed above, the majority of PD cases are sporadic by nature, indicating that there are environmental or lifestyle factors involved on develop of disease.

5.2.1 Risk factors

The first link of PD to the environment was a report indicating that intravenous injection of meperidine analogs resulted in chronic parkinsonism and degeneration of dopaminergic neurons in humans. A larger case series of drug users identified MPTP as a potent dopamine neuron toxicant (Langston and Ballard, 1983).

MPTP is a blood-brain barrier permeant compound. Auto-oxidation of this compound leads to the formation of the toxic 1-methyl-4-phenylpyridinium (MPP+), which is taken up by dopamine neurons via the dopamine transporter, where it presumably acts to inhibit mitochondrial complex I activity causing degeneration (reviewed in Mhyre et al., 2012).

After this initial discovery, a number of studies have reported the association between exposure to toxicants and increasing risk of developing PD. Pesticides such as rotenone, paraquat, dichlorodiphenyltrichloroethane, dieldrin, and organophosphates have been largely associated to PD (reviewed in Priyadarshi et al., 2000). A recent meta-analysis of 46 studies from around the world found a summary risk ratio of 1.62 (95% CI [1.40–1.88]) for pesticide exposure (ever versus never) (Van der Mark et al., 2012).

Besides pesticides, other toxicants were proposed to increase risk for PD, such as polychlorinated biphenyls, solvents, heavy metals (manganese) and air pollutant. Moreover, it seems that rural living, drinking well water, farming profession, head trauma (such as parkinsonism in boxers, american football and rugby players), viral infections (such as post-encephalitic parkinsonism) and exposure to electromagnetic radiation, might influence the risk to develop PD (reviewed in Mhyre et al., 2012).

5.2.2 Protective factors

Interestingly, recent studies have identified potential environmental protective factors that appear to confer some benefit to decrease the incidence of PD, including tobacco use, coffee and tea consumption, alcohol and circulating levels of uric acid (Wirdefeldt et al., 2011). Nicotine (Quik, 2004) and caffeine (Kalda et al., 2006) have been proposed to be neuroprotective in PD, and nicotine itself has been shown to inhibit α-syn fibrillization (Ono et al., 2007). Higher plasma urate levels have been related to a decreased rate of decline in PD patients (Noyce et al., 2012), but the biologic mechanisms for this possibly causal relation are poorly understood. Additionally, there are a number of medications for which negative associations with PD have been reported in observational studies, including calcium channel blockers, non-steroidal anti-inflammatories and statins (reviewed in Noyce et al., 2016).

6. PATHOGENESIS

The pathogenesis of PD includes a broad spectrum of pathways from oxidative stress and mitocondrial dysfunction to α-syn aggregation and abnormal protein metabolism, lysosomal dysfunction and neuroinflammation. Most of these pathophysiological links with PD are the results of studying the functional consequences of gene mutations implicated in familial or early onset PD. The genetic risk loci involved in sporadic PD are also being helpful in identifying novel pathways and raising new research questions.

6.1. Oxidative Stress and mitochondrial dysfunction

Oxidative stress (OS) is a condition of imbalance between free radicals production and antioxidant defenses, resulting in excessive accumulation of reactive species of oxygen (ROS) and reactive species of nitrogen. OS leads to damage in the cell, causing lipid peroxidation, changes in protein structure and function due to protein oxidation, and structural damage to DNA (Gandhi and Abramov, 2012).

The human body produces small amounts of ROS in healthy conditions, in order to undergo normal physiological processes such as inflammatory response, cell growth and signaling, synthesis of biological molecules and important roles in the immune system (Brieger et al., 2012). As the brain is one of the most metabolically active organs in the body, it is vulnerable to OS particularly because of the following reasons. First, the brain has a high oxygen demand, which constitutes 20% of the body oxygen consumption. Second, the redox-active metals such as iron or copper exist abundantly in the brain and they are actively involved to catalyze ROS formation. Third, the high levels of polyunsaturated fatty acids and phospholipids are found in the brain cell membranes and act as substrates for lipid peroxidation. Fourth, there are relatively low levels of glutation reductase in the brain, which plays a role as endogenous antioxidant in the elimination of ROS (Ferrerira et al., 2009) (reviewed in Kim et al., 2015).

Dopaminergic neurons are highly vulnerable to free radical-mediated injury due to their important oxidation metabolism (Halliwell, 1992). It has been widely suggested that dopamine metabolism itself contributes to neurodegeneration (Jenner and Olanow., 2006).

Tyrosine hidroxylase, responsible for the catalysis of tyrosine to L-Dopa, is known to stimulate the generation of hydroxyl radicals (Haavik, 1997). Moreover, dopamine is metabolised by MAO-A and B (monoamine oxidases) which catalyse its oxidative deamination promoting free radicals releasement.

Convincing evidence for increased oxidation has been found in postmorten brain tissue, cell culture and animal models of PD (reviewed in Varcin et al., 2012), as well as in human samples (Durán et al., 2011). Indeed, several studies have reported altered antioxidant defences (Sian et al., 1994, Nikam et al., 2009). *In vivo* and *in vitro* studies support the notion that OS promotes α-syn oligomerization and aggregation (Esteves et al., 2009), which in turn exacerbates ROS, creating a vicious cycle leading to neurodegeneration.

The mitochondrial free radical theory of aging suggests that mitochondria are a main source of ROS production (Harman, 1972). Among all the organelles that can generate ROS within the cell, mitochondria are the major sites responsible for more than 90% of the ROS generation. Mitochondrial dysfunction is closely related to the increased ROS formation in PD and dysregulation of calcium metabolism (Yan et al., 2013). The disruption of electron transfer, due to an error in the respiratory electron transport chain, causes a depletion in the synthesis of ATP and an increase of ROS resulting in apoptosis. Free radicals, including superoxide, are produced via oxidative phosphorylation and its progressive overabundance can result in neural degeneration.

The respiratory electron transport chain consists of five multi-subunit complexes including NADH-coenzyme Q (CoQ) reductase (NADH dehydrogenase, *Complex I),* succinate dehydrogenase *(Complex II),* coenzyme Q-cytochrome c reductase *(Complex III),* cytochrome C oxidase *(Complex IV),* and ATP synthase *(Complex V).*

The most definitive evidence of the implication of mitochondrial dysfunction in PD came from studies using mitochondrial complex-I inhibitors such as MPTP and its metabolite, MPP+, in which a loss of nigral dopaminergic neurons was found in animal models of PD (Thiruchelvam et al., 2003, Langston et al., 2004). In line with these studies, other complex I inhibitors such as the alkaloid rotenone (Sherer et al., 2003) and the acetogenin annonacin (Champy et al., 2004), can also induce irreversible lesions in dopaminergic neurons by the same mechanisms. Additionally, several research groups have reported a decrease in the activity of complex I in the SNpc, lymphocytes and platelets of PD patients (Heo et al., 2012, Schapira, 2008).

Major advances in the field of mitochondrial dysfunction have been made thank to the discovery that some of the PD-related genes detailed in previous sections, are involved in key biological processes that govern mitochondrial homeostasis and stress response. In particular, it is now well known that the recessive PD-linked genes *PINK1* (PTEN-induced putative kinase 1 gene) and *PARK2* (Parkin RBR E3 Ubiquitin Protein Ligase gene) likely play a central role in mitochondrial quality control and dynamics (Narendra et al., 2012). Mitochondrial quality control refers to cellular mechanisms aimed at ensuring proper functioning of the mitochondrial network, which is critical for cell physiology and survival.

It involves several functional aspects including autophagic elimination of dysfunctional/damaged organelles (known as mitophagy), damage prevention and repair mechanisms (reviewed in Michel et al., 2016).

The proteins PINK1 and Parkin (encoded by *PARK2*) have been shown not only to participate in the functional and morphological maintenance of the mitochondrial network (Gautier et al., 2008) but also to critically regulate the removal of dysfunctional mitocondria through mitophagy (Deas et al., 2011). Interestingly, in a recent study PINK1 kinase has been found to phosphorylate Parkin and ubiquitin, recruiting Parkin onto depolarized mitochondria, activating its E3 ligase activity (Koyano et al., 2014) and promoting the ubiquitinization of damaged mitochondrial proteins. These ubiquitinated proteins signal to the cell that the damaged mitochondria must undergo mitophagy. Remarkably, parkin and PINK1 fulfill these functions collectively.

Parkin is recruited to damaged mitochondria by PINK1 and acts to promote their degradation by autophagy (Narendra et al., 2010; Bertolin et al., 2013). Impairment of mitophagy by mutations of *PINK1* or *PARKIN* may become toxic by accumulation of dysfunctional mitochondria (Vives-Bauza et al., 2010).

Mutations in *SNCA* (α-syn gene) and *DJ-1* (Protein deglycase DJ-1 gene) have been also known to affect mitochondrial function and increase oxidative stress (Blesa et al., 2015). It has been demonstrated that α-syn inhibits complex I activity (Devi et al., 2008) and causes mitochondrial fragmentation (Nakamura et al., 2011) resulting in increased mitophagy (Choubey et al., 2011).

Moreover, functional studies have shown that the binding of α-syn to mitochondria is followed by cytochrome C release, increased calcium and ROS levels resulting in cell death (Parihar et al., 2008).

Finally, accumulation of point mutations and deletions in mitochondrial DNA may be also associated with mitochondrial dysfunction in PD (Hauser and Hastings, 2013).

6.2. α-Synuclein aggregation and protein impairment degradation

As mentioned above, one of the hallmarks in PD pathogenesis is the impairment of proteasome-mediated protein degradation and the formation and accumulation of α-syn aggregates in LBs.

The ubiquitin proteasome system (UPS) is the main non-lysosomal pathway which cells remove unwanted, short-lived and damaged proteins. UPS is also involved in the degradation of defective mitochondria. Structurally, it is a multiprotein complex including two different subunits: the 26S and the 20S proteasomes. UPS labels cellular proteins with ubiquitin for degradation, being helped by the ubiquitin-activating enzymes E1, E2 and E3 (parkin proteins encoded by *PARK2*).

Protein degradation constitutes a defense mechanism and the way to avoid that oxidizied proteins could form aggregates. The Aa products released during the degradation, can function as ROS scavengers protecting cellular components from oxidation (reviewed in Dasuri et al., 2012).

Mutations in *PARK2* and *UCH-L1* (ubiquitin carboxyl-terminal hydrolase L-1 gene) encoding two important components of the UPS, (the E3 ubiquitin ligase parkin and the ubiquitin carboxyl-terminal hydrolase L-1), shed some light into the implication of this molecular event in PD pathogenesis. In the SNpc of PD patients, impaired UPS has been found with structural proteasome alterations. There is also a reduction in proteasomal enzyme activity in the SNpc that is specific for PD and does not occur in other areas of the brain (Tofaris et al., 2003, McNaught et al., 2003).

Moreover, in culture, cell death induced by proteasome inhibitors leads to increased OS as well as alterations in mitochondrial function (Hyun et al., 2004). On the other way around, evidence suggests that OS can target the UPS and impair its ability to degrade proteins (Aiken et al., 2011), and that inhibition of mitochondrial complex I impairs proteasomal activity through oxidative modification of proteasome components (Shamoto-Nagai et al., 2003).

Maintaining physiological levels of α-syn in neurons is crucial for their survival. Toxic species of α-syn are usually more resistant to proteolysis by the UPS, and aggregation of α-syn is a key event involved in PD pathogenesis. Several studies have shown that oligomeric α-syn impairs UPS function (Lindersson et al., 2004, Tanaka et al., 2001). A vicious cycle occurs in which α-syn accumulation impairs its clearance, thus promoting further accumulation and ultimately leads to neurodegeneration. Posttranslational modifications of the protein could also contribute to disease pathogenesis through increasing neurotoxicity of the aggregating protein.

A critical process that leads to progressive neuropathology in PD is the prion-like propagation of α-syn. It seems that endocytosis of α-syn into healthy neurons triggers abnormal protein aggregation leading to a cytotoxic cascade which finishes in cell death (Braidy et al., 2014). However, how and why α-syn begins to misfold is not clear yet.

Genetic factors, such as *SNCA* point mutations, duplications or triplications enhance α-syn aggregation leading to early-onset and rapidly progressive forms of PD (Kim and Lee, 2008).

6.3. Autophagy and lysosomal dysfunction

Lysosomes are dynamic acidic organelles that contain hydrolytic enzymes capable of degrading intracelular components through several pathways, including autophagy. Autophagy is a term referring to "self-eating" and describes the cellular catabolic process in which cytosolic components, including mainly long-lived proteins and organelles are transported to lysosome for degradation. There are three main types of autophagy, including microautophagy, macroautophagy and chaperone-mediated macroautophagy (CMA). Emerging evidence has suggested impaired autophagy as one of the mechanisms for PD pathogenesis.

A decreased number of intraneuronal lysosomes and lysosomal-associated proteins have been observed in postmortem brain from patients with PD and in toxin and genetic rodent models of PD (reviewed in Dehay et al., 2013).

Although it was initially thought that α-syn was only degraded by the UPS, we now know that this protein can also be degraded through lysosomal-mediated autophagy.

It has been recently reported that α–syn can directly impair UPS and lysosome resulting in defective clearance and accumulation of abnormal species, both *in vitro* and *in vivo* (Winslow et al., 2010, Crews et al., 2010). Mutations in genes that encode lysosomal proteins, including the enzyme glucocerebrosidase (GCase) and lysosomal type 5 P-type ATPase (ATP13A2), support the idea that lysosomal impairment may play a primary pathogenic role in PD.

Recent studies indicate that GCase can influence α-syn processing through both gain-of function and loss-of-function mechanisms. GCase catalyzes the conversion of the glycolipid glucosylceramide into glucose and ceramide inside lysosomes. GCase dysfunction leads to the accumulation of glucosylceramide which is thought to stabilise α-syn oligomers. It seems that loss of GCase activity results in glucosylceramide accumulation, decreased lysosomal degradation, and subsequent accumulation of α-syn, which eventually impais GCase trafficking from endoplasmatic reticulum and Golgi to lysosomes (Mazzulli et al., 2011). This positive feedback loop was proposed to lead to neurodegeneration in PD. However, in another study, overexpression of several *GBA* (Glucocerebrosidase gene) mutants in cultured cell lines also resulted in α-syn accumulation, suggesting a gain-of function mechanism (Cullen et al., 2011).

ATP13A2 also confers protection against α-syn misfolding and attenuates its toxicity. Loss of function mutations are related to impaired lysosomal function and α-syn homeostasis in mice (Bras et al., 2012).

Other PD-related genes have been also linked to autophagy alterations. Mutations in *LRRK2* (Leucine-rich repeat kinase 2 gene) have been associated with impaired autophagy but its mechanism is still unknown (Gómez-Suaga et al., 2012). In addition, mutations in *PINK1* and *PARK2* have been shown to promote defective mitophagy, as it has been detailed above. Similarly, VPS35 (Vacuolar protein sorting 35) participates in vesicular trafficking and forms a functional network together with LRRK2 and proteins encoded by GWAS-identified genes *GAK* (Cyclin G Associated Kinase gene) and *RAB7L1* (member RAS oncogene family-like 1 gene), with a central role in the retromer complex pathway involved in endosomal-Golgi trafficking (Beilina et al., 2014). It was recently shown that the retromer complex has an important role in autophagosome elongation in autophagy (Orsi et al., 2012). Recently identified PD genes *SYNJ1* (Synaptojanin 1 gene), *DNAJC13* (DnaJ Heat Shock Protein Family (Hsp40) Member C13 gene) and *DNAJC6* (DnaJ Heat Shock Protein Family (Hsp40) Member C6 gene) also participate in vesicular trafficking and it is speculated that mutations in these genes could also be linked to autophagy dysfunction.

6.4 Neuroinflammation

Neuronal loss is associated with chronic neuroinflammation which is controlled mainly by microglia (Perry et al., 2012). Compelling evidence supports the notion that neuroinflammation is a critical component of the pathophysioloy of PD mediated by activated microglia. Microglia are brain phagocytes which in response to certain environmental toxins and endogenous proteins can shift to an over-activated state and release ROS causing neurotoxicity.

This process also leads to increased cytokine formation and decreased trophic factors secretion which in turn promote neurodegeneration in a neurotoxic vicious cicle (Iravani et al., 2012).

In 1988, McGeer et al., showed the presence of reactive microglia in the SNpc of human post-mortem brain tissue, which was the first evidence suggesting the involvement of neuroinflammation in PD pathogenesis. Subsequently, an over-activation of microglia was also detected in the SNpc and striatum of PD animal models (Stott and Barker, 2014, Tansey et al., 2007). Moreover, it has been seen infiltration and accumulation of immune cells from the periphery in and around the affected brain regions of PD patients (reviewed in Wang et al., 2015).

Aditionally, increased concentration of neuroinflammatory markers such as IL-2, IL-6, TNF-α and osteopontin among others, has been found increased in PD patients (Rentzos et al., 2007). Moreover, studies show that nonsteroidal anti-inflammatory drugs lower the risk of neurodegenerative diseases, such as PD (Hirsch et al., 2009).

Neuroinflammation historically was related to a response to neurodegeneration in PD. However, recent studies revealed that neuroinflammation could be the trigger or the key player in the onset of PD by creating a pathogenic environment. Interestingly, the finding of the human leukocyte antigen (*HLA*) as a risk factor for PD (Nalls et al., 2014) questions the possibility of a pro-inflammatory state in PD as a primary cause of neuronal loss or at least as an important disease-modifier risk factor. Also, α-syn, Parkin, LRRK2, and DJ-1 have recently been reported to mediate microglia activation (Wilhelmus et al., 2012).

What is still at issue is whether neuroinflammation is a primary process in PD, ie that inflammatory factors are the cause of PD or is a side effect to cell death that occurs in PD. Elevated presence of inflammatory molecules is a common component of the normal aging brain, and neuroinflammation appears to actively contribute to central nervous system (CNS) pathophysiology (Moore et al., 2010).

7. GENETIC BASIS OF PARKINSON'S DISEASE

7.1. Genetics: A general overview

In the following subsection, a general overview about the current panorama on genetics will allow to better understand the content of this thesis.

The diploid human genome is around 6 billion base pairs (bp) of DNA stored in 23 chromosome pairs. Only a small fraction of the total sequence of the genome called exome, encodes proteins. Although today the exact number of genes is still unknown, it is thought that there are approximately 21000 protein-coding genes (1%-2%) contained in the human genome **(Figure 4).** The remainder of the non-coding genome consists of RNA genes, regulatory sequences such as introns and untranslated regions (UTRs), and repetitive DNA in which the function is poorly understood (reviewed in Hardy and Pittman., 2013).

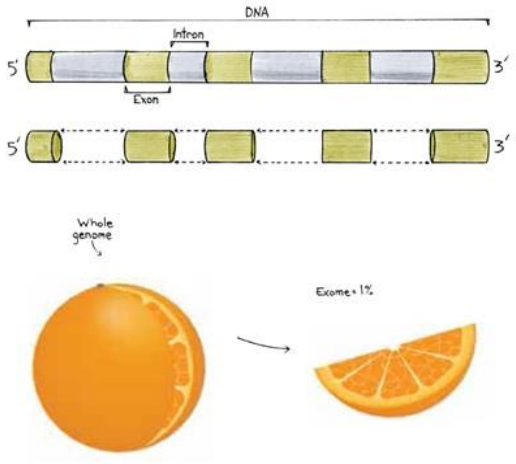


Figure 4. DNA illustration showing exome vs genome. (*Adapted from University of Washington, 2012)*

Several different classes of DNA variations can occur between the genomes of different individuals. The most common type of variation is the single-nucleotide polymorphism (SNP). There are approximately 3 million of such variants in any given individual compared with those of the reference sequence.

These single base substitutions or point mutations arise, every 1000 bp or so, and those present in more than 1% of the population are known as **common variants**. These are often located in noncoding regions of the genome and tend to have little or no phenotypic effect. The vast majority of these SNPs have been extensively studied in many ethnically diverse populations by initiatives such as the International HapMap Project (www.hapmap.org,) and constitute a valuable resource for genome-wide association studies (GWAS) (www.gwascentral.org), as it will be dicussed later in this thesis (reviewed in Hardy and Pittman., 2013).

It is thought that these SNPs must confer risk by altering the biologically relevant transcript either by affecting transcript expression levels, altering splicing, or changing its subcellular localization.

The SNPs that occur in less than 1% of the population are classified as **rare variants**, and some of these may have profound phenotypic effects, altering the sequence of a protein-coding gene. Genomic variation can also be caused by multiple base changes for insertion and deletion variants that range in size from 1 to 1000 bp, named Indels. Such variants can have a substantial effect in coding regions of the genome where they can result in deleterious alterations in the encoded protein or even a "frameshift" of the sequence resulting in a truncated protein. Larger insertions or deletions are referred to as copy number variants (CNVs) and can be both common and rare (reviewed in Hardy and Pittman, 2013).

Variation in DNA can also occur due to epigenetics. Epigenetic modifications provide phenotypic plasticity allowing adaptation to a change in the environment without modifying the genotype. Processes such as methylation, phosphorylation, acetylation and generation of micro-RNAs allow modulation of gene expression and translation in response to environmental stimuli.

To better understand the genetic architecture of complex diseases, Manolio et al., (2009) suggested that genetic variants with different effect size (low, moderate, high) and frequency (common or rare) are responsible of its etiology **(Figure 5).** Based on this concept, two distinctive hypothesis have been postulated which can be applied to the complicated nature of PD (Manolio et al., 2009).

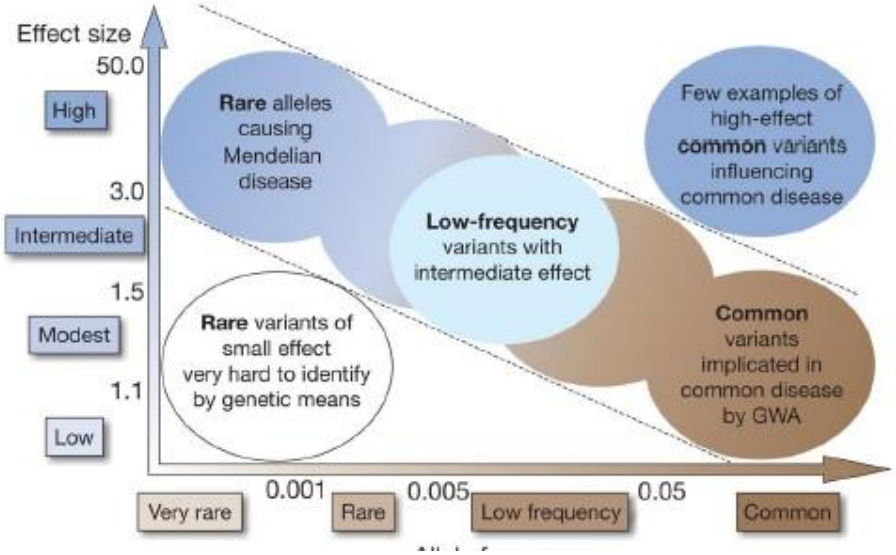


Figure 5. The genetic architecture of complex diseases. *(Reproduced from Manolio et al., 2009.)*

7.1.1. The common disease common variant hypothesis (CDCV)

The CDCV theory hypothesises that multiple, common risk variants of small effect size interact to cause common disease (Reich and Lander, 2001). By definition, these variants are common and have been within the population for a significant amount of time. Therefore, unlike rare variants, deleterious and highly functional alleles are more likely to have been selected out of the population.

This hypothesis would accept that the effect of individual common alleles on any deleterious trait related to PD is likely to be quite small, but collectively, numerous common alleles may contribute substantially to PD pathogenesis (reviewed in Singleton et al., 2010).

This hypothesis is the core for GWAS, whose methodology will be discussed later on this thesis.

7.1.2. The common disease rare variant hypothesis (CDRV)

It is also known as the disease heterogeneity hypothesis. The CDRV hypothesis speculates that a contributing risk component for complex disease will be rare genetic variants of small or moderate/big effect where highly functional, deleterious alleles might exist. This phenomenon may be particularly produced in late-onset diseases such sporadic PD, where selective pressures do not apply.

It is more difficult to efficiently study the genetic basis of PD following the CDRV hypothesis in powered sample series. First, GWAS cannot efficiently detect such variants due to lack of statistical power for sample size constraints.

Second, diseases such as PD, might be characterised by genetic heterogeneity where multiple rare variants exist within the same locus. Third, rare variants usually cannot be detected efficiently through imputation because of their rarity and moreover they are often absent from public databases. And finally, linkage analysis is not suitable because such variants are often not sufficiently penetrant.

The lower frequency of these alleles requires very large sample numbers to detect significant effects and thus it is expensive to execute well-powered studies. As a result, the CDRV hypothesis remains poorly tested in most common disorders such as PD, a situation that will change in the near future when next-generation sequencing (NGS) technologies become more accesible and cheaper to labs across the world. Exome sequencing and pooled analysis of large numbers of cases, as well as exon arrays of custom content are the most powerful approach to detect such rare variants (reviewed in Singleton et al., 2010).

7.2 Methods for gene discovery

In the recent years, we have witnessed the emergence of new technologies that have revolutionized our concepts to identify genetic mechanisms implicated in human health and disease. The publication of the human genome sequence in 2001 (Lander and Venter, 2001) followed by the description of detailed catalogs for common genetic variability (HapMap Project), marked an exciting starting point for studying the genomics of complex disease. As a consequence, the development of methodological strategies to study the genome in a systematic and rapid, cost-effective manner became a reality.

It took several thousand researchers and 13 years to sequence the first human genome at a cost of $3 billion. Today, one technician can sequence an entire genome in less than a week for less than $2000. We now can determine the genome structure and its variation and examine its effects on phenotype in an unprecedented manner. Understanding the pathogenetic mechanisms of a disease mainly depends on finding the causative gene and the variants associated with the phenotype. The application of novel technologies is changing the picture of medical genetic research. The discovery of disease-causing and disease-predisposing genes is accelerating, and the phenotypic spectrum associated with previously known genes is expanding.

Based on the proposed model by Manolio et al., to explain the genetics of complex diseases, (indexed at the beginning of the introduction), Singleton et al., (2010) reported the following graph of gene discovery strategies **(Figure 6).**

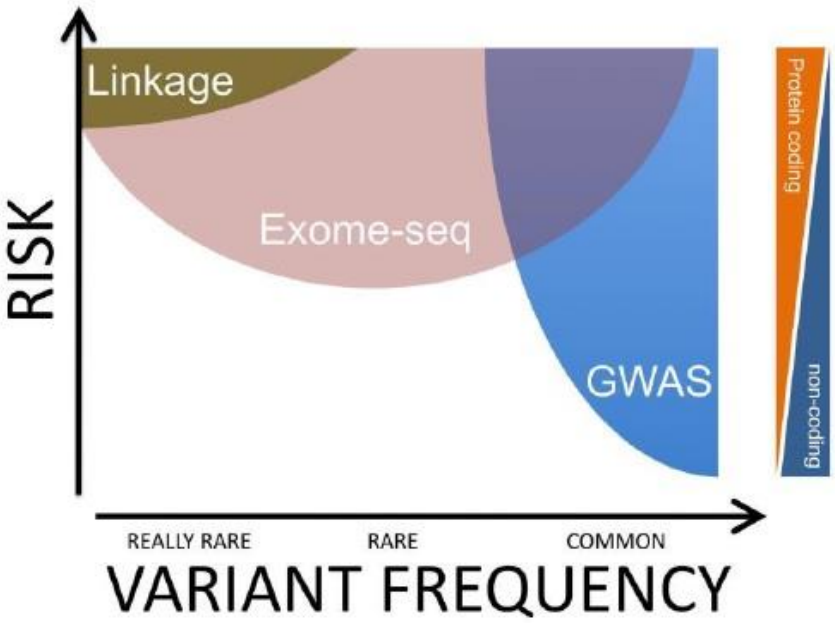


Figure 6. Methods for gene discovery based on the hypothesised model for genetic complex diseases *(Reproduced from Singleton et al., 2010).*

The invention and improvement of technological approaches have allowed for the identification of very rare causative mutations underlying Mendelian forms of disease through linkage analyses and sequencing aproaches, and of common variants with small effects contributing to sporadic, late-onset disorders through GWAS. These advances have greatly promoted the ability of scientists to find genetic variants that confer risk or cause disease. Thank to all this technology, the field of genetics is in a period of rapid discovery.

7.2.1 Linkage analysis

Linkage analysis (LA) was historically the primary tool used for the genetic mapping of Mendelian and complex traits with familial aggregation (Wijsman et al., 2012). When multiple affected individuals from the same family are available, the genomic region of interest can be narrowed down by linkage mapping analysis through locus identification, and the few genes located within the same locus can be quickly investigated. What is calculated is the likelihood that a certain locus, through genetic markers, segregate with disease.

LA is again emerging as a very useful method to elucidate the genetic basis of complex diseases, particularly for the identification of rare variants associated with a trait with high penetrance. LA has many advantages over sequencing approaches because it limits the number of genes which have to be analysed. It also takes into account possible phenocopies and reduced penetrance. However, LA are unable to adequately test variants that do not impart a very strong effect on disease and it requires a considerable number of individuals affected in a certain family.

Many new susceptibility genes have been identified using LA coupled with whole-genome sequencing (WGS) (reviewed in Ott et al., 2015). This strategy is thought to be widely used in the near future.

7.2.2 Genome-wide association studies (GWAS)

The concept of Genome-Wide Association Study (GWAS) was first formulated by Risch and Merikangas (Risch and Merikangas, 1996), but this methodological approach has been available and relatively affordable since 2005.

A GWAS evaluates large numbers of cases and controls by studying hundreds of thousands of markers throughout the genome without any prior knowledge, using high throughput gene-chip arrays (McCarthy et al., 2008) **(Figure 7)**. Allele and genotype frequencies at each of these genetic variants are then compared between the case and the control group to detect alleles or genotypes that are over-represented in one group versus the other.

A statistically significant association implies that either the associated marker is the risk variant or is close to the risk variant. The allele for which an association is found, can be directly involved in the manifestation of the disease or can be in Linkage Disequilibrium (LD) with a 'nearby' susceptibility disease allele. The aim of a GWAS is to identify genetic variants that modulate the risk rather than cause disease, testing the CDCV. As explained previously in the introduction, such variants are common in the population and predispose to disease with small effect sizes (odds ratio < 1.5).

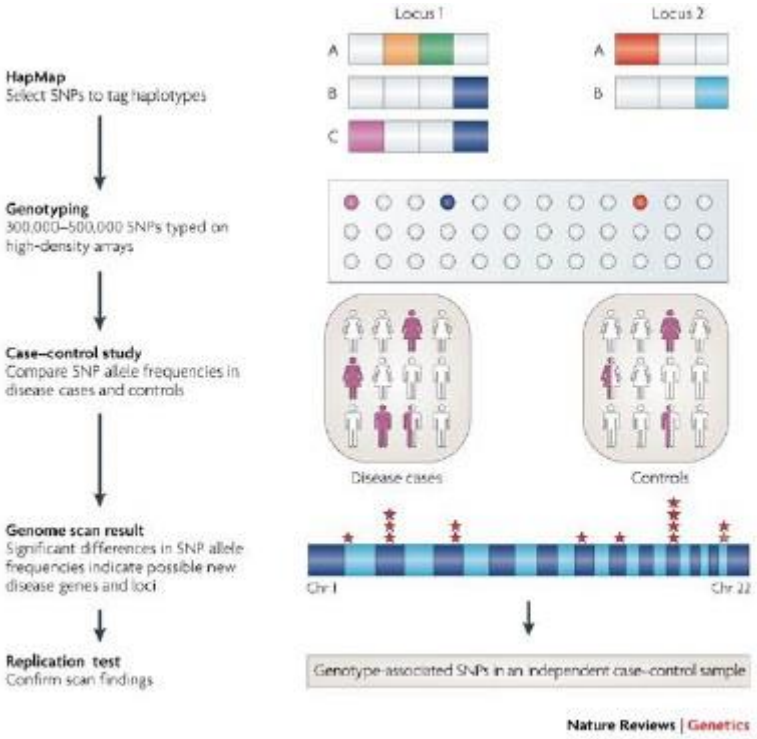


Figure 7. Experimental and analytical design of a GWAS. (*Reproduced from Mathew, 2008).* A GWAS includes the following steps; a) Selection of haplotype-tagging SNPs. b) Genotyping: Determine the genotypes of the individuals included in the study through microarrays. c) Compare allele/genotype frequencies between cases and controls. d) Graphical representation of results as a Manhattan plot. e) Replication of significant results in an independent case-control cohort.

The first step of a GWAS is selection of an appropriate case-control group. Accurate phenotyping of cases, which is related to the etiological heterogeneity of disease, is a requirement for the success of a GWAS. Solutions to this problem include deep endophenotyping as close as possible (Simon-Sanchez and Singleton, 2008).

It should be pointed out that the power to detect association for a variant with a particular effect size and minor allele frequency (MAF) depends on the number of cases and controls included in the study. In theory, the larger the sample size is, the more sensitive is the identification of variants with small effect sizes. Selection of appropriate markers for genotyping is also important. LD patterns in the human genome allow selection of a subset of informative markers that capture most of the genetic variability, thus reducing genotyping costs (Singleton et al., 2010). This means that genotyping a few hundred thousand SNPs in a subject will allow us to predict by imputation the genotype at more than a million additional variants with high confidence.

After performing the genotyping experiment, stringent quality control (QC) procedures are followed to ensure exclusion of samples and SNPs that could result into false associations. It is important to check the presence of population stratification, since allele types and frequencies for a substantial proportion of SNPs differ between ethnicities. Later, the association is conducted in a process called "discovery phase", in which is important to adjust for diferent covariates, controlling population stratification. Finally, correction for multiple testings is crucial to avoid false positive results (type I error) which could occur by chance. It has been generally accepted that the cut-off for significant association at a GWAS level should be $5x10^{-8}$.

Significant SNPs are then genotyped in a second independent cohort for validation of the association, and this is called "replication phase".

In order to assess whether the associated markers are truly disease-causing or are proxies of the causative variants, it is common to subject the associated loci to deep resequencing and/or to fine map the region (Balding, 2006).

GWAS are a major step forward from candidate gene studies. In the traditional candidate case-control studies, the gene(s) chosen to be examined are already biologically associated to the studied disease. In these 'candidate gene' studies, sample sizes are generally small and the variants assayed are limited to a few, often yielding associations that are difficult to replicate (Reviewed in Hardy et al., 2009). GWAS, on the other hand, are hypothesis-free approaches. The remarkable power of this genome wide assessments, is that it produces an unbiased analysis yielding to surprising findings that would have been overlooked by hypothesis-based approaches.

However, GWAS has also limitations. First, it is only able to study relatively common types of variants, those that occur at a frequency of more than 5% in the general population. It is not designed for and is inefficient at finding rare genetic variants which contribute to disease. Second, although GWAS are a powerful approach to identify new pathways for PD, such assessments identify loci not genes. A positive signal from a GWAS is not always within the known functional unit of a gene. The major problem associated with the discovery of risk-associated variants is the interpretation of the risk in the context of disease pathogenesis. These variants are most of the times noncoding, so it is not clear what the target gene or functional consequence is.

It is expected that in the field of PD, mega meta-analysis of GWAS will continue to detect new associations over the next few years.

Despite the considerable success reached up to date in PD genetics, GWAS have identified only one-tenth of the heritable component, suggesting that there is much left to find (Singleton and Hardy, 2016). There will likely be more that can be extracted from the already created data, seeking deeper into the sub-significant hits and through alternative approaches, such as pathways-based analysis. The post-genomic era in genetics of PD now accepts that real advances can only be made by Consortiums applying global standards of scientific methods and collaborative spirit.

7.2.3 Next generation sequencing strategies

DNA sequencing in the laboratory has been possible since the 1970s, when the Sanger method (first generation sequencing) was first developed. However, the technique remains too laborious and expensive for the routine sequencing of whole genomes. Over the past 10 years, a number of new NGS technologies, also called second and third generation technologies, have been developed and have significantly reduced the cost and time required for sequencing. Depending on the regions of interest to be sequenced, we can distinguish whole-exome sequencing and whole-genome sequencing.

Whole exome *versus* Whole-genome sequencing

Whole exome-sequencing (WES) is an application of NGS to determine the variation of all coding regions of known genes. It is estimated that around 85% of the disease-causing mutations are located in coding and functional regions of the genome (reviewed in Rabbani et al., 2014). Sequencing the whole exome has the potential of uncovering the causes of rare, monogenic disorders where the number of available individuals for study is small.

More recently, it is also being used to explore every type of genetic variants (rare, intermediate and common) in large-scale case-control studies (Price et al., 2010). One of the main advantages of WES is that it promises to condense the time required for the identification of novel mutations from years to weeks. It also reduces the costs to sequence, storage and analyse a targeted region at a high depth, which makes it possible to increase the number of samples to be sequenced, enabling large population based comparisons.

However, there are also certain limitations that should be considered. WES focuses on only around 1% of the genome and is limited to the coding and splice-site variants in annotated genes. Moreover, the current technology does not allow detect every base pair of the protein-coding regions of each gene. For instance, GC-rich target regions and homopolymers (large repetitions of the same nucleotide) are difficult to sequence. In addition, large deletions, duplications and rearrangements, may easily be missed.

It is also the case that exon capture can potentiate bias along the procedure giving false positives and negatives, because capture probes tend to preferentially enrich reference alleles. One way to reduce the amount of false negative and false positive results is to increase the depth of coverage of the sequencing reaction, which leads to higher sequencing costs.

On the other hand, WGS is the most comprehensive method to explore the entire genome, and it is expected to become the method of reference in the following years. It allows you to look for variants in both the ~1% part of the genome that encodes protein sequences and the ~99% of remaining non-coding sequences. It allows examination of single-nucleotide variants (SNVs), large insertions, duplications and deletions in coding and non-coding regions of the genome, as well as regulatory regions such as promoters and enhancers. Moreover, WGS has more reliable sequence coverage and PCR amplification is not required during library preparation, reducing the potential of GC bias. Sequencing read length is not a limitation for WGS, thus large deletions, duplications and rearrangements can be detected.

WGS has great expectation, however it has an important limitation. The cost is considerable not only for the sequencing procedure but also for the data storage, and interpreting that enormous amount of data is challenging. Therefore, WES constitutes a "temporary alternative" to study the genetics of complex diseases, and it is clear that in the future we will perform extremely large-scale WGS analyses in PD.

7.3 Genetic architecture of Parkinson's disease

Keeping in mind the model proposed by Manolio et al., (2009) it can be clearly extrapolated to explain the genetic architecture of PD **(Figure 8).** As it will be comprehensively described in the following section, PD can be caused by rare, very high risk and fully penetrant variants (i.e pathogenic mutations in *SNCA*), moderate risk variants with intermediate allele frequency in the general population (i.e in the *GBA* gene), and very common variants of low effect size (i.e in the *STK39* loci). Notably, some of these genes carry multiple types of variants (pleomorphic risk loci such as the *SNCA* and *LRRK2* genes) (Singleton and Hardy, 2011).

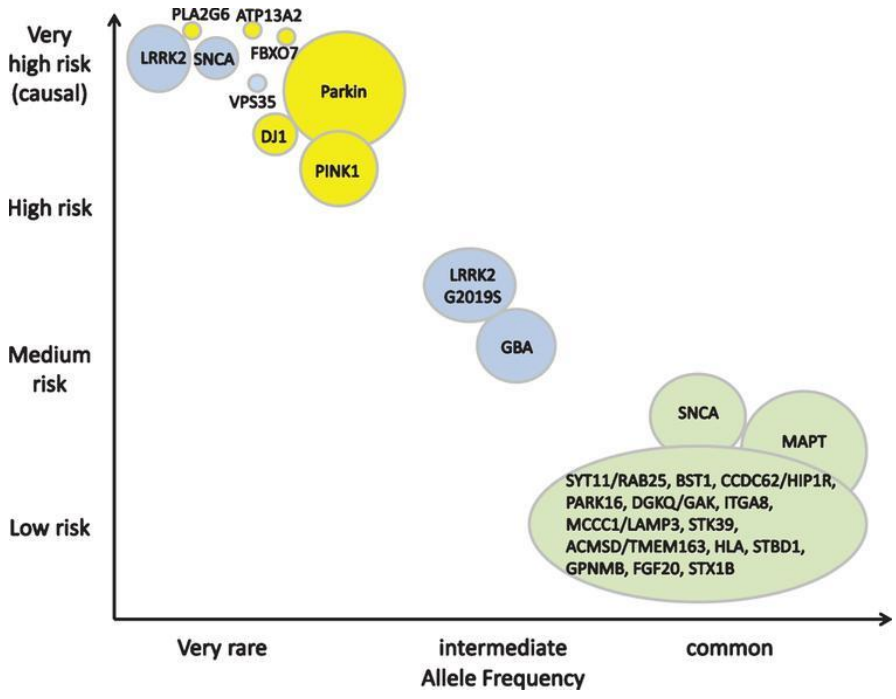


Figure 8. Genetic architecture of Parkinson's disease (*Reproduced from Gasser, 2015*). The size of the bubbles roughly corresponds to population allele frequencies. Colors symbolize modes of inheritance: dominant (blue), recessive (yellow), risk loci (green).

As mentioned before, important success in understanding the genetics of PD arose from monogenic forms of disease with classic "Mendelian", observable patterns of inheritance. Mendelian PD is caused by highly, fully penetrant mutations affecting multiple members per family and being inherited generation to generation. Only between 5-10 % of PD patients develop this rare form of the disease.

The pattern of inheritance can be autosomal dominant or recessive. Dominant alleles can act in many ways. They can generate gain of function, implying that the toxicity is caused by an amplification of the normal function of the protein or through the gaining of a new toxic function. They can generate loss of function too, through the loss of activity of the protein. In this case, some of the pathogenic mutations would be expected to be nonsense mutations. Or they can cause loss of function through a dominant negative mechanism, whereby the mutant allele somehow interferes with the function of the wild-type allele, leading to partial or complete loss of function.

On the other hand, nearly all recessive alleles are loss of function alleles. Many recessive alleles are clearly pathogenic, because they would be predicted to lead to the production of no protein or inactive protein (reviewed in Hardy et al., 2009).

7.3.1 Autosomal dominant Parkinson's disease genes

SNCA (PARK1/PARK4)

The year 1997 marks the starting point for PD genetics with the discovery of mutations in *SNCA,* encoding α-syn. The first mutation underlying PD (p.A53T), was identified in a large Italian family and then in three families from Southern Greece with autosomal dominant pattern of inheritance (Polymeropoulos et al., 1997). This landmark discovery revealed the first indisputable, heritable component of PD. Later on, some additional families from Greece were also found to have mutations in *SNCA*, suggesting the presence of a founder mutation (Spira et al., 2001, Athanassiadou et al., 1999,). A decade later, two Korean and one Swedish family were shown to have the same mutation (Ki et al., 2007, Puschmann et al., 2009, Choi et al., 2008). This mutation is considered as the most frequent in the *SNCA* gene throughout the world.

Just after the discovery of *SNCA* mutations causing a rare familial form of PD, α-syn was identified as the major component of LBs and the main pathological hallmark of PD (Spillantini et al., 1997). This finding distinctly tied together the pathogenesis of genetic forms of PD with sporadic cases. Subsequently, two new *SNCA* mutations, p.A30P in a German family (Kruger et at., 1998) and p.E46K in a Spanish family from the Basque country (Zarranz et al., 2004) were identified **(Figure 9)**.

Interestingly, whole gene duplications (Chartier-Harlin et al., 2004, Ibanez et al., 2004, Ibanez et al., 2009, Nishioka et al., 2006, Fuchs et al., 2007, Ikeychi et al., 2008, Troiano et al., 2008, Uchiyama et al., 2008) and triplications (Singleton et al., 2003, Farrer et al., 2004, Ibanez et al., 2009) have also been found to cause a very deleterous form of PD.

More recently, three novel pathogenic mutations have been described; the p.H50Q mutation was reported in two sporadic PD cases sharing a common haplotype on the *SNCA* locus (Appel-Cresswell et al., 2013, Proukakis et al., 2013), the p.G51D mutation in two familial cases with the most severe clinical presentation known to date (Kiely et al., 2013, Lesage et al., 2013), and the p.A53E mutation in a patient with atypical PD (Pasanen et al., 2014).

Figure 9. *SNCA* gene with the p.A30P, p.E46K and p.A53T mutations and phosphorilation sites. The three novel mutations p.H50Q, p.G51D and p.A53E are not highlighted *(Reproduced from Hardy et al., 2009)*

Clinical phenotype

The age at onset of *SNCA* mutation carriers is early but variable. Age at onset for families with the p.A53T mutation is 46 years with full penetrance (Papapetropoulos et al., 2001). In contrast, families with the p.A30P mutation have an age at onset close to 50 years and the disease is not fully penetrant (Kruger et al., 1998), while the p. E46K mutation causes PD at the ages between 50-65 years with early dementia (Zarranz et al., 2004). Regarding the two recently described *SNCA* mutations, p.G51D presents a clinical phenotype similar to p.A53T, and p.H50Q has been detected in an early onset PD patient with a positive history for parkinsonism and dementia (Kiely et al., 2013, Lesage et al., 2013) and in one patient with late-onset sporadic PD (Proukakis C et al., 2013, Appel-Cresswell S et al., 2013).

SNCA duplication/triplication carriers display a broad clinical spectrum. Severity and age at onset seem to correlate with the number of copies of *SNCA*, indicating a gene-dosage effect. Patients with four copies of the gene (triplication), develop the disease about a decade earlier than those with three copies, and the disease progression is generally more aggressive (Ferese et al., 2015).

SNCA-linked PD patients usually present with progressive L-Dopa responsive parkinsonism, rapid disease progression, cognitive decline, dementia, automonic dysfunction and hallucinations (Houlden and Singleton, 2012).

Protein function

Despite the clear role of α-syn in the pathogenesis of PD, its functions remain poorly understood. *SNCA* encodes a small 140 Aa protein that exists in a range of quaternary states, from monomeric to low molecular weight oligomers and finally to the weight amyloid fibrils found in LBs. It has been reported that α-syn interacts with members of both Rab and SNARE families, poiting to a role in vesicular trafficking (Thayanidhi et al., 2010). It has been suggested that this protein also plays an important role in membrane interactions and synaptic activity through regulation of synaptic vesicle release (Bendor et al., 2013). α-Syn is mainly found in the citosol where is bound to lipids in an interaction that is required for the synapse (Fortin et al., 2004). Many observations suggest that α-syn is responsible for neurodegeneration by interfering with multiple signalling pathways altering membrane permeability to ions. It can also associate with mitochondria and cause mitochondrial dysfunction and interfere with autophagy regulation as it will be discussed later on this thesis.

LRRK2 (PARK8)

In 2004, the discovery of mutations in *LRRK2*, encoding the leucine-rich repeat kinase or also called dardarin, was a real breakthrough in the field of PD, since for the first time a genetic defect was not only very frequent but also found in both familial and sporadic forms (Paisán-Ruiz et al., 2004, Zimprich et al., 2004). Linkage of PD to a region in chromosome 12 was originally mapped in a family from Japan showing incomplete penetrance (Funayama et al., 2002).

Within two years, positional cloning allowed to identify the most common mutations responsible for familial PD. Today, more than 100 distinct mutations have been reported (Rubio et al., 2012). However, only a small proportion of them are considered to be damaging with varying degrees of proof pathogenicity (p.R1441C, p.R1441G, p.R1441H, p.G2019S, p.Y1699C, p.I2020T, p.I2012T, p.N1437H and p.S1761R) (reviewed in Hernandez et al., 2016). These pathogenic mutations are present in exons encoding the Ras of complex proteins ROC, C-terminus of ROC, or kinase domains of the protein **(Figure 10).**

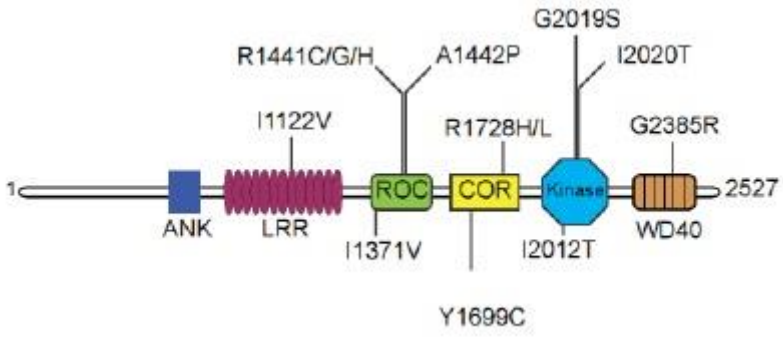


Figure 10. *LRRK2* gene with the main mutations and domains. *(Reproduced from Hardy et al., 2009)*

Based on available data, the prevalence of *LRRK2* mutations varies markedly across populations (Gorostidi et al., 2009, Papapetropoulos et al., 2008, Schlitter et al., 2006, Kalinderi et al., 2007, De Rosa et al., 2009, Toft et al., 2007). The most studied mutation, p.G2019S, is responsible for 40% of familial and sporadic PD in Arab samples from North Africa, 30% of familial PD in Ashkenazi Jewish populations, and up to 6% of familial cases in Europe and 3% of apparently sporadic PD in Europe and North America. However, it has been described as a very rare genetic cause in Asian populations.

It is believed that most *LRRK2* p.G2019S mutation carriers come from a few founder events passed down from many centuries (Lesage et al., 2010). The penetrance of this mutation is age-dependent and varies from 28% at 59 years of age, to 51% at 69 years and 74% at 79 years of age (Healy et al., 2008). The remaining seven pathogenic mutations are less frequent worldwide, although p.R1441G is common in the Basque country with a prevalence of 15% in PD patients from this region (Mata et al., 2005).

Clinical phenotype

Overall, *LRRK2* mutation carriers are indistinguishable from sporadic PD showing mid to late onset disease with slow progression, ocasional dystonia and a good response to levodopa therapy. Dementia in these individuals is rare (Haugarvoll and Wszolek, 2009).

Protein function

Leucine-rich repeat kinase is a large, multidomain protein of 2527 Aa of the ROCO protein family (Greggio et al., 2009). It is widely expressed in the brain and localized in LBs where it is associated with the endoplasmic reticulum of dopaminergic neurons (Vitte et al., 2010, Alegre-Abarrategui et al., 2008). The protein has an enzymatic core where the proven autosomal dominant pathogenic mutations are present. It is formed by a GTPase ROC, whose C terminus as well as the Ser/Thr kinase domain, act as the enzymatic core. The flanking ankryn, leucine rich repeat and WD40 domains allow for interaction with numerous other proteins related to PD including the GAK, and Rab7l1 proteins (Beilina et al., 2014).

Among the most common mutations, p.G2019S has been shown to consistently increase kinase activity (Greggio, 2012). LRRK2 has been implicated in several cellular processes including cytoskeletal dynamics, autophagy, kinase cascades, mitochondrial function, and vesicular trafficking (reviewed in Hernandez et al., 2016). It is widely suggested that this protein is involved in multiple cellular processes and may be a central component of signalling pathways crucial for the proper function of neurons. The many diverse activities of LRRK2 make difficult a clear view of its exact functions, but further ongoing investigations to elucidate its exact role.

VPS35 (PARK17)

In 2011, Zimprich et al., were the first to use NGS methods to detect a PD causing gene. They identified the p.D620N mutation in *VPS35*, encoding the vacuolar protein sorting 35, in an Austrian family with late-onset PD (Zimprich et al., 2011). Simultaneously, another group identified the same mutation also as a cause of late-onset PD in a large family from Switzerland (Vilarino-Guell et al., 2011).

Sequencing of the entire coding portion of *VPS35* revealed other mutations in these studies (p.G51S, p.M57I, p.T82R, p.I241M, p.P316S, p.R524W, and p.L774M), however, these mutations have not been proven to be pathogenically relevant. Since then, several studies have screened for the p.D620N mutation, finding it to be more frequent in Yemenite Jews (1.67 %), French (1.2 %) and Tunisian people (0.5 %).

Interestingly, it has not been identified in many other ethnicities. Overall, *VPS35* mutations constitute a rare cause of PD accounting for about 1% of familial parkinsonism and 0.2% of sporadic PD (Lesage et al., 2012, Zimprich et al., 201, Nuytemans et al., 2013).

Clinical phenotype

VPS35-linked PD reminds typical idiopathic disease with a mean age of onset at 50 years, bradykinesia, rigidity, resting tremor, dyskinesia, dystonia, and good response to levodopa therapy (Deng et al., 2013).

Protein function

Vacuolar protein sorting 35 is a highly conserved 796 Aa protein. Experiments performed in yeast demonstrated that the protein is a member of the retromer complex, which also contains Vps26p and Vps29p, and is responsible for the retrograde transport of proteins in endosomes to the trans-Golgi network (Seaman et al., 1998). The human homologs of the retromer complex were later cloned and shown to function in the same endosome–trans-Golgi network pathway (Haft et al., 2000). VPS35 is located at the center of the complex and is required for the binding of the cytosolic domains of the retromer complex for retrograde transport (Nothwehr et al., 2000). Recent research links the p.D620N mutation to dysfunction of the retromer complex by redistributing retromer bound-endosomes to the perinuclear region, in cell lines and PD patient-derived fibroblasts (reviewed in Hernandez et al., 2016).

The study also shows that the mutant VPS35 protein alters the trafficking of a protein implicated in the degradation of α-syn named Cathepsin D (Follett et al., 2014, Cullen et al., 2009). Although mutations in *VPS35* are not frequent, its high level of conservation has made it one of the best understood genes associated with PD. *VPS35* is related to *SNCA* and *LRRK2*, through endosomes and vesicular trafficking and claims the importance of studying these pathways in PD.

TMEM230

Mutations in *TMEM230*, also known as *C20orf30,* have been recently linked to familial and early onset PD in a set of patients from China (Deng et al., 2016). The authors found several mutations cosegregating with PD; the p.R141L in thirteen patients with familial PD, the p.*184Wext*5G codon-stop mutation in a patient with familial PD, and the p.Y92C mutation in an early onset sporadic PD. They also identified another p.*184PGext*5 codon-stop mutation, in nine familial PD cases from seven unrelated families. Notably, the last five amino acids (HPPHS) replaced by the TAG stop codon, are identical in the *184Wext*5 and *184PGext*5 mutants. However, incomplete penetrance of the *184PGext*5 mutation has been seen in the heterozygous state.

Clinical phenotype

The phenotype of *TMEM230* mutation carriers is heterogeneous. Age at onset has been found to be variable. It ranges from 48 to 85 years, and symptoms were those of typical PD, including bradykinesia, resting tremor, rigidity and postural instability.

Most of the patients had good response to L-dopa, and a case developed dementia (Deng et al., 2016).

Protein function

Due to the recent discovery, the precise functions of the Transmembrane Protein 230 and pathogenic mechanisms in PD mediated by mutations in this gene, remain to be elucidated. It has been proposed that TMEM230 is a trafficking protein of secretory/recycling vesicles, primarily involved in exocytosis, endocytosis and recycling of synaptic vesicles in neurons. Dysfunction of synaptic vesicles, such as impaired trafficking and recycling, as mentioned before, underlies the pathogenesis of PD (Deng et al., 2016).

7.3.2 Autosomal recessive Parkinson's disease genes

PARKIN (PARK2)

One year after the discovery of *SNCA* mutations, a homozygous deletion of exons 3–7 in *PARKIN*, was identified in a Japanese family with autosomal recessive juvenile parkinsonism (ARJP) (Kitada et al., 1998). This study also identified four families showing a homozygous deletion of exon 4. Mutations in *PARKIN* have been found in patients of different ethnicity and have been considered as the major mutant factor for ARJP.

Several mutations in all 12 exons of *PARKIN* have been identified **(Figure 11),** consisting of point mutations and exon rearrangements, including deletions and duplications (Foroud et al., 2003, West et al., 2002, Hedrich et al., 2001 and 2004, Lesage et al., 2007, Nuytemans et al., 2010).

To date, about 150 different exonic mutations have been described of which 30% are SNV, 10 % are minor deletions, and 50% are larger deletions or duplications (Grunewald et al., 2010). Mutations are present in approximately 50% of patients with autosomal recessive PD in the age range of 7–58 years of age and present in up to 77% of sporadic cases with age at onset younger than 20 years (Lucking et al., 2000).

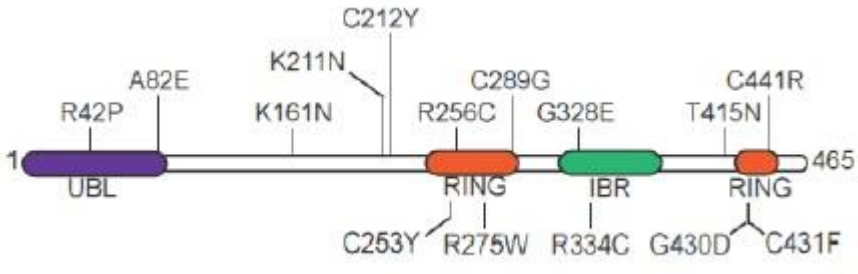


Figure 11. *Parkin* gene with the main mutations and domains. *(Reproduced from Hardy et al., 2009).*

Clinical phenotype

PARKIN-linked disease has been reported to include similar symptoms to those with typical early-onset PD without *PARKIN* mutations (Periquet et al., 2003). It is generally characterised by a very early age at onset, foot dystonia, psychiatric symptoms, and extremely good response to levodopa (Khan et al., 2003). Interestingly, these patients present with very slow and benigh course and sleep benefit.

Protein function

PARKIN is the second largest gene in the genome being mainly expressed in the nervous system. It encodes the E3 ubiquitin ligase, a large protein with a crucial role in the degradation system of proteasome (Shimura et al., 2000). The loss of function effect of mutations in *PARKIN* results in inactivation of its E3 ligase function, failure of ubiquitination of the targeted proteins and therefore a toxic build-up of proteins that are no longer effectively degraded by the parkin-dependent ubiquitin/proteosome pathway (reviewed in Kalindieri et al., 2016). The formation of these toxic aggregates in neurons of the SNpc has an important effect on the pathogenesis of PD. Also, *PARKIN* mutations are of paramount importance because they have been implicated in mitochondrial quality control in the pathogenesis of PD, as will be discussed later on this thesis.

PINK1 (PARK6)

The PARK6 locus was first associated with PD in a consanguineous family from Sicily (Valente et al., 2001). Subsequently, Valente et al., identified two homozygous mutations, (the p.G309D missense mutation and the p.W437X truncating mutation) in the PTEN-induced putative kinase 1 (*PINK1*) gene in a Spanish family and two Italian families, respectively **(Figure 12).** The Italian families shared a common haplotype, showing a shared ancestry (Valente et al., 2004). Later on, a number of additional missense, nonsense, frameshift mutations and large deletions of multiple exons have been reported in *PINK1* in families of different ethnicity (Bonifati et al., 2005, Ibanez et al., 2006). *PINK1* mutations are detected in 2–4% of early onset PD (EOPD) in Caucasian populations and 4–9% in Asian populations (Schulte et al., 2011).

An exception occurs in the Philippines, where one founder mutation has a high carrier frequency (Bonifati et al., 2005). To date, more than 10 different mutations have been associated with EOPD. The combination of compound heterozygous or homozygous loss of function mutations in *PINK1* is considered as the second most common cause of autosomal recessive EOPD, present in about 3.7 % of patients (Healy et al., 2004, Klein et al., 2005, Valente et al., 2004, Li et al., 2005, Bonifati et al., 2005, Marongiu et al., 2007).

Clinical phenotype

It reminds classic idiopathic PD with levodopa response and no reports of dementia (Ibanez et al., 2006). It presents with slow progression and often atypical features such as dystonia, sleep benefit, pyramidal signs and psychiatric co-morbidities such as anxiety and depression (Bonifati et al., 2005).

Protein function

PINK1 encodes a 581 Aa Ser/Thr kinase located in the mitochondria. It phosphorylates Parkin to regulate mitophagy of damaged mitochondria (see section "Pathogenesis"). Additionally, over-expression of wild-type *PINK1*, has been seen to rescue stress-induced apoptotic death via the mitophagy pathway (Valente et al., 2004). The role of *PINK1* and *PARK2* in the mitophagy pathway confirms that it is essential for neuronal health and survival, being a good therapeutic alternative in a group of EOPD patients.

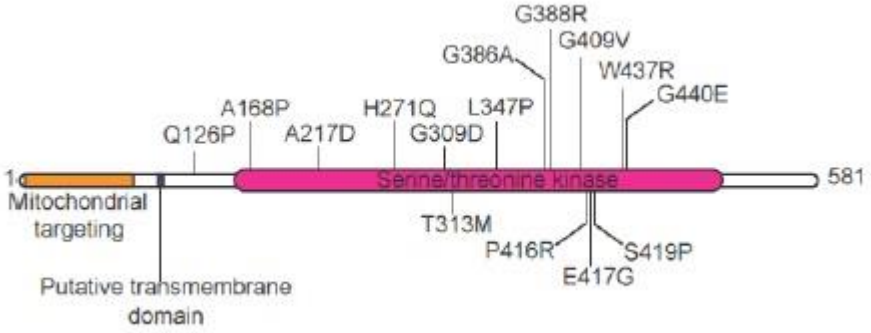


Figure 12. *PINK1* gene with the main mutations and domains. *(Reproduced from from Hardy et al., 2009)*

DJ-1 (PARK7)

In 2003, homozygosity mapping and positional cloning was performed on consanguineous pedigrees from a genetically isolated population in Netherlands revealing that a homozygous deletion of several exons in *DJ-1* caused disease. Bonifati et al., identified recessively inherited missense and exonic deletions in this gene in two families making it the third gene associated with autosomal recessive PD **(Figure 13)** (Bonifati et al., 2003). Subsequently, a missense mutation in a highly conserved residue (p.L166P) was shown to cause disease in an Italian family with recessive pattern of inheritance. Mutations in *DJ-1* are extremely infrequent, identified in 1–2% of EOPD cases. The mutations are found in both compound heterozygous and homozygous states.

Clinical phenotype

It presents a wide clinical spectrum including motor neuron disease phenotype with levodopa response (Annesi et al., 2005). *DJ-1* mutations cause disease in the twenties-thirties, similar to *PARK2* and *PINK1* forms (Canet Aviles et al., 2004, Mitsumoto and Nakagawa, 2001, Abou-Sleiman et al., 2003, Bonifati et al., 2003, Pankratz et al., 2006, Zhou et al., 2006).

Protein function

The *DJ-1* gene encodes a protein of 189 Aa that was first identified in cancer (Nagakubo et al., 1997). DJ-1 is part of the peptidase C56 family and has been seen to protect cells against OS preserving normal dopaminergic function in the nigrostriatal pathway (Canet-Aviles et al., 2004).

It has been reported to have a role as chaperone having the ability to inhibit α-syn aggregation (Shendelman et al., 2004). More recent work has linked DJ-1 to the Parkin/PINK1 pathway again through transcriptional regulation of PINK1 (Fitzgerald et al., 2008, Requejo Aguilar et al., 2015). In fact DJ-1 was indicated as a transcriptional positive regulator of PINK1 by binding to its promoter. Increased glycolysis upon DJ-1 loss of function was suggested to contribute to the OS and dopaminergic neurodegeneration in PD (Requejo-Aguilar et al., 2015).

This study adds a new perspective in the PD pathogenesis; however whether DJ-1 loss of function has a deleterious effect on dopaminergic neurons by altering glucose metabolism and the exact role of DJ-1 in modulating the function of the PINK1–parkin pathway still remains elusive.

It has also been suggested that DJ-1 may be implicated in transcriptional regulation of antiapoptotic or neuroprotective genes (Xu et al., 2005).

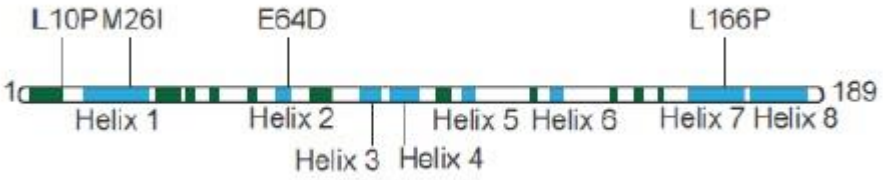


Figure 13. *DJ-1* gene with the main mutations and domains. (*Reproduced from Hardy et al., 2009)*

Moreover, other genes have been associated with atypical forms of PD, such as PARK9 (*ATP13A2*) (Ramirez et al., 2006), PARK14 (*PLA2G6*) (Paisan-Ruiz et al., 2009) and PARK15 (*FBX07*) (Shojaee et al., 2008).

ATP13A2 (PARK9)

Pathogenic mutations in *ATP13A2* **(Figure 14)** cause a rare, juvenile-onset disorder named Kufor–Rakeb syndrome, which is characterized by atypical features of PD such as dystonia and supranuclear palsy with a low response to levodopa (Ramirez et al., 2006, Bruggemann et al., 2010). It was first described in a Jordanian family and later in several families with numerous different mutations.

Clinical phenotype

The phenotype of the syndrome reminds to the lysosomal storage diseases (Williams et al., 2005, Najim et al., 1994). Severity is quite variable between patients and seems to be related to the mutation inherited (Park et al., 2015).

Protein function

ATP13A2 encodes the ATPase type 13A2, a 1175 Aa member of the P5 family of ATPases. ATP13A2 is a multifunctional protein with 10 transmembrane domains, which is hypothesized to play a role in mitochondrial health (Grunewald et al., 2012) and endosome–lysosome dynamics (Usenovic et al., 2012), as well as in protecting cells from metals (Mn2+ and Zn2+) induced toxicity (Kong et al., 2014). These studies also indicate that ATP13A2 is present in LBs of PD patients (Dehay et al., 2012) and over-expression of *ATP13A2* rescues neurons from α-syn accumulation (Kong et al., 2014).

Mutations in *ATP13A2* are a very rare cause of parkinsonism, but represent an important link between autophagy–lysosomal and mitochondrial pathways.

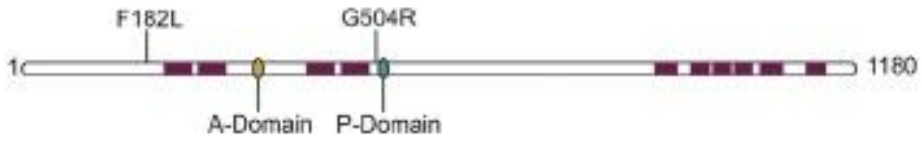


Figure 14. *ATP13A2* gene with the main mutations and domains. *(Reproduced from Hardy et al., 2009)*

PLA2G6 (PARK14)

Pathogenic mutations in the gene *PLA2G6* cause autosomal recessive, levodopa-responsive parkinsonism with dystonia, neurodegeneration with brain iron accumulation and Karak syndrome **(Figure 15)** (Malik et al., 2008, Mubaidin et al., 2003). Brain iron accumulation is found in the vast majority but not all postmortem brain tissues of affected individuals (Paisan-Ruiz et al., 2009).

PD associated with *PLA2G6* is very rare and it is caused by the compound heterozygous or homozygous inheritance of different missense mutations (Paisan-Ruiz et al., 2009, Yoshino et al., 2010, Sina et al., 2009).

Protein function

PLA2G6 encodes an 806 Aa protein called phospholipase a2 group VI with a lipase domain, seven ankryn repeats and a calmodulin-binding domain. This enzyme hydrolyzes glycerophospholipids producing free fatty acids and 2-lysophospholipids (Balsinde and Balboa, 2005).

Research studies performed in fibroblasts from a patient with the PD-associated mutation p.R747W have implicated this protein in mitochondrial dysfunction and neurodegeneration (Kinghorn et al., 2015).

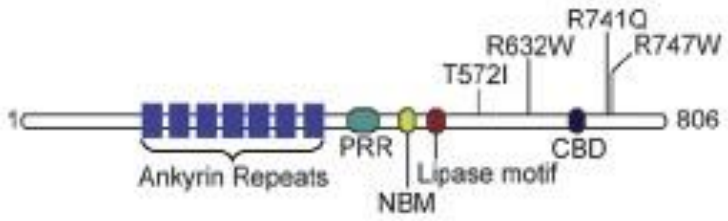


Figure 15. *PLA2G6* gene with the main mutations and domains. *(Reproduced from Hardy et al., 2009)*

FBXO7 (PARK15)

Mutations in *FBXO7* are a rare cause of ARJP and were first idenfied through linkage mapping followed by gene sequencing in a Persian family **(Figure 16)** (Shojaee et al., 2008).

Later on, two families from Italy with similar symptoms were shown to have three different mutations in *FBXO7* in either the compound heterozygous or homozygous state (Di Fonzo et al., 2009).

Clinical phenotype

The affected individuals first showed early-onset spastic paraplegia and later displayed dopa-responsive parkinsonism, with a complex phenotype of a parkinsonian pallidal syndrome (Laman et al., 2006).

Protein function

FBXO7 encodes F-box only protein 7 and is made up of 443 Aa. It is a part of an E3 ubiquitin ligase (Laman et al., 2006) and it has been shown to interact with Parkin and PINK1 in mitophagy and mitochondrial maintenance (Burchell et al., 2013, Zhou et al., 2015).

These studies show that FBXO7 contributes to translocation of Parkin to the mitochondria in response to cell stress and a PD-associated mutation (p.T22M) leads to mislocalization of FBXO7 to the cytosol (reviewed in Hernandez et al., 2016).

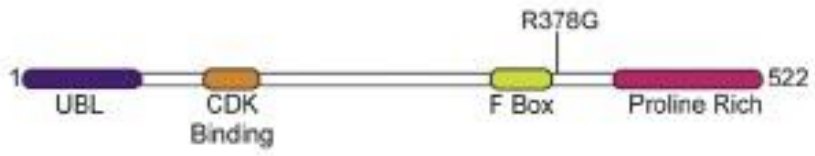


Figure 16. *FBX07* gene with the main mutations and domains. (*Reproduced from Hardy et al., 2009)*

VPS13C

It has been recently reported that homozygous or compound heterozygous truncating mutations in the *VPS13C* gene cause autosomal recessive PD (Lesage et al., 2016). A total of five truncating mutations have been identified in three unrelated PD cases by a combination of homozygosity mapping and exome-sequencing.

Moreover, the largest and more recent meta-analisis from GWAS detected *VPS13C* as a susceptibility locus risk for PD (Nalls et al., 2014), although such association was not related with either methylation or expression levels.

Clinical phenotype

VPS13C mutations are associated with a distinct form of early-onset parkinsonism characterized by rapid and severe disease progression, early cognitive decline and good levo-dopa response. The pathological features are in concordance with diffuse LB disease.

Protein function

VPS13C belongs to a family of large proteins crucial for vesicular transport (Bankaitis et al., 1986). It has been shown that VPS13C has a direct impact in mitocondria morphology, transmembrane potential and respiration (Lesage et al., 2016). Silencing of *VPS13C* was associated with lower mitochondrial membrane potential, mitochondrial fragmentation, increased respiration rates, exacerbated PINK1/Parkin-dependent mitophagy, and transcriptional upregulation of PARK2 in response to mitochondrial damage.

Further studies are necessary to dissect the mechanisms by which loss of VPS13C function affects the survival of dopaminergic neurons.

ADORA1

It has been recently proposed that pathogenic mutations in *ADORA1*, encoding the adenosine A1 receptor A_1R, might cause an early-onset form of parkinsonism with a recessive pattern of inheritance (Jaberi et al., 2016). The homozygous missense mutation p.G279S was found to segregate with disease in two siblings of a consanguineous Iranian family, suggesting causality.

However, a subsequent comprehensive analysis using whole exome sequencing data of 1354 PD patients and 111 Dementia of Lewy Bodies cases of European ancestry, has not identified any possible common risk factor or causative mutation among the screened individuals (Blauwendraat et al., 2016).

Clinical phenotype

ADORA1 mutation carriers are affected with a very early onset form of parkinsonism with cognitive dysfunction. Both siblings refer parkinsonism symptoms, including bradykinesia, resting tremor, rigidity and postural instability with a good response to L-dopa. Mental and psychomotor retardation, as well as psychiatric manifestations are features present in these individuals.

Protein function

Adenosine receptors are known to be involved on neural activity and influence release of neurotransmitters including dopamine.

A_1R has been shown to have neuroregulatory roles in a wide spectrum of brain functions including neuroplasticity, motor function, cognition and emotion–related behaviours (reviewed in Jaberi et al., 2016). In fact, p.G279S is positioned within a highly conserved transmembrane domain, important for receptor activation.

There is also evidence for an interaction between the adenosine and dopamine receptors (Fuxe et al., 2010, Franco et al., 2011), and it should be pointed out that drugs targeting A_1R have been considered therapeutic tools for treatment of PD.

Apart from the above mentioned genes a number of additional genes and loci have been associated with Mendelian PD such as PARK3 (Gasser et al., 1998), PARK5 (*UCHL1*) (Leroy et al., 1998), PARK10 (Hicks et al., 2002), PARK11 (Pankratz et al., 2003), PARK12 (Pankratz et al., 2003), PARK13 *(OMI/HTRA2*) (Strauss et al., 2005), PARK16 (Satake et al.,2009), PARK18 *(EIF4G1*) (Chartier-Harlin et al., 2011), PARK19 *(DNAJC6*) (Edvarson et al., 2012), PARK20 *(SYNJ1)* (Krebs et al., 2013, Quadri et al., 2013), PARK21 *(DNAJC13)* (Vilariño-Guëll et al., 2014), however either the responsible gene has not been identified or the results are currently poorly replicated and their link to PD remains uncertain. It should be noted that studies on these atypical PD genes are very useful as they can pave the way for the identification of new pathways implicated in PD pathogenesis.

DNAJC6 (PARK19)

Mutations in this gene have been found in a family with juvenile parkinsonism and in a family with complex parkinsonism. This gene is thought to be involved in clathrine-mediated endocytosis (Edvardson, et al., 2012, Koroglu, et al., 2013). Follow up studies on large PD cohorts are necessary to understand the role of this gene in the pathogenesis of PD.

SYNJ1 (PARK20)

Mutations in this gene have been reported in three families with complex parkinsonism (Krebs et al., 2013, Olgiati et al., 2014, Picillo et al., 2014, Quadri et al., 2013), but no mutations have been found in typical PD (Winkler et al., 2014). SYNJ1 is important for synaptic endocytic activity (Krebs et al., 2013).

DNAJC13 (PARK21)

Mutations in this gene were first discovered in a multigenerational family with autosomal dominant PD. However, the very recent finding of the *TMEM230* gene as the unequivocal cause of PD in this family, questions the role of *DNAJC13* in the pathogenesis of PD (Deng et al., 2016). Follow-up analysis of this gene in large PD cohorts identified 5 additional mutations, although it is still debatable if it could be in a certain way related to PD etiology. DNAJC13 is involved in the function of the endosomes and clathrin-mediated endocytosis (Vilarino-Guell et al., 2014).

OMI/HTRA2

Mutations in this locus were first reported to be associated with PD in 2005 with the identification of two different novel mutations in a large case-control sequencing analysis. However, these studies were not replicated in a subsequent study (Simon-Sanchez and Singleton, 2008). *OMI/HTRA2* mutation carriers present with LB pathology and the pathogenic mutations reported are associated to mitochondrial dysfunction (Plun-Favreau et al., 2012). Moreover, the knock-out mouse model demonstrates parkinsonian features (Martins et al., 2004).

GIGYF2

GIGYF2 mutations were first found to be associated to PD in 2008 with the identification of 10 mutations present in PD patients but not in controls. However, this finding was not replicated by several follow up studies (Bonetti et al., 2009, Bras et al., 2009, Cao et al., 2010, Di Fonzo et al., 2009, Li et al., 2010, Meeus et al., 2011, Nichols et al., 2009, Vilarino-Guell et al., 2009, Zhang et al., 2009, Zimprich et al., 2009).

UCHL-1

UCHL-1 mutations were initially thought to be associated to PD (Leroy et al., 1998, Maraganore et al., 2004) but this was refuted after a large metaanalysis (Healy et al., 2006).

EIF4G1

Mutations in *EIF4G1*, that encodes a transcription factor involved in the regulation of the initiation of mRNA translation, have also been reported to be associated with PD (Chartier-Harlin et al., 2011) but with incomplete penetrance (Nuytemans et al., 2013). However, recently it has been shown that mutations in this gene are more frequent in controls than cases, inferring that *EIF4G1* is not related to PD (Nichols et al., 2015).

As mentioned above, generally PD is considered as a genetic complex disease. It usually lacks a clear Mendelian pattern of inheritance, since the vast majority of affected individuals are idiopathic sporadic PD cases, with an unknown etiology possibly caused by a combination and interaction of genetic and environmental-lifestyle risk factors. In the following section, the main risk genes/loci mplicated in PD will be addressed.

7.3.3 Risk genes/loci

In the last ten years, GWAS have been a successful key approach to identify risk loci related to sporadic PD. The first GWAS performed in PD failed to convincingly identify any susceptibility loci, due to insufficient sample size to detect such associations (Maraganore et al., 2005, Fung et al., 2006). In 2009, two independent studies performed in Caucasian and Asian population revealed significant associations with PD (Simón-Sanchez et al., 2009, Satake et al., 2009). *SNCA, LRRK2* and the *PARK16* locus (including the genes *NUCKS1, RAB7L1* and *SL41A*) were linked to PD in both studies. *BST1* was found to be linked to PD in the Asian study and the *MAPT* locus in the Caucasian study.

Later on, two additional risk loci, *GAK* and *HLA-DRB5* were identified (Pankratz et al., 2009, Hamza et al., 2010). Subsequently, meta-analysis of the previous GWAS with larger patient-control cohorts, confirmed previous findings and revealed additional risk loci (Saad et al., 2011, Simón-Sánchez et al., 2011, Nalls et al., 2011, IPDGC and WTCCC2 et al., 2011, Lill et al., 2012, Sharma et al., 2012, Pankratz et al., 2012).

The largerst and most recent mega meta-analysis (Nalls et al., 2014) pooled SNP data from a total of 15 European GWAS including approximately 14000 patients and 95000 controls. Up to date, there have been identified a total of 28 independent risk loci **(Figure 17)**.

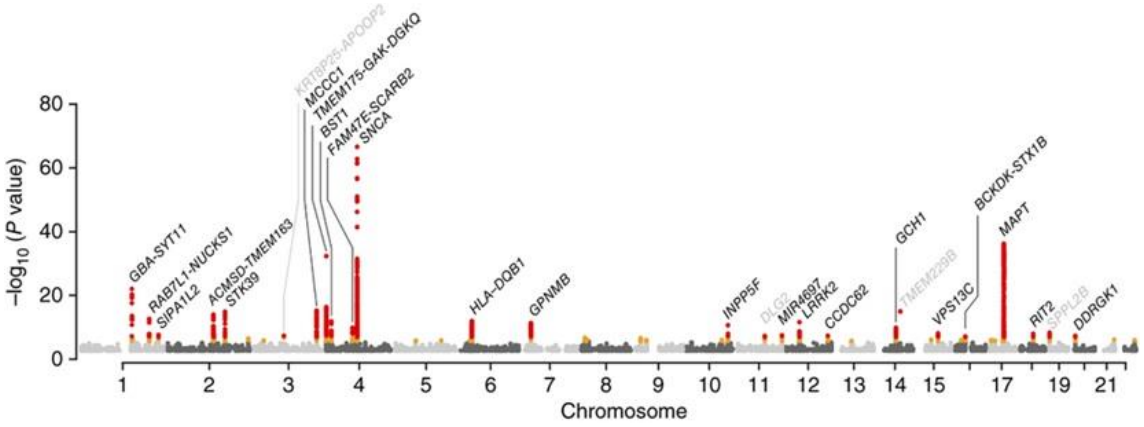


Figure 17. Manhattan Plot showing top hits from the largest and most recent meta-analysis in PD genetics up to date *(Reproduced from Nalls et al., 2014).*

Interestingly, there are loci containing multiple risk alleles which act as pleiomorphic risk loci (reviewed in Singleton and Hardy, 2011). *SNCA* and *LRRK2* are clear examples. As it has been detailed above, pathogenic mutations in these genes are responsible for autosomal dominant PD.

However, it has also been widely demonstrated that non-coding variability within these loci, predisposes to idiopathic PD. The idea of pleiomorphism can be extrapolated to several PD related loci, where we can find more than one independent risk allele.

Regarding *SNCA*, point mutations, duplications and triplications cause familial PD. On the other hand, Maragarone et al., (2006), reported an association between the REP1 polymorphism in the promoter region of *SNCA* and PD, and following GWAS signals at *SNCA* showed an association with PD from intron 4 to after the 3′ UTR region (Simón-Sanchez et al., 2009).

Similarly, *LRRK2* seems to be a pleiomorphic locus. As highlighted before, well-known pathogenic mutations cause monogenic PD. However, common variability has also been examined in several populations. Two variants, p.G2385R and p.R1628P, were first identified and linked to PD in the Asian population (Mata et al., 2005, Ross et al., 2008). It was later shown that more than being deleterious pathogenic mutations, they were susceptibility alleles doubling the risk for developing PD (Di Fonzo et al., 2006). The *LRRK2* locus has been continuously detected in GWAS, confirming the hypothesis that is related to both, familial and sporadic PD (Nalls et al., 2014). Remarkably, recent studies corroborate the link between familial and sporadic etiologies. Lesage et al., (2016), have recently found that rare deleterious variants in *VPS13C* are responsible for a very aggresive form of early onset PD. This locus has also been found associated with sporadic PD in the largest meta-analysis (Nalls et al., 2014).

Among the most relevant and recent found loci (reviewed in Trinh et al., 2013) **(Figure 17)**, it should be pointed out the cyclin G associated kinase–diaclyglycerol kinase (*GAK–DGKQ*) locus. This genomic region consists of a block containing three genes encoding three proteins; GAK, a protein that is involved in clathrin mediated endocytosis, DGKQ and phosphatidylinositol kinase PIK3CD, both with a central role in membrane curvature and signalling.

Additionally, the *PARK16* locus contains several genes (*NUCKS1, RAB7L1* and *SL41A)* in which, as previously mentioned, variability has been associated with susceptibility to PD. Within this locus, *RAB7L1* has been shown to interact with *LRRK2* and *VPS35*, and seems to play a part in endosomal–lysosomal trafficking.

Moreover, *HLA-DRA* and *HLA-DRB* encode the α and β chains of major histocompatibility complex class II DR. These proteins are cell surface molecules that interact with T-cell receptors implicated in immune response and inflammation. Finally, the *BST1* locus encodes the bone marrow stromal cell antigen 1, a cell-surface glycosylphosphatidylinositol-anchored glycoprotein that mediates neutrophil adhesion and migration (reviewed in Trinh et al., 2013).

The following section will focus on the most relevant risk loci related to PD.

GBA

Homozygous mutations in the *GBA* gene lead to Gaucher's disease (GD), a lysosomal storage disorder with an autosomal recessive pattern of inheritance. The clinical observation in relatives of patients with GD showed an increased incidence of PD, raised interest in *GBA* as a causative factor for PD (Neudorfer et al., 1996, Halperin et al., 2006).

It has been proved that, in GD families, relatives carrying heterozygous *GBA* variants have an increased incidence of PD (Lwin et al., 2004). Additionally, a recent multicenter study confirmed the genetic association and calculated a five-fold increase in PD risk (Sindransky et al., 2009).

Heterozygous *GBA* mutation-carriers are of particular interest because it does not appear to exhibit the absolute penetrance of the other monogenetic forms of PD. Approximately 5-10% of PD patients carry *GBA* mutations, making these mutations numerically the most important genetic predisposing risk factor for the development of PD identified to date. The frequency of the *GBA* mutations, however, varies according to the different ethnicities. *GBA* mutations are particularly frequent in some populations like Jews of Ashkenazi origin, but its prevalence varies too in non-Ashkenazi PD populations (Bandrés-Ciga et al., 2016, Beavan and Schapira, 2013, Bras et al., 2009).

To stimate its prevalence is however complicated by the existence of a *GBA* pseudogene named *GBAP*, in effect a non-functioning duplicate of *GBA.* The pseudogene has 96% identity to the coding regions of the functional gene.

This complicates sequencing, as many mutations are identical to sequences ordinarily found only in the pseudogene. Interestingly, because *GBA* genetic variants can appear with frequencies < 5%, it was initially omitted from GWAS analyses. It was only after a candidate gene approach when GWAS were able to confirm its clear significance as a PD risk factor.

So far, 300 pathogenic mutations throughout the *GBA* gene have been identified (Hruska et al., 2008), including point mutations, insertions, deletions, frameshift mutations and splice-site alterations. The c.1226A>G (N370S) mutation is the commonest *GBA* mutation in the literature followed by c.1448T>C (L444P).

The N370S variant, although frequent among those of European, American, or Middle Eastern origin, is not typically seen in Chinese or Japanese populations (Mitsui et al., 2009, Huang et al., 2011). Other mutations such as c.1093G>C (E326K), and c.1223C>T (T369M) have been reported as single disease-causing mutations (Durán et al., 2013, Clark et al., 2007), but both genetic variants have been found in patients mainly in cis phase (Park et al., 2002, Walker et al., 2003).

Clinical phenotype

GBA-related parkinsonism result in a phenotype that is virtually indistinguishable clinically, pharmacologically, and pathologically from sporadic PD, but it has been characterized by some slight differences, for instance a younger age of onset, a higher frequency of cognitive decline, bradykinesia (Gan-Or et al., 2009), olfactory dysfunction (Goker-Alpan et al., 2008), and a lower frequency of rigidity (Clark et al., 2007) (reviewed in Beavan and Schapira, 2013).

Protein function

GBA encodes the lysosomal enzyme GCase, one of the over a hundred lysosomal hydrolases found within the lysosomal membrane. These enzymes have the role of breaking down the waste products of autophagy under acidic conditions. GCase itself converts the sphingolipid glucosylceramide to ceramide.

The mechanisms underlying the association between *GBA* mutations and PD remain elusive, but proposed theories include the effects of reduced GCase and lysosomal dysfunction with the following accumulation of toxic substrates, such as α-syn, as it was previously discussed in the section "Pathogenesis-Autophagy and lysosomal disfunction".

GCH1

Mutations in this gene are known to cause both autosomal dominant (Ichinose et al., 1994) and recessive (Bruggemann et al., 2012, Nardocci et al., 2003) dopa-responsive dystonia (DRD), a disease with an impressive, sustained response to L-dopa administration (Trender-Gerhard et al., 2009).

Recently, mutations in *GCH1* were found to segregate in families with a combination of members with typical PD or DRD following an autosomal dominant pattern of inheritance with incomplete penetrance (Hagenah et al., 2005, Mencacci et al., 2014). Prompted by this observation, a follow-up large exome sequencing study in over 1,000 PD patients and 1,000 controls showed that known *GCH1* pathogenic mutations and novel variants are more frequent in PD patients than in controls and are associated to a 7-fold increase in the risk for developing PD (Mencacci et al., 2014).

Clinical phenotype

GCH1 mutation-carriers develop parkinsonian syndrome in the absence of dystonia with very good levo-dopa response. The age at onset is older than 40 years. They present with clinically relevant motor complications of chronic levodopa treatment, including wearing off, motor fluctuations and dyskinesias and classic non-motor features, strongly supporting nigrostriatal cell loss as the underlying pathology.

Protein function

GCH1 encodes GTP cyclohydrolase 1, an essential enzyme for dopamine production in nigrostriatal cells. It catalyses the synthesis of tetrahydrobiopterin that serves as a cofactor for the tyrosine hydroxylase, enzyme which catalyses the synthesis of dopamine.

MAPT

It encodes tau, a component of the microtubule-binding proteins. Tau was first found to be involved in AD, when it was identified as a part of the neurofibrillary tangles (Goedert et al., 1988). *MAPT* mutations have been linked to a wide variety of diseases known as tauopathies, and the first ones were reported to underlie frontotemporal dementia with parkinsonism-17 (Hutton et al., 1998). The main mutations are clustered in exons 1, 9-13 and segregate with an autosomal dominant pattern of inheritance.

The association between PD and *MAPT* has been controversial since tau pathology has been mainly associated to dementias. Moreover, a recent analysis from GWAS performed in AD and PD failed to reveal the coexistence of common pleiotropic alleles shared between these two diseases (Moskvina et al., 2013).

However, there are clear links between *MAPT* and parkinsonisms. Tauopathies such as progressive supranuclear palsy, frontotemporal dementia and corticobasal degeneration present with parkisonism. In addition, Dementia of Lewy Bodies reminds clinically as a mix between AD and PD, and it should be pointed out that PD occasionally has tau pathology (Charlesworth et al., 2012). These observations might indicate that the association is true and not due to misdiagnosed PD cases. There are two different haplotypes at the *MAPT* locus, H1 and H2 (Baker et al., 1999). The H1, the directly oriented haplotype, and the H2, which has an inverted chromosomal sequence. The H1c subhaplotype has been linked to an increased risk for AD and PD (Myers et al., 2005, Pittman et al., 2005), related to an increased expression of the exon 3 of *MAPT* (Trabzuni et al., 2012).

Additional regions within H1 have been linked to various forms of PD (Skipper et al., 2004). *MAPT* is one of the top hits found in GWAS (Nalls et al., 2014), but it seems to be limited to Europeans but not Asian populations (Satake et al., 2009).

Protein function

There are 6 different tau isoforms created by alternative splicing (Wade-Martins, 2012). Tau, similarly as α-syn, can undergo different post-translational modifications as phosphorylation and glycosilation (Baudier and Cole, 1987). Its function depends on the isoform and is not completely understood. Its role has been attributed to microtubule assembling stabilization and spacing, cellular signalling, axonal transport and protein fibrilization **(Figure 18)**.

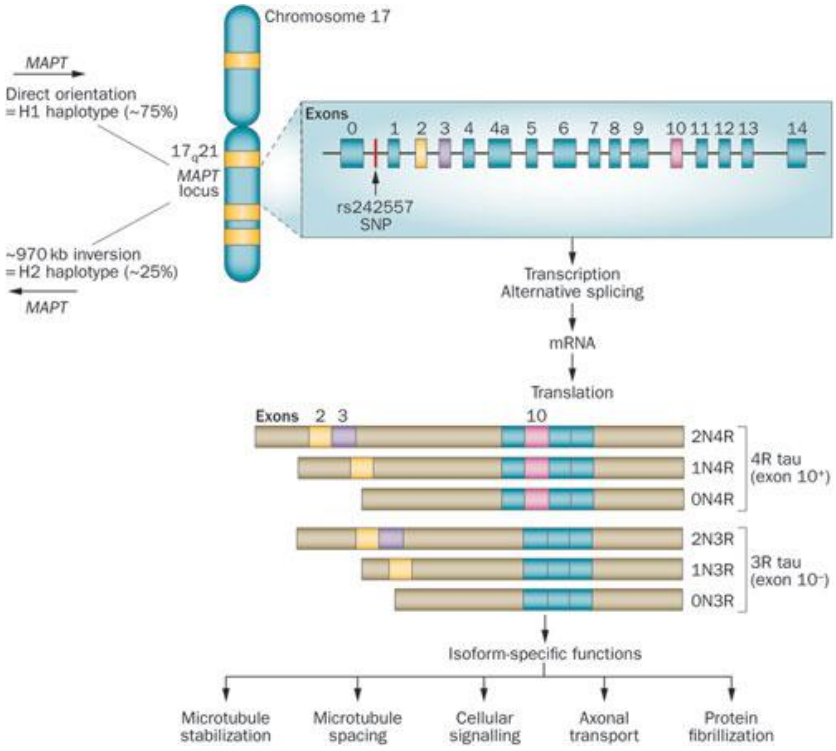


Figure 18. *MAPT* locus and the tau protein isoforms. *(Reproduced from Wade-Martins, 2012).* The *MAPT* locus has two haplotypes: the directly oriented H1 and the inverted H2. H1 gives relatively increased expression of exon 10, the exon found in protein tangles in several neurodegenerative diseases, whereas H2 is linked to increased expression of exon 3, which is proposed to be protective against the formation of tau tangles. Alternative splicing of exons 2, 3 and 10 of the *MAPT* gene gives rise to six protein isoforms, each of which may have distinct functions in cell biology.

II. Hypothesis and Objectives

Over the last 20 years, numerous studies have confirmed that genetic factors contribute to the complex pathogenesis of PD. Despite consistent effort, only a small portion of the heritable component for PD has been explained, suggesting there is a substantial unknown genetic basis to be discovered.

We hypothesised that Andalucia, given its geographical location on the southernmost region of Spain, might be the result of a particular genetic landscape coming from multiple historical migrations and the settlement of different civilizations. Up to date, despite its historical background, this population has been poorly studied in this context, and it is still unknown which genetic risk variants or pathogenic mutations contribute significantly to the development of PD. To test this hypothesis, the aims listed below were chosen as the focus of this research.

1) To assess the contribution of 8 known PD-related genes in a cohort diagnosed with either familial PD (FPD) or early-onset sporadic PD (EOPD) through next- generation sequencing.

2) To screen for common pathogenic mutations in the PD risk genes *LRRK2* and *GBA* in a cohort of late-onset sporadic PD (LOPD) through Sanger sequencing.

3) To perform a case-control association study by assessing 64 single nucleotide polymorphisms (tag-SNPs) selected from recent PD risk loci in a representative and homogeneous sample of PD cases and controls.

4) To identify novel putative candidate loci associated with idiopathic PD by performing the first Spanish PD GWAS, and to investigate whether SNPs previously identified as risk variants contribute to PD risk in our population.

5) To calculate a genetic risk profiling across the previously established risk loci in the biggest and most recent PD genetic meta-analysis (Nalls et al., 2014) and to analize whether this genetic risk is associated with age at disease onset.

6) To identify recessive founder risk variants by exploring runs of homozygosity enriched in PD cases versus controls.

7) To screen for novel disease-associated rare variants (MAF < 1 %) and to explore whether previous PD disease-associated rare variants are present in our cohort.

8) To estimate PD heritability attributable to genetic variation assessed by our genotyping platform.

9) To evaluate the role of structural genomic variations as risk factors/causing factors of PD in these subjects.

III. Analysis of the genetic variability in Parkinson's disease from Southern Spain

1. ABSTRACT

To date, a large spectrum of genetic variants has been related to familial and sporadic PD in diverse populations worldwide. However, very little is known about the genetic features of PD in Southern Spain, despite its particular genetic landscape coming from multiple historical migrations.

134 patients were included in the study of which 97 individuals were diagnosed with late-onset sporadic PD (LOPD), 28 with early-onset sporadic PD (EOPD) and 9 with familial PD (FPD). Genetic analysis was performed through a next-generation sequencing panel to sequence 8 PD-related genes (*LRRK2, SNCA, PARKIN, PINK1, DJ-1, VPS35, GBA* and *GCH1)* in EOPD and FPD groups and direct Sanger sequencing of *GBA* exons 8-11 and *LRRK2* exons 31 and 41 in the LOPD group.

In the EOPD and FPD groups, we identified 11 known pathogenic mutations among 15 patients (40.5 %). *GBA* (E326K, N370S, D409H, L444P) mutations were identified in 7 patients (18.9 %); *LRRK2* (p.R1441G and p.G2019S) in 3 patients (8.1 %); bi-allelic *PARK2* mutations (p.N52fs, p.V56E, p.C212Y) in 4 cases (10.8%) and *PINK1* homozygous p.G309D in one patient (2.7 %). An EOPD patient carried a single *PARK2* heterozygous mutation (p.R402C) and another had a novel heterozygous mutation in *VPS35* (p.R32S), both of unknown significance. Moreover, pathogenic mutations in *GBA* (E326K, T369M, N370S, D409H, L444P) and *LRRK2* (p.R1441G and p.G2019S) were identified in 13 patients (13.4 %) and 4 patients (4.1 %) respectively in the LOPD group.

A large number of known pathogenic mutations related to PD have been identified. In particular, *GBA* and *LRRK2* mutations appear to be considerably frequent in our population, suggesting a strong influence from the Jewish population. Further research is needed to study the contribution of the novel found mutation p.R32S in *VPS35* to the pathogenesis of PD.

2. INTRODUCTION

In the last decade, several loci and risk variants have been identified and linked to the pathology of familial and sporadic PD in diverse populations worldwide (Singleton et al., 2013).

Given its geographical location on the southernmost region of Spain, the population from Granada is the result of a particular genetic landscape coming from multiple historical migrations and the settlement of different civilizations. Its complex history over the last millennia has involved the long-term residence of 2 very different populations with distinct geographical origins: North African Muslims and Sephardic Jews. Southern Spain represents a potential migration network and the major cross-link between Europe and Africa. These remarkable interactions across the Mediterranean Sea and the North of Africa have contributed to a genetic enrichment and might have shaped a unique genetic profile.

The long period of coexistence between North Africa and Southern Spain during the 8 centuries of the Islamic invasion suggests a marked genetic relationship. The Jewish presence has also been widespread and long-established in Granada, and admixture analysis indicates a substantial proportion of ancestry from Sephardic Jews sources (Adams et al., 2008). Moreover, Southern Spain has been subject to other important influences coming from eastern Mediterranean populations such as the Greek and the Phoenician colonization (Zalloua et al., 2008). However, despite its historical background, the population from Granada has been poorly studied, and it is still unknown which genetic variants contribute significantly to the development of PD.

Our aim was to assess the contribution of known genes in a cohort diagnosed with either familial PD (FPD) or early-onset sporadic PD (EOPD) from Southern Spain, predominantly Granada and its area of influence. The genes of interest have been those traditionally associated with autosomal-dominant or -recessive forms and include *LRRK2, SNCA and VPS35, PARKIN, PINK1,* and *DJ-1*, respectively. Other risk genes recently linked to the disease such as *GBA* (Sidransky et al., 2009) and *GCH1* (Mencacci et al., 2014) have been also studied. Additionally, we screened for *LRRK2* and *GBA* common pathogenic mutations in a cohort of late-onset sporadic PD (LOPD).

3. MATERIAL AND METHODS

a) Patient cohort

We included a group of 134 PD patients. 97 were diagnosed with LOPD, 28 with EOPD and 9 unrelated FPD, all treated at the Movement Disorders Unit of the Service of Neurology in the both Hospital Clínico San Cecilio and Hospital Virgen de las Nieves of Granada (Spain). PD was diagnosed at least by two experienced neurologists in the field of movement disorders following the criteria of the UK PD Society Brain Bank (Gebb et al., 1999). EOPD defined by an age of onset ≤ 50 years. Patients who had at least one first-degree PD affected relative were classified as familial. The study was approved by the local ethic committee and written informed consent was taken from each participant.

b) DNA extraction from blood samples

DNA extraction from blood was done with the QIAmp DNA Blood Maxi kit. First 500 µl of Protease K were added to the bottom of a 50 mL plastic tube, followed by the addition of 5mL of blood sample. When that volume was not available, Phosphate-Buffered Salina (PBS) was added to bring up the volume to 5 mL. PBS was prepared making up a tablet (Gibco, Life Technologies) to 500 mL with distilled MilliQ water. After adding 6 mL of AL buffer and vigorously shaking up to 20 times by inversion, the tube was incubated at 70ºC for 10 min in a water bath. Later, 5 mL of 100% ethanol was added and vigorously shook up to 10 times by inversion.

The solution was then transferred to the QIAamp column inside a 50 mL tube and was centrifuged at 3000 rpm for 3 min.

After discarding the filtrate, 5 mL of AW1 washing buffer were added to the column and the tube was centrifuged at 4000 rpm for 1 min. Then, the column was washed with 5 mL of AW2 buffer and centrifuged at 4000 rpm for 15 min. The filtrate was discarded and the tube was incubated for 15 min at 50ºC in an incubator in order to evaporate residual ethanol. Finally, DNA was eluted in a clean tube from the column with 1mL of distilled MilliQ water, incubated at room temperature for 5 min and then centrifuged at 4000 rpm for 3 min. DNA concentration was measured with a Nanodrop.

c) DNA extraction from saliva samples

DNA extraction from saliva was performed following the protocol provided by the OG-500 Oragene-DNA collection kit (DNA Genotek, Life Technologies) **(Figure 19).** First, the tube collecting the sample was incubated at 50ºC in a water bath for an hour to ensure that nucleases were permanently inactivated. Secondly, the saliva sample was transferred into a 15 mL centrifuge tube and 1/25x volume of purification enzyme was added according to the volume of saliva (i.e. 160 µl of enzyme for 4 mL of sample, 140 µl for 3.5 mL, 120 µl for 3.0 mL). Then, the sample was mixed by inversion up to 10 times, incubated on ice for 10 min and centrifuged at room temperature for 15 min at 4000 rpm.

In order to discard the white pellet on the bottom of the tube, the sample was carefully transferred by pouring it into a fresh 15 mL centrifuge tube. After adding the equivalent volume of 100% ethanol than the one present in the tube, the clear supernatant was mixed gently by inversion up to 10 times. The sample was incubated at room temperature for 10 min allowing the DNA to fully precipitate and later centrifuged for other 10 min at 3000 rpm.

Subsequently, the supernatant was discarded without disturbing the pellet and 1 mL of 70% ethanol was added to the tube, which was incubated for 1 min at room temperature. The tube was centrifuged and the supernatant discarded immediately. After the ethanol wash step, the sample was centrifuged once again at 3000 rpm for 1 min to facilitate a complete ethanol removal.

Figure 19. Collection kit for purification of genomic DNA from saliva. *(Reproduced from Oragene, Genotek)*

LOPD patients were sequenced for *GBA* exons 8-11 and *LRRK2* exons 31, 41 and their flanking intronic sequences by Sanger sequencing since most pathogenic mutations are within these exons (Durán et al., 2013; Lesage et al., 2006).

d) Polymerase-chain reaction (PCR)

First, primers were optimised to ensure efficiency of the reaction, which was confirmed through electrophoresis. Specific conditions are detailed below:

GBA: 3rd fragment (exons 8-11)

Primers for *GBA* amplification:

Forward primer: 5'-TGTGTGCAAGGTCCAGGTCAG-3'

Reverse primer: 5'-ACCACCTAGAGGGGAAAGTG-3'

PCR reagents (per sample)

7.5 µL of Roche Fast Start PCR master (Roche, UK)

0.5 µL of 10mM Forward primer

0.5 µL of 10mM Reverse primer

5 µL of double distilled water

1.5 µL of genomic DNA

Termocycling conditions (corresponding to programme 64td37)

Temperature (º C)	Time (min)	Number of Cycles
94	15	
94	00:45	
60-62	00:45	20
72	3	
94	00:45	
60	00:45	18
72	3	
72	7	
4	Hold	

LRRK2: (exons 31-41)

Primers for *LRRK2* amplification:

Exon 31:

Forward primer: 5'-CCCAGTTTGAAAGCAAACAC-3'

Reverse primer: 5'-ACATTTCTCTACCAGCCTACCA-3'

Exon 41:

Forward primer: 5'-TTTTGATGCTTGACATAGTGGAC-3'

Reverse primer: 5'-CACATCTGAGGTCAGTGGTTATC-3'

PCR reagents (per sample)

10 µL of Roche Fast Start PCR master (Roche, UK)

2 µL of 5pM Forward primer

2 µL of 5pM Reverse primer

5 µL of double distilled water

1.0 µL of genomic DNA

Termocycling conditions (corresponding to programme 60td50)

Temperature (ºC)	Time (min)	Number of Cycles
94	5	
94	00:60	
60-62	00:45	20
72	3	
94	00:45	
60	00:45	18
72	3	
72	7	
4	Hold	

e) Agarose gel electrophoresis

An agarose gel was prepared for a subsequent electrophoresis to ensure that the PCR was working successfully. 10x Tris Borate Ethylenediaminetetraacetic acid (TBE) buffer was prepared by adding 121.1 gr of Trizma base, 61.8 gr of Boric acid and 7.4gr of EDTA to a final volume of 1L with double distilled water. 10x TBE buffer was then diluted to 1x.

To prepare a 2% agarose gel, a plastic pot was filled up to 200 mL with 1x TBE and 4gr of agarose were added. The solution was then heated in the microwave for 2min. When the solution was clear of particles, we added 20 µl of gel red dye and mixed up by shaking until the solution was homogeneous. Once cooled, the gel was poured into a mould and red combs were placed on the top. Finally, the gel was left at room temperature until it got solid. The gel was loaded as follows; First, 3 µl from the PCR product of each sample were mixed with 3µl of Orange Dye. Subsequently, the mix was loaded on each well of the agarose gel and 5 µl of DNA ladder (1Kb) were added in the first well of the lane. The electrophoresis was carried out at 120V for 30min and the DNA bands were visualised under UV light.

f) Sanger sequencing

It is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication.

A DNA polymerase, a primer (forward or reverse), and four types of deoxynucleotide triphosphates (dNTPs) are used to synthesize the complementary strand to the template sequence.

dNTPs usually contain deoxyadenosine triphosphate (dATP), deoxythymin triphosphate (dTTP), deoxyguanin triphosphate (dGTP) and deoxycytosin triphosphate (dCTP). All dNTPs have a 3' hydroxygroup that is required by the DNA polymerase to attach the phosphate group of the following nucleotide during strand synthesis.

Dideoxynucleotide triphosphates (ddNTPs) lack this hydroxygroup. If a ddNTP is incorporated into the sequence, synthesis is terminated. This effect is used in this technique, where a small proportion of ddNTPs is added to the sequencing reaction. The result is a number of fragments with different lengths and different terminal ddNTPs (hence, the name "chain-termination sequencing").

The ddNTPs are labelled radioactively or fluorescently. The sequencing reaction is performed in a single vessel with different fluorescently labelled ddNTPs (i.e ddATP may be green, ddCTP may be red and so on). The DNA fragments are separated by size through capillary electrophoresis.

A laser and a detector are utilized to first excite and then read the fluorochromes of each sequence fragment at the end of the capillary, typically producing a chromatogram with four different colours.

The different steps of Sanger sequencing protocol are described below:

- ✓ ***Purification of PCR products***

The aim of this step was to remove excess of primers and dNTPs avoiding interferences with the sequencing reaction. The Exo-Sap method was used to clean-up as follows.

First, we prepared a stock solution including 50 µl of Exonuclease I enzyme (to remove ssDNA from PCR products), 200 µl of Fast-Alkaline phosphatase (which removes unused dNTPs) and 750 µl of double distilled MilliQ water. Once the mix was gently vortexed, 5 µl of Exo-Sap were added to 12 µl of PCR product in a new plate for *GBA* purification. For *LRRK2* purification, 2 µl of Exo-Sap were added to 5 µl of PCR product. The conditions for the purification process (Exo-Sap program) are detailed below:

Temperature (ºC)	Time (min)
37	30
80	15
4	Hold

- ✓ ***Sanger sequencing reaction:***

  Specific conditions of primers design are described below.

  Primers for *GBA* sequencing:

  Exon 8

  Forward primer: 5'-AGTTCCAGAAGCCTGTGTGC-3'

  Reverse primer: 5'-CTTCTGTCAGTCTTTGGTGAAA-3'

  Exon 9

  Forward primer: 5'-CCCACATGTGACCCTTACCT-3'

  Reverse primer: 5'-TGTAGGAGATGATAGGCCTGGT-3'

  Exon 10-11

  Forward primer: 5'-GGGTCCGTGGGTGGGT-3'

  Reverse primer: 5'-TGCTGTGCCCTCTTTAGTCA-3'

Primers for *LRRK2* sequencing (see *LRRK2* primers for amplification)

The mix was prepared by adding the following reagents into a 1.5 mL Eppendorf. Then 7 µl of the mix were added to a new 96-well plate:

Sequencing reagents (per sample)

2 µl of sequencing buffer (BigDye terminator v1.3 Cycle sequencing kit, Applied Biosystems, USA)

1 µl of Primers forward or reverse (5pM)

3.5 µl of double distilled water

0.5 µl of BigDye (BigDye terminator v1.3 Cycle sequencing kit, Applied Biosystems, USA)

3 µl of purified PCR product

Termocycling conditions (corresponding to programme 3730)

Temperature (ºC)	Time (min)	Number of Cycles
94	01:00	
94	00:30	25
50	00:15	
60	04:00	
4	Hold	

* We sequenced both forward and reverse strands for confirmation.

- ***Sanger sequencing reaction purification***

The purpose of this step is to remove excess of fluorescently labelled ddNTPs, primers and salts from the sequencing reaction. We performed this step with the Sephadex method. First, we mixed 40 mL of autoclaved distilled water and 2.9gr of Sephadex G-50 Bioreagent for molecular biology in a 50 mL tube. We allowed this solution to hydrate at room temperature for 30 min prior to use. After shaking vigorously, we added 350 μl of Sephadex solution per well on a Corning FiltrEX 96-well filter plate placed on an empty collection plate. We then prepared a balance of equal mass and centrifuged them at 750xg for 3 min.

The water collected on the plate was discarded and the filter plate placed on a new 96-well collection plate. We then transferred the sequencing reaction product (10 μl) onto the filter plate without touching the tips on the Sephadex matrix.

The plate was centrifuged at 910xg for 5 min producing the purified product. We added the same volume of double distilled water to the empty wells prior to putting it into the sequencher ABI 3730xl DNA analyzer (Applied Biosystems, USA). Each filter plate could be used up to 5 times, as long as we cleaned it 4 times with double distilled water and centrifuge it at 910xg for 3 min to remove residual water. In the case we were not intended to place the plate directly into the sequencing machine, we used to seal it and then cover it with foil before putting it in the freezer. When the ABI3730xl DNA analyzer was available, we sealed the plate with transparent paper by heating the corners and put it into a black, plastic holder with the cut corner of the plate on the opposite corner to the cut corner of the black base.

Finally, we put the plate into the ABI3730xl DNA analyzer (Applied Biosystems, Foster City, CA) so the sequencing read could happen. Results were analysed using the CodonCode Aligner v.6.0.2 software.

g) Target sequencing

EOPD and FPD patients were screened for potential mutations employing *target sequencing*, a next-generation sequencing (NGS) technique. The NGS method used was a PCR amplicon-based called TruSeq Custom amplicon Assay (TSCA) V1.6 from *Illumina*. This technique enables rapid design and sequencing with flexibility in selecting the areas of the genome to be covered. In this study, we aimed to screen for variants across the coding exons of the following 8 PD genes; *SNCA, LRRK2, VPS35, PINK1, PARK2, DJ-1, GBA* and *GCH1*.

Probes were designed using Illumina TSCA Design Studio v1.6 online (http://www.illumina.com/applications/designstudio.ilmn).

This technique is based on the Sequencing by Synthesis (SBS) technology, where DNA synthesis-terminating fluorescently-labelled nucleotides bind to the DNA emitting a light which can be detected at each base along the chain. Each nucleotide will discharge a different colour which is imaged and then, the dye is removed **(Figure 20a).** This process is completed in a massively parallel way with thousands of DNA amplicons being read simultaneously.

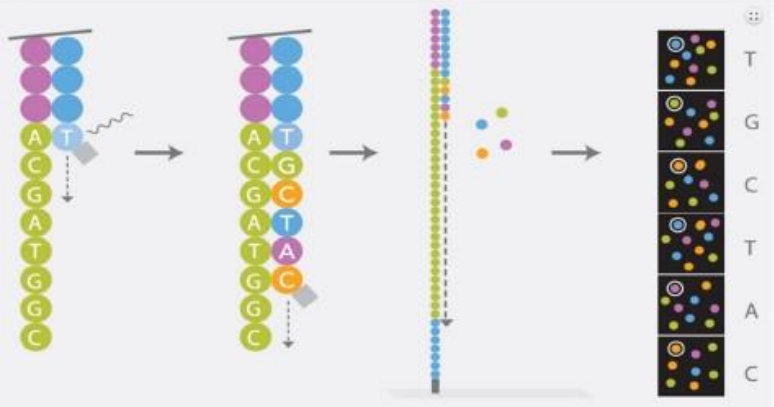


Figure 20a. TruSeq Custom Amplicon Workflow *(Reproduced from Illumina)*

The assay was performed according to the following protocol. First, two designed probes are incubated with each DNA sample to enable hybridisation upstream and downstream of the region of interest **(Figure 20b).** Unlike Sanger sequencing, hundreds of primers can be added to the same well containing one DNA sample. Each probe contains unique, target-specific sequence as well as a universal adapter sequence that is used in a subsequent amplification reaction.

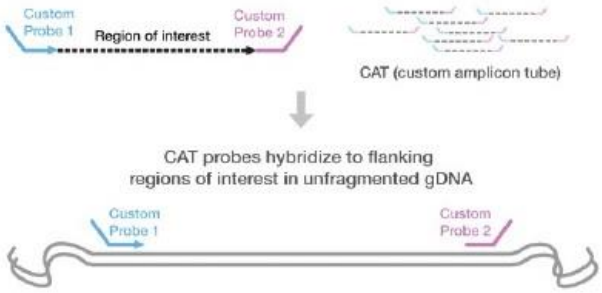


Figure 20b. TruSeq Custom Amplicon Workflow *(Reproduced from Illumina)*

Next, the samples are washed using a filter plate to remove any primers which have not bound to the DNA. Subsequently, the extension-ligation step allows to bridge the two probes using DNA polymerase and DNA ligase to create copies of the target region. Extension-ligation templates are PCR-amplified and two unique, sample-specific indexes are incorported. Here, each patient is barcoded with a unique section of genetic code enabling massively parallel sequencing because multiple patients can be pooled in the same mixture **(Figure 20c).**

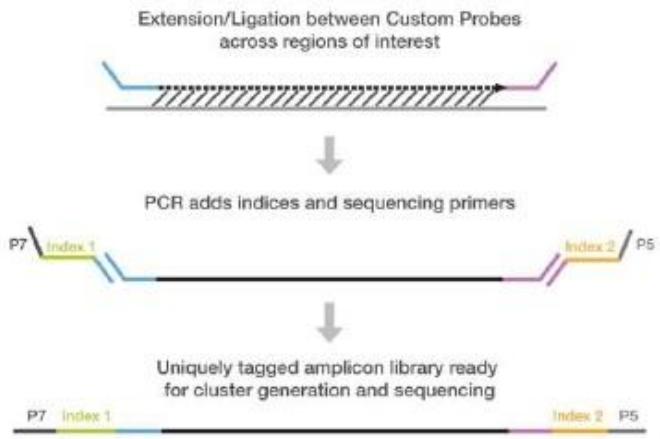


Figure 20c. TruSeq Custom Amplicon Workflow *(Reproduced from Illumina)*

After PCR clean-up, library normalisation is achieved by using magnetic beads at a set concentration which bind to a limited amount of DNA allowing wash-off of all other products. Lastly, samples are then pooled together and denatured for use on the MiSeq. On the MiSeq instrument, a flow cell is used, which is a glass slide with primers projecting from the surface. These primers bind specifically to the amplicons which undergo bridge amplification and cluster generation **(Figure 21).**

The former describes the process of an amplicon bending over and attaching at both ends to the flow cell. A copy of this amplicon is created and both amplicons make new bridges for further amplification. The resulting mass of amplicons is called a *cluster.* The clonal amplification of template DNA to generate *clusters* of identical DNA is followed by sequencing through a stepwise incorporation of fluorescently labelled nucleotides or oligonucleotides. The reason for generating clusters of the same amplicon is to produce a higher fluorescent signal which is much easier to image.

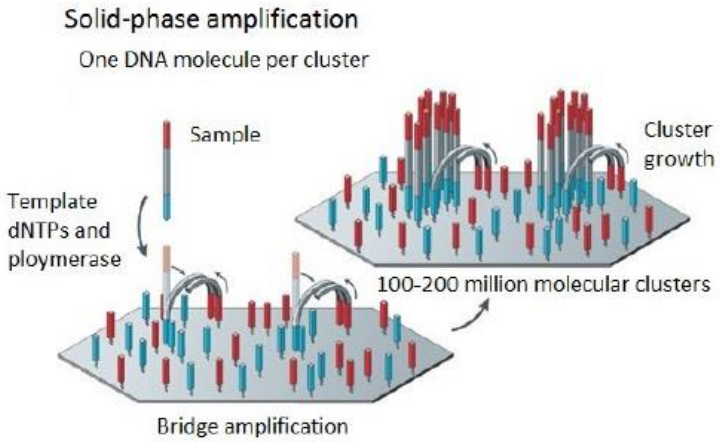


Figure 21. Bridge amplification. *(Reproduced from Metzker et al.,2010)*

The sequencing panel was tested for 37 patients. FASTQ files created by MiSeq were aligned using Novoalign to the reference genome (UCSC hg19). Variants were filtered using the following criteria: Artefacts common to multiple wells and with a low read balance as well as variants that resulted in no alteration of the Aa sequence and variants with a minor allele frequency ≥ 1 % in general population according to 1000 Genome Project (http://www.1000genomes.org/) were excluded from the analysis. Targeted exons with a coverage of less than 10 reads were subsequently screened by Sanger Sequencing.

h) Multiple Ligation dependent Probe Amplification (MLPA)

PD cases that carried point mutations in *PARK2* and *PINK1* were screened for exon rearrangements through multiplex ligation-dependent probe amplification (MLPA).

MLPA is a semi-quantitative technique that is used to determine CNVs in a single multiplex PCR-based reaction. The principle of MLPA is based on the amplification, by use of a single PCR primer pair, of up to 60 probes, each of which detecting a specific DNA sequence of approximately 60 nucleotides in length. After denaturation of the DNA, a mixture of MLPA probes is added to the sample. Each MLPA probe consists of two oligonucleotides that must hybridise to immediately adjacent target sequences in order to be ligated into a single probe. Each probe in an MLPA probemix has a unique amplicon length, typically ranging between 130 and 500 nucleotides. During the subsequent PCR reaction, all ligated probes are amplified simultaneously using the same PCR primer pair. One PCR primer is fluorescently labelled, enabling the amplification products to be visualised during fragment separation. This is done on a capillary electrophoresis instrument, yielding a specific electropherogram.

The relative height of each individual probe peak, as compared to the relative probe peak height in various reference DNA samples, reflects the relative copy number of the corresponding target sequence in the sample. A deletion of one or more target sequence thus becomes apparent as a relative decrease in peak height, while an increase in relative peak height reflects an amplification **(Figure 22).**

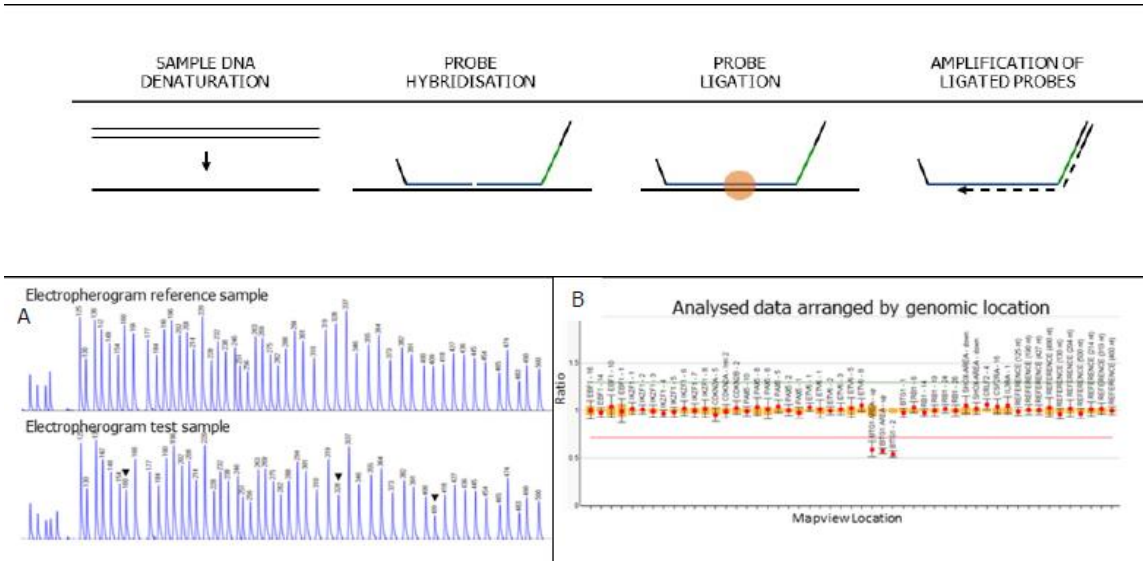


Figure 22. Multiple Ligation dependent Probe Amplification. (*Reproduced from MRC Holland, Amsterdam, the Netherlands).* A) Electropherogram of a test sample (bottom) is compared to that of a reference sample (top) showing a relative decrease of three probes in the test sample (arrows). B) Calculated probes ratios of the same test sample after analysis of these two samples: arranging probes by chromosomal location shows a reduced copy number for these three adjacent probes in the test sample.

We used the P051-C3 Salsa MLPA Parkinson probe set (MRC Holland, Amsterdam, the Netherlands). This set includes probes that detect exonic rearrangements in *PARK1* ([SNCA]; exons 2-7), *PARK2* ([PRKN]: exons 1–12), *PARK6* ([PINK1]; exons 1–8), *PARK7* ([DJ-1]; exons 1b, 3, 5, and 7) and *PARK9* ([ATP13A2]: exon 2, 9), *PARK8* ([LRRK2]: exon 41). The experimental procedure was performed as described below:

- ✓ **Denaturation**

First, we added 5 µL of DNA sample at 50-100 ng. DNA was diluted when necessary in TE 0.1 (10nm Tris-HCl + 0.1 mM EDTA). Denaturation of DNA was performed for 5 min at 98ºC followed by cooling at 25ºC in a thermocycler.

- ✓ **Hybridisation**

Hybridisation master mix was prepared for each reaction by adding 1.5 µL of MLPA buffer to 1.5 µL of SALSA probe mix. 3 µL of the mix was then added per each sample well and mixed properly by pipetting up and down. Finally, the plate was incubated for 1 min at 95 ºC followed by 16-20 hours at 60ºC.

- ✓ **Ligation**

Ligation master mix was prepared by adding 25 µL of dH_2O, 3µL of Ligase-65 Buffer A and 3 µL Ligase-65 Buffer B. Then, 1 µL of Ligase-65 enzyme was added and mixed by pipetting gently up and down. Subsequently, the plate was incubated at 54ºC and 32 µL of ligase mix was added to each well while the samples were in the thermocycler.

At this point, incubation continued for 15 min at 54ºC for ligation followed by 5 min at 98ºC for heat inactivation of Ligase-65 enzyme and paused at 20ºC.

- ✓ **PCR reaction**

Polymerase master mix was prepared by mixing 7.5 µL of dH_2O, 2 µL of SALSA PCR primer mix and 0.5 µL of SALSA polymerase and stored on ice until use. SALSA PCR master mix was prepared by adding 4 µL of SALSA Buffer, 26 µL of dH_2O and 10 µL of SALSA polymerase reaction. Then, 30 µL of SALSA PCR master mix was pipetted to each well of a new plate and mixed with 10 µL of MLPA ligation reaction at room temperature. Finally, data analysis was performed using Genemarker v. 2.6.2 software.

Termocycling conditions for MLPA-PCR reaction

Temperature (ºC)	Time (min)	Number of Cycles
95	00:30	35
60	00:30	
72	1	
72	20	1
15	Hold	

4. RESULTS

Characteristics of the patient cohort

		SP	
	FPD	**LOPD**	**EOPD**
Age at onset (years)	46.3	65.8	49.0
Disease Duration (months)	150.8	74.0	97.8
Female : Male	3:6	35:62	11:17
Disease severity			
(H&Y score) %			
I	26.7	29.3	11.2
II	26.7	47.6	50.4
III	26.7	19.5	30.1
IV	20.0	3.7	8.5

Table 1. Demographic and clinical characteristics of the groups under study. SP: Sporadic Parkinson's disease; EOPD: Early-onset Parkinson's disease; LOPD: Late-onset Parkinson's disease; FAM PD: Familial Parkinson's disease; H&Y score: Hoehn y Yahr score

Mutational screening

For the genes assessed by NGS sequencing in the FPD and EOPD group, a total of eleven known PD-related mutations were identified among 15 patients (40.5%) **(Table 2).**

Gene	Exon	rsID	Variant	NT change	Zygosity	Patients		
						FPD	SP	
GBA							EOPD	LOPD
	10	rs1064651	D409H	c.1342G>C	Het	-	2	2
	11	rs35095275	L444P	c.1448T>C	Het	1	-	2
	10	rs76763715	N370S	c.1226A>G	Het	1	2	4
	9	rs2230288	E326K	c.1093G>A	Het	-	1	2
	8	-	T369M	c.1223T>C	Het	-	-	2
					Hom	-	-	1
LRRK2								
	41	rs34637584	p.G2019S	c.6055G>A	Het	1	1	3
	31	rs33939927	p.R1441G	c.4321C>G	Het	1	-	1
PARK2								
	2	N/A	p.N52fsX80	c.154delA	Hom	1	1	N/A
					[a)]Het	1	-	N/A
	11	rs55830907	p.R402C	c.1204 C>T	[b)]Het	-	1	N/A
	2	rs137853059	p.V56E	c.167 T>A	Het	1	-	N/A
	6	rs137853058	p.C212Y	c.635 G>A	Het	1	-	N/A
PINK1	4	rs74315355	p.G309D	c.926G>A	Hom	1	-	N/A
VPS35	2	N/A	**p.R32S ****	c.96A>T	Het	-	1	N/A

Table 2. Genetic PD-related variants identified in PD patients from Southern Spain: U: Unknown; N/A: Not applicable since it has not been studied; Het: Heterozygous; Hom: Homozygous; ** Novel mutation; a) *PARK2* heterozygous deletion of exons 3 and 4.b) Single heterozygous state.

Interestingly, one EOPD patient carried a novel mutation in VPS35 (p.R32S). Four different heterozygous *GBA* mutations were detected among six patients. Two sporadic (SP) cases with EOPD carried the mutation D409H and one familial case carried the L444P. The mutation N370S was found in two SP cases with EOPD and in one FPD case, and the E326K was identified in one SP case with EOPD. Regarding *LRRK2*, we found two pathogenic mutations in the heterozygous state among three patients. One familial and one SP case with EOPD carried the mutation p.G2019S. The mutation p.R1441G was identified in one FPD case. Moreover, four mutations were identified in *PARK2* among five patients.

Three independent cases carried the frameshift deletion p.N52fs, two in the homozygous and one in the heterozygous state. MLPA analysis revealed the presence of a *PARK2* deletion of exon 3 and 4 in the latter case.

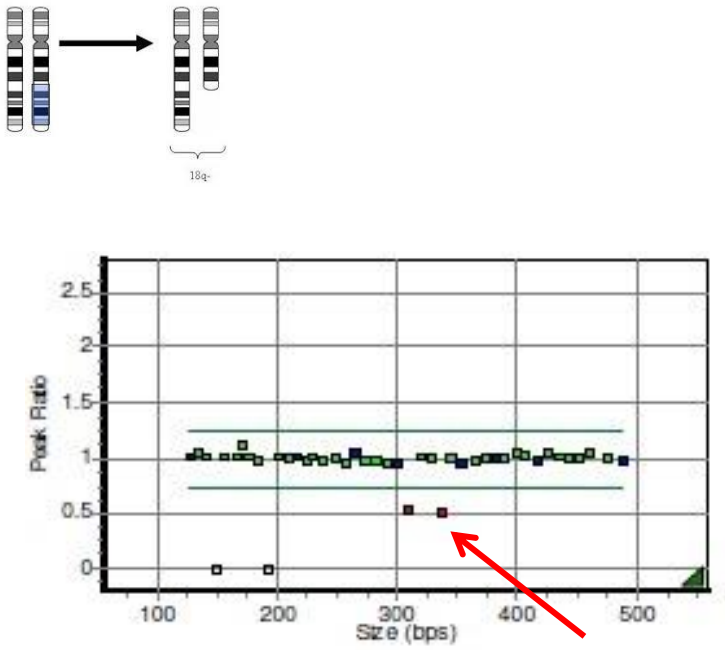


Figure 23. MLPA showing heterozygous deletion of exons 3 and 4 in *PARK2.*

The heterozygous mutation p.R402C of uncertain significance was identified in one SP case with EOPD. However, no rearrangements were found in the single heterozygous *PARK2* mutation carrier. One FPD case carried the heterozygous point mutations p.V56E and p.C212Y simultaneously. The *PINK1* homozygous mutation p.G309D was identified in one FPD case.

The remaining 22 patients (59.5%) did not have any identifiable genetic risk variant. We found genetic variants related to the disease in 28.6 % of EOPD and 88 % of FPD cases. Four additional SNPs of unknown significance were detected in *LRRK2* (p.I1371V, p.N2081D, p.M1646T, p.R1514Q) in two EOPD cases and two FPD cases, as well as one *DJ-1* variant (p.R98Q) in a FPD. These variants are most likely benign as they are all reported at relatively high frequencies in the ExAc database (minor allele frequency > 0.001). No mutation carriers were found for *SNCA* and *GCH1.*

In the LOPD group, we identified six variants in *GBA* and two in *LRRK2* among an overall of 17 patients (17.5%) **(Table 2).** The *GBA* heterozygous mutation D409H was found in two patients, the heterozygous mutations L444P and E326K were identified simultaneously in two patients, the heterozygous mutation N370S was detected in four patients and the variant T369M was found in three patients (two in the heterozygous state and one in the homozygous state). Two examples of the identified variants are represented with sequencing chromatograms in **Figures 24** and **25.** For the remaining 80 patients (82.5 %) we did not find any genetic alteration.

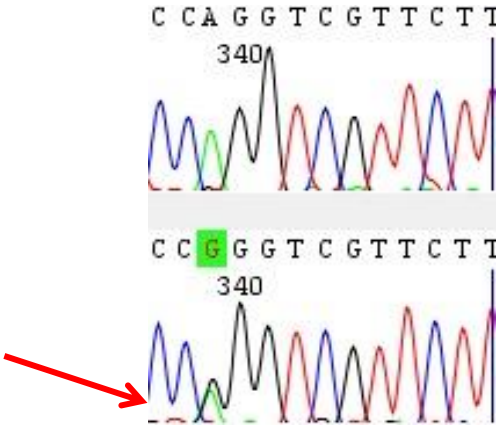


Figure 24. Chromatogram showing wild-type (upper image) versus heterozygous risk variant N370S, (c.1226A>G) in the *GBA* gene (lower image). The red arrow shows the nucleotide change (*Generated with CodonCode Aligner).*

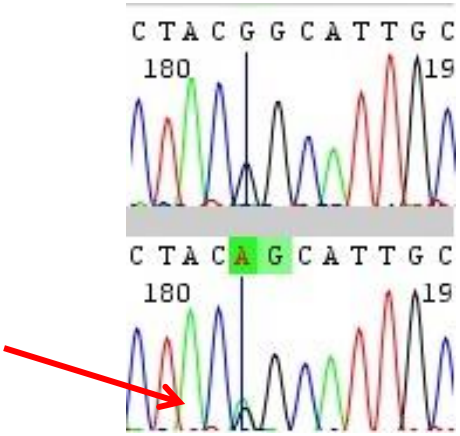


Figure 25. Chromatogram showing wild-type (upper image) versus heterozygous pathogenic mutation G2019S (c.6055G>A) in the *LRRK2* gene (lower image). The red arrow shows the nucleotide change *(Generated with CodonCode Aligner).*

Frequencies of pathogenic mutations in the genes under study are documented below for each group.

Gene	Variant	EOPD	FPD	LOPD	State	Controls
GBA	D409H	0.07	-	0.02	Het	1 x 10 $^{-4}$
	L444P	-	0.11	0.02	Het	U
	N370S	0.07	0.11	0.04	Het	3 x 10 $^{-3}$
	E326K+L444P	-	-	0.02	Het	U
	E325K	0.03	-	-	Het	1.2 x 10 $^{-2}$
	T369M	-	-	0.03	Hom/Het	9 x 10 $^{-3}$
LRRK2	p.G2019S	0.03	0.11	0.03	Het	9 x 10 $^{-3}$
	p.R1441G	-	0.11	0.01	Het	U
PARK2	p.N52fsX80	0.03	0.11	N/A	Hom/Het	U
	p.R402C	0.04	-	N/A	Het	2 x 10 $^{-3}$
	p.V56E	-	0.11	N/A	Het	2.9 x 10^{-5}
	p.C212Y	-	0.11	N/A	Het	1.5 x 10^{-5}
PINK1	p.G309D	-	0.11	N/A	Het	U
VPS35	p.R32S	0.04	-	N/A	Het	-

Table 3. Allele frequency of found mutations in PD patients from Spain and European controls compiled from ExAx database. (U: Unknown, Het: Heterozygous, Hom: Homozygous).

a) In silico analysis of the novel VPS35 mutation

Since the variant *VPS35* p.R32S raises further questions as to the structural effect, protein stability and malfunction on the vacuolar protein sorting-associated protein 35, we performed an *in silico* analysis using the SIFT bioinformatics tool (Kumar et al., 2009) and the HOPE web server (Venserlaar et al., 2011).

HOPE prediction results

The original wild-type residue and newly introduced mutant residue differ in size, charge and hidrofobicity. The mutant residue is small and neutral while the wild-type is bigger and positively charged. Moreover, the mutation introduces a more hydrophobic residue resulting in loss of hydrogen bonds and disturbing its correct folding. It should be pointed out that the mutation is located within a stretch of residues annotated as a special region of interaction with SNX3 which could disturb the protein and affect to its function.

SIFT prediction results

The mutant amino acid was predicted to be damaging.

Aa position	Aa Reference	Aa substitution	SIFT	
			Score	Prediction
32	R	S	0	DAMAGING

Table 4. SIFT prediction for the *VPS35* R32S mutation. SIFT scores range from 0 to 1 and represent the scaled probability of an AAS (Aminoacid substitution) to be tolerated. AASs with scores that either math with or fall below 0.05, are predicted to affect protein function and are considered as damaging or intolerant. While that AASs with scores ≥ 0.05 are predicted to be tolerated.

IV. Genetic association study of Parkinson's disease related-loci in Southern Spain

1. ABSTRACT

It has been widely accepted that genetic factors contribute to the complex pathogenesis of PD. We aimed to perform a case-control association study by assessing 64 SNPs in 113 individuals with PD and 374 controls from Southern Spain.

SNPs were selected from the following PD-related loci (*SNCA, LRRK2, PARK2, PINK1, DJ-1, VPS35, MAPT, GBA, HLA, STK39, ACMSD, GAK*). Genotyping was carried out using Taqman assays in an OpenArray Real-Time PCR platform. Logistic regression and haplotype analyses were performed assuming an additive model and adjusted by covariates to test association with PD.

Our study revealed significant differences (adjusted significance threshold: $p=1 \times 10^{-3}$) for the following SNPs: *SNCA* rs2736990-G (OR=1.8, 95% CI [1.3-2.5], $p=1 \times 10^{-4}$) and *SNCA* rs356204-T (OR=1.7, 95% CI [1.3-2.3], $p=3 \times 10^{-4}$). *GBA* haplotypes TTGG and CCAA (rs2990245-rs2049805-rs914615-rs206698), and the *SNCA* haplotype TGGT (rs356204-rs356219-rs2736990-rs356220) were found to be significantly overrepresented in cases versus controls (*GBA*-TTGG; OR= 2.6, 95% CI [1.1-6.3], $p= 4.1 \times 10^{-11}$; *GBA*-CCAA; OR= 7.7, 95% CI [2-29.3], $p= 1.2 \times 10^{-8}$), (*SNCA*-TGGT; OR=1.4, 95% CI [1-2], $p= 6 \times 10^{-4}$).

Common SNPs in the *SNCA* and *GBA* loci are risk factors for idiopathic PD in the Southern Spanish population.

2. INTRODUCTION

Although the etiology of PD still remains unclear, approximately 5%-10% of the patients are known to have monogenic forms of the disease, as it has been reported in the introduction of this thesis. To date, a considerable number of genes have been associated with both autosomal dominant and autosomal recessive PD (Singleton et al., 2013). However, these cannot explain the etiology of the vast majority of patients with an apparently sporadic PD which is thought to result from complex interactions between genes and environmental factors.

Recently, several GWAS (Edwards et al., 2010, Hernandez et al., 2012, Pihlstrom et al., 2013, Saad et al., 2011, Satake et al., 2009, Simón-Sánchez et al., 2009, Simón-Sánchez et al., 2011) and large scale meta-analyses (Nalls, et al., 2014, Nalls et al., 2011, Do et al., 2011, International Parkinson's Disease Genomics Consortium and Welcome Trust Case Control Consortium 2, 2011, Lill et al., 2012, Pankratz et al., 2012) have provided new insights into the genetic landscape of PD, identifying a number of susceptibility loci considered as risk factors. However, replication studies in independent populations are absolutely necessary to test the robustness of such association reports. It has been widely suggested that studies in populations with limited genetic heterogeneity are valuable for studying the genetic basis of disease (Hernández et al., 2012). Here, we perform a case-control association study to examine the relationship between SNPs in the most significant loci reported in recent GWAS and the risk of developing PD in a representative and homogeneous sample from Southern Spain. Moreover, we establish haplotype analyses to study whether there is any SNPs combination associated significantly with PD.

3. MATERIALS AND METHODS

a) Patient cohort

The study population consisted of 113 PD patients from Southern Spain recruited in the Movement Disorder Unit of the University Clinical Hospital "San Cecilio" and the Hospital "Virgen de las Nieves" from Granada (Spain) and 374 healthy subjects from the same region with no family history of PD. The recruitment of the whole sample was held between 2006 and 2012. PD was diagnosed by at least two expert neurologists in the field of movement disorders under the criteria of the UK PD Society Brain Bank (Hughes et al., 1992). Controls were extensively assessed to rule out any sign of neurological or psychiatric condition. Cognitive impairment was evaluated using the scale "Screen for Cognitive Impairment in Psychiatry (SCIP)". Studies comparing cognitively-impaired individuals to those with adequate functioning propose a cut-off point of the SCIP at 70, what is associated with a sensitivity of 87.9 and specificity of 80.6 (Pino et al., 2008). None of our control subjects showed cognitive deterioration. This study was approved by the local ethic committee and written informed consent was taken from each participant.

b) SNPs selection

64 SNPs were selected from recent reported GWASs or candidate studies in PD-related genes/loci as documented in the following table.

CHR	Locus	SNP	BP	A1/A2	MAF
1	*PARK7*	rs12727642	8046672	A/C	0.165
1	*DDOST;PINK1*	rs2298299	20974802	G/A	0.1717
1	*DDOST;PINK1*	rs2298300	20975463	C/T	0
1	*DDOST;PINK1*	rs2070660	20980450	G/T	0.219
1	*MUC1;MIR92B;THBS3;MTX1*	rs2066981	155172379	G/A	0.434
1	*MTX1;GBAP1;THBS3*	rs914615	155175892	A/G	0.435
1	*GBAP1;GBA*	rs2049805	155194980	T/C	0.415
1	*GBAP1;GBA*	rs2990245	155197462	C/T	0.456
1	*GBAP1;GBA*	rs1045253	155201235	A/G	0.338
1	*GBAP1;GBA*	rs9628662	155206341	G/T	0.344
1	*GBA*	rs11264345	155213124	A/T	0.344
2	*ACMSD*	rs6430544	135602511	C/T	0
2	*ACMSD*	rs2166480	135637338	G/A	0.463
2	*STK39*	rs6749447	169041386	G/T	0.274
4	*GAK*	rs1564282	852313	T/C	0.131
4	*GAK*	rs11248051	858332	T/C	0.127
4	*SNCA*	rs356219	90637601	G/A	0.384
4	*SNCA*	rs11931074	90639515	T/G	0.070
4	*SNCA*	rs356220	90641340	T/C	0.485
4	*SNCA*	rs3857052	90643857	A/G	0.069
4	*SNCA*	rs7684318	90655003	C/T	0.061
4	*SNCA*	rs356204	90663542	T/C	0.482
4	*SNCA*	rs2736990	90678541	G/A	0.482
4	*SNCA*	rs104893878	90756731	C	NA
6	*HLA-DOA;BRD2*	rs206769	32961104	T/C	0.211
6	*HLA-DOA*	rs3128947	32965062	G/A	0.179
6	*HLA-DOA*	rs12216336	32967741	C/G	0.306
6	*HLA-DOA*	rs12190787	32967914	C/G	0.306
6	*HLA-DOA*	rs2395300	32968276	A/G	0.305
6	*HLA-DOA*	rs2894311	32968339	C/G	0.067
6	*HLA-DOA*	rs2395301	32968693	T/C	0.306
6	*HLA-DOA*	rs12199692	32968929	G/T	0.305
6	*HLA-DOA*	rs172274	32969457	T/C	0.325
6	*HLA-DOA*	rs206762	32970450	G/A	0.466
6	*HLA-DOA*	rs3130602	32972207	T/C	0.020
6	*HLA-DOA*	rs3129304	32973743	C/T	0.142
6	*HLA-DOA*	rs3129303	32973878	C/T	0.139
6	*HLA-DOA*	rs2581	32974401	T/G	0.439
6	*HLA-DOA*	rs399604	32975014	C/T	0.426
6	*HLA-DOA*	rs365066	32975257	T/C	0.426
6	*HLA-DOA*	rs381218	32977420	T/G	0.255
6	*HLA-DOA*	rs429916	32978587	A/C	0.084
6	*HLA-DOA*	rs1367731	32985199	T/C	0.164
6	*HLA-DOA*	rs423639	32987774	T/C	0.073

6	*PARK2*	rs17573347	161981289	G/T	0.062
6	*PARK2*	rs17651866	161989970	G/A	0.062
6	*PARK2*	rs992037	162001436	T/C	0.354
6	*PARK2*	rs16893009	162169954	C/T	0.009
6	*PARK2*	rs9295167	162221296	G/A	0
6	*PARK2*	rs3016539	162236075	C/T	0.124
6	*PARK2*	rs6930532	162967096	C/T	0.145
6	*PARK2*	rs2077934	163057269	T/C	0.245
12	*LRRK2*	rs28903073	40653510	A/G	0.003
12	*LRRK2*	rs34995376	40704237	G	NA
12	*LRRK2*	rs2404834	40729007	T/C	0.162
12	*LRRK2*	rs34637584	40734202	A/G	0
12	*LRRK2*	rs10784522	40740365	T/G	0.343
12	*LRRK2*	rs10878405	40742254	A/G	0.343
12	*HIP1R;VPS37B*	rs7306948	123345347	G/A	0.069
17	*MGC57346;C17orf69*	rs393152	43719143	G/A	0.232
17	*MAPT*	rs17564780	44005413	G/A	0.231
17	*MAPT*	rs2316784	44021699	T/G	0.231
17	*MAPT*	rs1800547	44051846	G/A	0.231
17	*MAPT;STH*	rs8070723	44081064	G/A	0.230

Table 5. 64 SNPs included in our case-control genotyping study. (A1: Allele 1, A2: Allele 2, BP: Base pair, SNP: Single nucleotide polymorphism, MAF: minor allele frequency).

c) Genotyping

Genomic DNA was isolated and purified from peripheral blood leucocytes or saliva as described in Chapter III. DNA quantification and quality control tests were carried out using Nanodrop.

Genotyping of selected SNPs was performed using TaqMan OpenArray technology (Thermo Fisher Scientific, Waltham, MA, USA) and subsequent allele assignation using the software Taqman genotyper v1.2 (Thermo Fisher Scientific).

Briefly, this technology utilizes a microscope slide–sized plate with a great number of throughholes. Each TaqMan® OpenArray® Genotyping Plate contains 3,072 through-holes arranged in 48 subarrays of 64 through-holes each **(Figure 26)**. Each through-hole is 300 µm in diameter with a depth of 300 µm. Proprietary processes are used to coat the surfaces of the plates so that they are hydrophobic, while rendering the interiors hydrophilic and biocompatible. When processed, each of the 3,072 through-holes contains 33 nL of fluid held in place by means of surface tension **(Figure 26).**

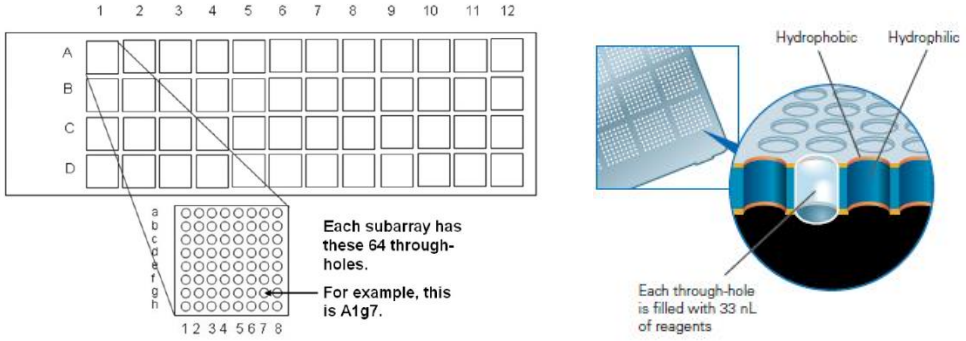


Figure 26. 64 SNPs TaqMan® OpenArray format used in our study. Cross-section of several through-holes present in the array. *(Reproduced from Thermo Fisher Scientific)*

This array can be customised and allows to screen 64 SNPs per sample at the same time. It is recommended screening up to 46 samples including 2 negative controls. The technical basis of the genotyping experiment concurs with an "allelic discrimination experiment". It is an endpoint experiment used to determine the genotype of unknown samples differentiating two alleles of a certain SNP. In TaqMan® genotyping experiments, the PCR includes a specific fluorescent-dye-labeled probe for each allele of the target SNP.

The probes contain different fluorescent reporter dyes to differentiate each allele (usually VIC™ and FAM™). Therefore, a genotyping experiment determines if unknown samples are homozygous or heterozygous for a certain allele.

Each assay contains:

- A reporter dye at the 5′ end of each probe: VIC ™ dye is linked to the 5′ end of the allele 1 probe and FAM ™ dye is linked to the 5′ end of the allele 2 probe, a forward primer and a reverse primer

- A minor groove binder which increases the melting temperature of probes without increasing probe length, thereby allowing the design of shorter probes. Consequently, the TaqMan minor groove binder probes exhibit greater differences in melting temperature values between matched and mismatched probes; greater differences in Tm values provide accurate genotyping.

- A non-fluorescent quencher at the 3′ end of the probe. Because the quencher does not fluoresce, real-time PCR systems can measure reporter dye contributions accurately.

During PCR, each probe anneals specifically to its complementary sequence between the forward and reverse primer sites. The DNA polymerase can cleave only probes that hybridize to their specific SNP allele (match). Cleavage separates the reporter dye from the quencher dye, substantially increasing fluorescence of the reporter dye. Thus, the fluorescence signals generated during PCR amplification indicate the alleles that are present in the sample **(Figure 27).**

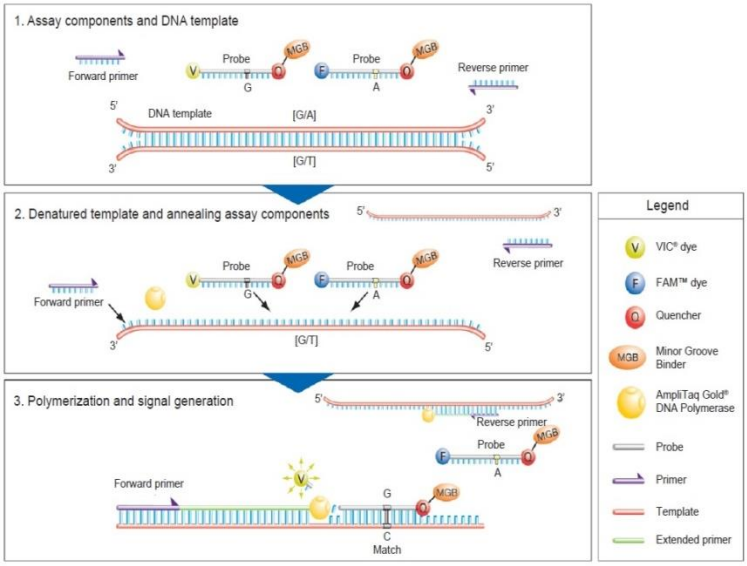


Figure 27. Genotyping process with TaqMan® OpenArray probes *(Reproduced from Thermo Fisher Scientific)*

The following figure illustrates results from matches and mismatches between target and probe sequences in TaqMan SNP Genotyping Assays. A mismatch between a probe and a SNP allele greatly reduces the efficiency of probe hybridization. Furthermore, the DNA polymerase is likely to displace the mismatched probe rather than to cleave it to release reporter dye. In other words, matches generate signal; mismatches do not generate signal.

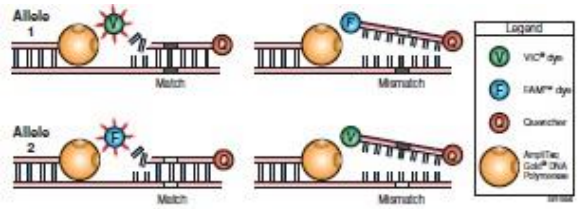


Figure 28. Example of match and mismatch of different TaqMan® OpenArray probes *(Reproduced from Thermo Fisher Scientific)*

d) Statistical analysis

To guarantee the genotyping quality, each array included negative controls. Stringent quality control criteria were applied to both SNP and individual data using PLINK 1.07 (Purcell et al., 2007). SNPs were excluded if they had a missing genotype rate > 10 % or showed departure from the Hardy-Weinberg equilibrium (HWE) ($p<0.01$). Individuals with genotypic data showing a missing rate > 10 % were also excluded for subsequent analysis. After quality control procedures, 64 SNPs and 487 individuals were finally included in the study.

Each SNP was assessed for HWE in patients and controls separately by using PLINK 1.07. The same statistical package was used to test the association between PD and the genotyping data by a logistic regression assuming an additive model adjusted by sex and age.

The number of effective-independent tests performed was calculated with the single nucleotide polymorphism spectral decomposition (SNPSpD) method. This method corrects for non-independence of SNPs in linkage disequilibrium (LD) with each other (Nyholt et al., 2004). The effective number of independent marker loci was 41, and the experiment-wide significance threshold required to keep type I error rate at 5 % was $1x10^{-3}$.

We further estimated the haplotype frequencies with the standard expectation-maximization algorithm and performed simple tests of association based on the distribution of probabilistically inferred set of haplotypes in cases versus controls. Haplotype- specific odds ratio (OR) and 95 % CI was calculated. Statistical significance was set at $p < 0.05$ for haplotype association analysis.

4. RESULTS

Information on demographic and clinical characteristics is summarized:

	Cases	Controls
Age	61.19 ± 12	60.1 ± 11.6
F: M	43 : 70	154 : 220

H&Y score	n (%)	F:M	AAO	DD
I	29(25.6)	10:19	62.38±12.12	35.34±29.8
II	50(44.3)	20:30	63.84±9.99	52.14±41.34
III	28(24.8)	9:19	55.58±11.90	126.85±86.77
IV	6(5.3)	4:02	59.50±15.41	153.17±120.40

Table 6. Demographic and clinical characteristics of the Spanish cohort. F:M: Female, Male. AAO: Age at onset, DD: Disease duration (months), H&Y: Hoehn and Yahr score (index of disease severity).

None variants deviated significantly from HWE in both groups under study. SNPs in LD ($r^2 > 0.8$) are summarized as follows:

			Distance	R^2	D Prime
SNCA	rs356204	rs2736990	14999	1.000	1.000
	rs356220	rs356219	3739	1.000	1.000
	rs3857052	rs11931074	4342	1.000	1.000
	rs7684318	rs3857052	11146	1.000	1.000
	rs7684318	rs11931074	15488	1.000	1.000
GAK	rs11248051	rs1564282	6019	1.000	1.000
GBAP1;GBA	rs2990245	rs2049805	2482	1.000	1.000
	rs2990245	rs914615	21570	0.872	1.000
	rs2990245	rs2066981	25083	0.842	1.000
	rs914615	rs2066981	3513	0.966	1.000
	rs914615	rs2049805	19088	0.872	1.000
	rs2066981	rs2049805	22601	0.842	1.000

			Distance	R^2	D Prime
LRRK2	rs10784522	rs10878405	1889	0.965	1.000
GBA;FAM189B	rs11264345	rs9628662	6783	1.000	1.000
	rs11264345	rs1045253	11889	0.928	1.000
	rs9628662	rs1045253	5106	0.928	1.000
PARK2	rs17573347	rs17651866	8681	1.000	1.000

Table 7. SNPs in LD ($r^2 > 0.8$)

Two out of the sixty-four SNPs analyzed were significantly associated with PD after adjustment **(Table 8).** We showed that rs2736990 and rs356204 in the *SNCA* gene were related to PD. A trend towards association was detected for *SNCA* rs356219, a tagging SNP in LD with the last two variants of the *SNCA* gene, and for the intronic *ACMSD* rs2166480 variant. We could not establish any other association with PD for the remaining tested variants.

Although rs28903073 (*LRRK2*), rs11248051 (*GAK*), rs356220 (*SNCA*), rs1564282 (*GAK*) and rs6749447 (*STK39*) were significantly associated with PD in our unadjusted model, they did not surpass the stringent threshold.

Table 8: Results of association analysis of the top SNPs and PD

CHR	SNP	Gene	BP	A1/A2	OR (95%CI)	L95	U95	p-add (UNADJ)	Cases	Controls
*4	rs2736990	*SNCA*	40653510	A/G	1.85	1.35	2.56	0.0001491	0.5825	0.4234
*4	rs356204	*SNCA*	90663542	T/C	1.75	1.29	2.39	0.0003334	0.5664	0.4236
4	rs356219	*SNCA*	90637601	G/A	1.8	1.26	2.57	0.001148	0.48	0.3259
2	rs2166480	*ACMSD*	135637338	G/A	1.69	1.23	2.33	0.001317	0.455	0.3425
12	rs28903073	*LRRK2*	90678541	G/A	10.16	2.11	53.80	0.004209	0.02703	0.002695
4	rs11248051	*GAK*	858332	T/C	1.9	1.18	3.09	0.00889	0.1339	0.07609
4	rs356220	*SNCA*	90641340	T/C	1.52	1.16	3.01	0.01588	0.4545	0.3411
4	rs1564282	*GAK*	852313	T/C	1.86	1.08	2.15	0.01027	0.1372	0.07995
2	rs6749447	*STK39*	169041386	G/T	1.369	0.99	1.89	0.05896	0.3839	0.3147

CHR: Chromosome, SNP: Single nucleotide polymorphism, BP: Base pair position, A1/A2: Alleles, OR: Odds ratio, 95%CI: 95% Confidence interval, L95, U95 :lower/upper intervals, p-add (UNADJ):unadjusted p value under an additive model.

* SNPs significantly associated with PD after adjustment for the SNP spectral decomposition method ,** OR values refer to Allele A1.

Haplotype analyses identified the *GBA* haplotypes TTGG and CCAA (rs2990245-rs2049805-rs914615-rs206698) to be significantly overrepresented in PD cases versus controls **(Table 9)** (*GBA*- TTGG; OR= 2.6, 95% CI [1.1-6.3], p= 4.1 x 10^{-11}; *GBA*-CCAA; OR= 7.7, 95% CI [2-29.3], p=1.2 x 10^{-8}.). Similarly, the *SNCA* haplotype TGGT (rs356204-rs356219-rs2736990-rs356220) was found to increase significantly the risk for PD in our cohort (OR=1.4, 95% CI [1- 2], p= 6 x 10^{-4}) **(Table 9).** This cohort was previously sequenced for the *GBA* exons 8-11, identifying a total of 5 different pathogenic mutations among 19 patients (Bandrés-Ciga et al., 2016). We performed extra analyses to explore the relationship between the occurrence of *GBA* mutations and the two *GBA* risk haplotypes (TTGG and CCAA) reported in the current work. Our results showed that the two *GBA* risk haplotypes are not overrepresented in *GBA* mutation carriers in comparison with non-carriers (TTGG: χ^2 = 1.5, p = 0.21; CCAA: χ^2 = 0.24, p = 0.62).

Locus	Haplotype	F_A	F_U	OR	CI	p-value	SNPs
SNCA	TGGT	0.45	0.32	1,4	(1-2)	0.0006	A
GBA	TTGG	0.08	3.2E-02	2.6	(1.1-6.3)	4,1E-11	B
	CCAA	0.06	1.4E-02	7.7	(2-29.3)	1,2E-08	C

Table 9. Estimated *SNCA/GBA* haplotype frequencies in cases and controls. F_A: Freq in cases; F_U: Freq in controls; OR: Odds ratio; 95 % CI: 95 % Confidence Interval; SNPs: Single nucleotide polymorphisms forming the different haplotypes. A: rs356204, rs356219, rs2736990, rs356220; B: rs2990245, rs2049805, rs914615, rs2066981; C: rs2990245, rs2049805, rs914615, rs2066981.

V. Genome wide assessment of Parkinson's disease in a Southern Spanish population

1. ABSTRACT

Here, we set out to study the genetic architecture of Parkinson's disease (PD) through a GWAS in a Southern Spanish population.

About 240 PD cases and 192 controls were genotyped on the NeuroX array. We estimated genetic variation associated with PD risk and age at onset (AAO). Risk profile analyses for PD and AAO were performed using a weighted genetic risk score (GRS). Total heritability was estimated by genome-wide complex trait analysis. Rare variants were screened with single-variant and burden tests. We also screened for variation in known PD genes. Finally, we explored runs of homozygosity and structural genomic variations.

We replicate PD association (uncorrected p-value < 0.05) at the following loci: *ACMSD/TMEM163, MAPT, STK39, MIR4697* and *SREBF/RAI1.* Subjects in the highest GRS quintile showed significantly increased risk of PD versus the lowest quintile (OR=3.6, p-value < $4e^{-7}$), but no significant difference in AAO. We found evidence of runs of homozygosity in two PD-associated regions: one intersecting the *HLA-DQB1* gene in six patients and one control; and another intersecting the *GBA-SYT11* gene in one PD case. The *GBA* N370S and the *LRRK2* G2019S variants were found in 8 and 7 cases respectively, replicating previous work. A structural variant was found in one case in the *PARK2* gene locus.

This current work represents a comprehensive assessment at a genome-wide level characterizing a novel population in PD genetics.

2. INTRODUCTION

Over the last several years, genetic investigation of PD has successfully identified many disease-causing mutations (Polymeropoulos et al., 1997, Kitada et al., 1998, Paisán-Ruiz et al., 2004, Zimprich et al., 2004, Bonifati et al., 2003, Valente et al., 2004, Zimprich et al., 2011, Vilarino-Guell et al., 2011) providing considerable insight into the molecular mechanisms underlying the etiology and pathogenesis of this multifactorial and complex disease.

Importantly, GWAS (Edwards et al., 2010, Hernandez et al., 2012, Pihlstrom et al., 2013, Saad et al., 2011, Satake et al., 2009, Simón-Sánchez et al., 2009, Simón-Sánchez et al., 2011) and large scale meta-analyses (Nalls et al., 2014, Nalls et al., 2011, Do et al., 2011, International Parkinson's Disease Genomics Consortium and Welcome Trust Case Control Consortium 2, 2011, Lill et al., 2012, Pankratz et al., 2012) have been applied to identify and replicate risk loci that fit the common disease, common variant hypothesis in PD (Reich et al., 2001). Despite the significant number of novel risk loci identified so far, only a small portion of the heritable component for PD has been explained, suggesting there is a substantial unknown genetic component to be discovered (Keller et al., 2012). The polygenic nature of the illness and the hypothetically large number of loci involved with risk variants of small effect size make analysis of the genetic contribution to disease phenotype particularly difficult.

It has been widely suggested that studies in populations with limited genetic heterogeneity are valuable for studying the genetic basis of disease (Hernandez et al., 2012).

Moreover, replication studies in independent populations are absolutely necessary to test the robustness of such association reports. As highlighted in previous chapters, Andalusia given its geographical location on the southernmost region of Spain, represents a cross-link between Europe and Africa. The interactions and interbreeding across the Mediterranean Sea and North Africa have contributed to shape a genetic profile which to date has been poorly studied in the context of PD genetics. Motivated by these considerations, we set out to study the genetic architecture of PD in this population by performing a genome-wide association study.

The purpose of this project was to identify novel putative candidate loci associated with PD and to investigate whether SNPs previously identified as risk variants contribute to PD risk in the Southern Spanish population. Furthermore, we use genetic risk profiling to aggregate risk across the previously established risk loci. In the same way, we aim to understand whether this genetic risk is associated with age at disease onset. We also attempt to identify recessive founder variants by exploring runs of homozygosity enriched in cases and explore whether disease-associated rare variants are present in our cohort. Finally, we estimate PD heritability attributable to genetic variation assessed by our genotyping platform and evaluate the role of CNVs as risk factors for PD in these subjects.

3. MATERIAL AND METHODS

a) Patient cohort

We included a group of 240 PD patients treated at the Movement Disorders Unit of the Service of Neurology in both Hospital Clínico San Cecilio and Hospital Virgen de las Nieves of Granada (Spain). PD was diagnosed at least by 2 experienced neurologists in the field of movement disorders following the criteria of the UK PD Society Brain Bank (Gelb et al., 1999). Our control group was comprised of 192 healthy individuals with no signs or symptoms of parkinsonism who were extensively assessed to rule out any sign of neurological condition. The study was approved by the local ethics committee, and written informed consent was taken from each participant.

b) Genotyping

Samples were genotyped using the NeuroX Array (Illumina), a powerful and reliable tool for the investigation of genetic factors associated with neurodegenerative disorders (Nalls et al., 2015a). It comprises standard Illumina exome content of approximately 240,000 variants densely covering previously published PD GWAS-associated loci, ancestry informative markers, markers for determination of identity by descent (IBD), X chromosome SNPs for sex determination and over 24,000 custom content variants focusing on rare variants related to neurodegenerative diseases. The genotyping experimental procedure began denaturing genomic DNA in 0.1N NaOH, followed by amplification at 37ºC for 20-24 hours.

Subsequently, the DNA was enzymatically sheared at 37º C for 1 hour to form fragments of around 300bp. After precipitating with 2-propanolol and resuspending in RA1 (Illumina), the DNA fragments were denatured at 95 º C for 20 minutes, dispensed upon the BeadChips using a robot, and hybridised for 16-20 hours at 48 ºC. After incubation, the BeadChips were washed to remove redundant DNA fragments and were stained allele-specifically with the robot. This was followed by a second step of washing, vacuum-drying and imaging of the BeadChips **(Figure 29).**

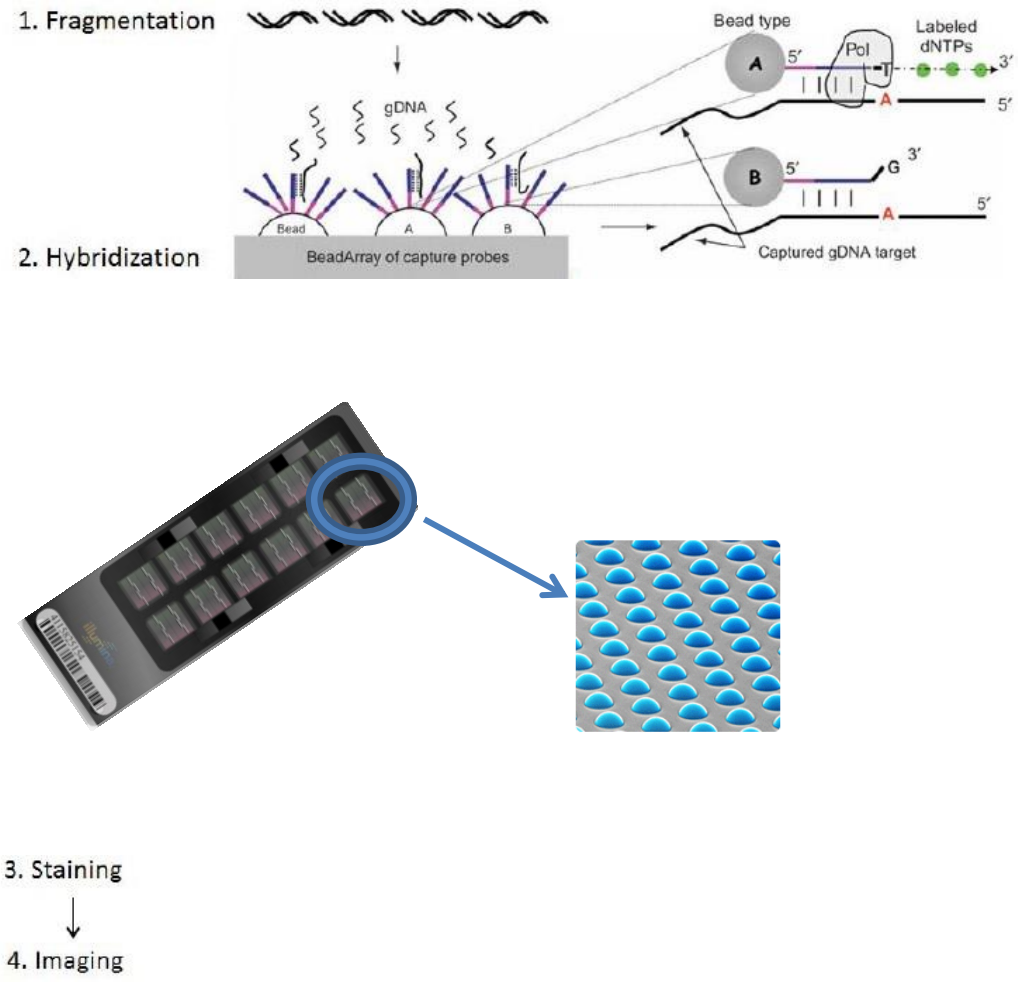


Figure 29. Genotyping process. *(Reproduced and modified from Gunderson et al., 2005).*

c) Bioinformatic analysis

After genotyping, genotypes were clustered using Illumina Genome Studio using a previously developed cluster file for NeuroX (Nalls et al., 2015a). A final report was generated to create the binary files before input in plink.

- ✓ **Quality control**

Stringent QC analysis was performed using either plink (Purcell et al., 2007) or R as follows:

Exclusion criteria

- Samples with call rates of less than 95% and whose genetically determined sex from X chromosome heterogeneity did not match that from clinical data.
- Samples exhibiting heterozygosity greater than 6 standard deviations from the population mean.
- SNPs with MAF < 0.01.
- SNPs with HWE p-value < 1E-5.
- SNPs with missingness rates > 5 %.
- Palindromic SNPs.
- SNPs thought to be in LD in a sliding window of 50 adjacent SNPs which scrolled through the genome at a rate of 5 overlapping SNPs.

Next, samples were clustered using principal component analysis (PCA) to evaluate European ancestry as compared to the HapMap3 CEU/TSI populations (International HapMap Consortium, 2003).

Confirmed European ancestry samples were extracted and principal components (PCs) 1 and 2 were used as covariates in all analysis. Those samples cryptically related at the level of first cousins or closer (sharing proportionally more than 12.5 % of alleles) were dropped from the following analysis. Samples passing QC numbered 206 cases and 172 controls.

- ✓ **Genome-wide association study (GWAS)**

For all SNPs (non-LD pruned) and samples passing QC as detailed above, a logistic regression model adjusted for sex, age and first and second genotype PCs as covariates was used to estimate risk associated with the disease. A linear regression model adjusted for the same covariates was used to explore the influence of genetic variation on the AAO.

- ✓ **Genetic risk score**

Risk profile analysis for PD and AAO was performed as described in detail elsewhere (Kara et al., 2014, Nalls et al., 2015b). Briefly, a cumulative GRS was calculated incorporating 30 SNPs previously associated with PD (Nalls et al., 2014). Risk allele dosages were counted and a GRS was generated across all loci. All SNPs were weighted by their published odds ratios, giving greater weight to alleles with higher risk estimates.

The dataset was divided into quintiles based on the GRS. A regression was performed regressing disease (logistic regression) or AAO (linear regression) against quintile membership, and odds ratios were reported comparing the reference group (lowest risk quintile) to the remaining 4 quintiles. Risk profiling analysis was adjusted for PCs 1 and 2 to account for population substructure.

- ✓ **Genome complex trait analysis**

In an attempt to estimate PD heritability attributable to genome-wide assayed genetic variation, we used the genome-wide complex trait analysis (GCTA) package (Yang et al., 2011). This analysis has the ability to detect contribution to disease risk of variants which do not reach genome significance because of small effect size, though not to identify the specific variants contributing to disease risk. First, a genetic relationship matrix is calculated by estimating pairwise genetic relations between the individuals in the cohort. Then, a linear mixed model is applied to estimate phenotypic variance explained by genome-wide SNPs passing QC, correcting for PCs 1 and 2, age, and gender.

- ✓ **Identity by state comparisons**

For IBS comparisons, PLINK 1.07 was used to calculate case and control specific rates of proportional allele sharing both genome-wide and within defined segments. We used a pairwise approach to estimate overall allelic similarity between each pairing of subjects.

Then, case-control labels were permuted 2000 times to assess whether case/control pairings are more different in the actual data than in permuted data, suggesting segregation of cases and controls into distinct populations. In these analyses, cryptically related PD individuals removed in previous steps were included in order to identify shared rare recessive variants.

- ✓ **Rare variant tests**

Subsequently, we tested for association between rare variants (MAF < 0.05) and PD using a single-variant test under an additive genetic model. This approach evaluates if there is any association between each independent variant and PD, employing a significance threshold of 5×10^{-8}. The minimum minor allele count considered for the analysis was 3, and we adjusted for the top 20 PCs, age, and sex. In addition to testing each variant individually, we evaluated cumulative effects of multiple genetic variants by collapsing rare variants within a gene level using the SKAT-O algorithm at default settings as part of the EPACTS package (http://genome.sph.umich.edu/wiki/EPACTS) (Lee et al., 2012).

Moreover, we screened for variants with MAF <1% in known PD-causing genes **(Table 10),** checking all variants with two approaches: a putative dominant model (allele present only in cases and not in controls) and a putative recessive model (allele present with two copies only in cases and not in two copies in controls).

Table 10. Parkinson's disease known genes

Gene	Gene name	Model of inheritance	Reference
SNCA	Synuclein, alpha	Dominant	Polymeropoulos et al., 1997
LRRK2	Leucin-rich repeat kinase 2	Dominant	Paisán-Ruiz et al., 2004, Zimprich et al., 2004
PARK2	Parkin E3 ubiquitin protein ligase	Recessive	Kitada et al., 1998
PINK1	PTEN- induced putative kinase 1	Recessive	Valente et al., 2001
GBA	Glucocerebrosidase	Dominant*	Sidransky et al., 2009
VPS35	Vacuolar protein sorting 35 homolog	Dominant	Zimprich et al., 2011, Vilariño-Guell et al., 2011
DJ-1	Parkinson protein 7	Recessive	Bonifati et al., 2003
ATP13A2	ATPase type 13A2	Recessive**	Ramirez et al., 2006
GIGYF2	GRB10 interacting GYF protein 2	Dominant**	Lautier et al., 2008
PLA2G6	Phospholipase A2 group VI	Recessive	Morgan et al., 2006
FBXO7	F-box protein 7	Recessive	Shojaee et al., 2008
SYNJ1	Synaptojanin1	Recessive	Krebs et al., 2013, Olgiati et al., 2014, Quadri et al., 2013
DNAJC13	DnaJ Homolog Subfamily C ,Member 13	Dominant	Vilariño-Guell et al., 2014
GCH1	GTP cyclohydrolase 1	Dominant*	Mencacci et al., 2014

* Reduced penetrance

** Putative PD linked genes

- ✓ ***Structural Genomic Variation analysis***

Detection of structural genomic variations was performed using PennCNV-1.0.3 (Illumina Inc., San Diego, CA) and visualised with R. Two metrics were assessed with this utility: B allele frequency and log R ratio. These two statistics allow visualization of copy number changes and are described in detail elsewhere (Matarin et al., 2008).

Briefly, log R ratio gives an indirect measure of copy number of each SNP by plotting the ratio of observed to expected hybridization intensity. An R value above 1 is indicative of an increase in copy number (duplication or triplication), and an R value below 1 suggests a decrease (deletion). B allele frequency plots the proportion of times an allele is called A or B at each genotype: thus the expected ratios are 1.0 (B/B), 0.5 (A/B) and 0.0 (A/A). Significant deviations from these figures in contiguous SNPs are indicative of a CNV. While this metric exhibits a high level of variance for individual SNPs, it does provide a measure of CNV when log R ratio values for numerous adjacent SNPs are visualized.

4. RESULTS

a) Patient cohort

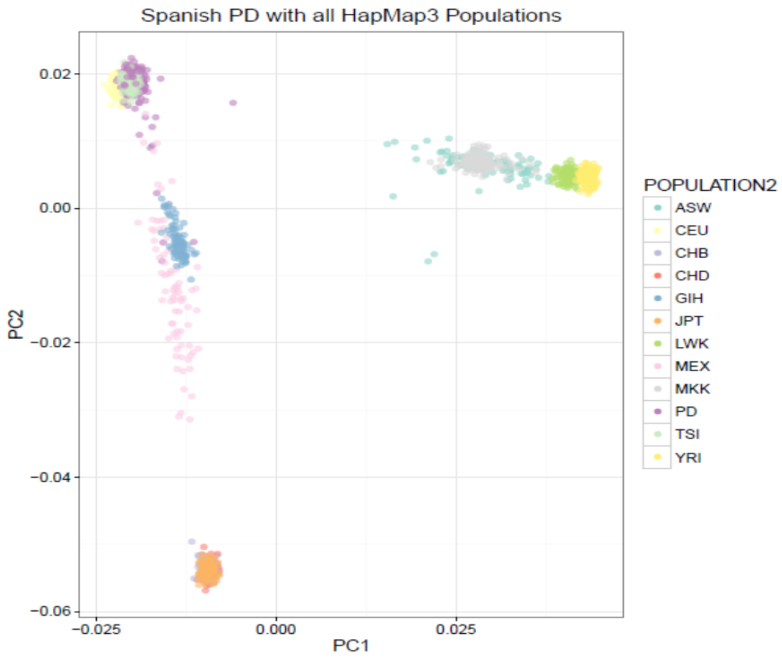
Descriptive statistics of the Spanish cohort are summarized below.

	Samples passing QC	Male/Female	*Age	*AAO
Whole sample	378	210/168	63.14 ± 13	
PD cases	206	133/73	67.38 ± 10.51	61.88 ± 13.77
Controls	172	77/95	58.07 ± 13.99	

Table 11. Demographic characteristics of the Spanish cohort. *Expressed as mean age ± standard deviation. *Age: Age at study enrolment; AAO: Age at onset

b) Bioinformatic analysis

As we can see in the following images, visualization of the top 2 genetic PCs of the Spanish samples show them to be similar overall to HapMap European populations.



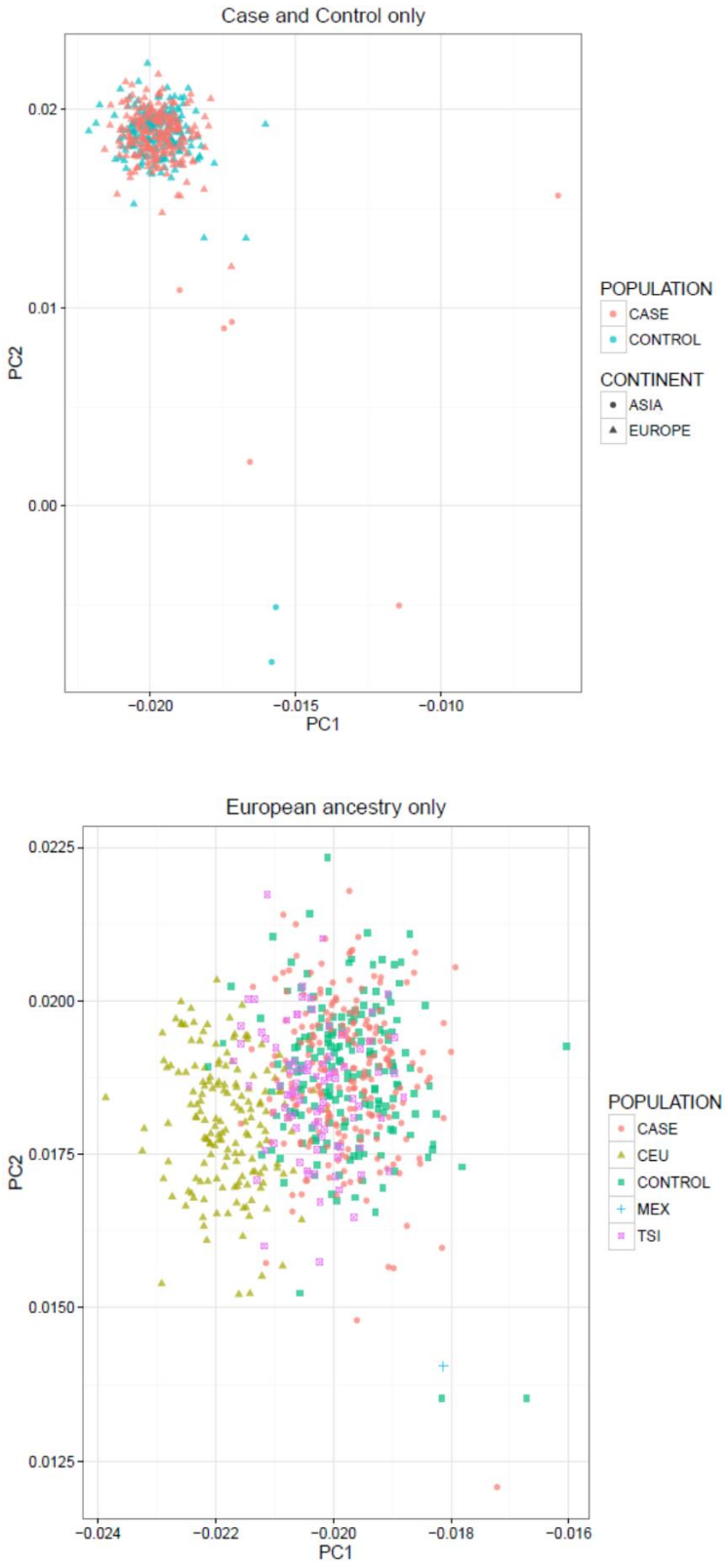


Figure 30. Spanish cohort with HapMap3 populations; Spanish cohort ancestry; Spanish cohort with European CEU/TSI/MEX populations

QQ Plots show no potential problems of population stratification as expected as the sample comes from a relatively homogeneous ancestry background (see **figures 31** and **32**).

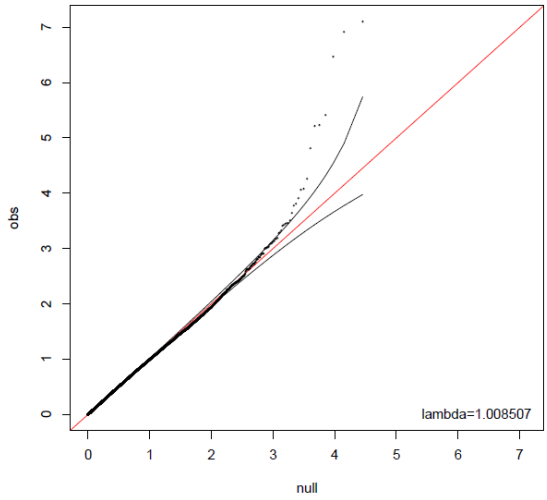


Figure 31. QQ plot of Genome-Wide association with PD risk. Genomic inflation factor = 1.008

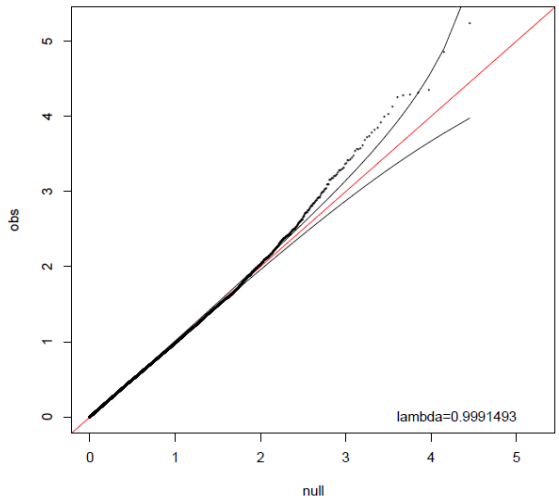


Figure 32. QQ plot of Genome-Wide association with AAO. Genomic inflation factor = 0.9914

✓ ***Genome Wide Association Study***

Genetic variants passing QC numbered 33074 SNPs, 28393 of which remained after LD pruning. Our GWA failed to detect any genetic variant significant at $p < 5 \times 10^{-8}$ **(Figure 33)**. Similarly, genome-wide assessment for AAO of PD did not reveal any genetic variant to be associated at p values $< 5 \times 10^{-8}$ **(Figure 34)**.

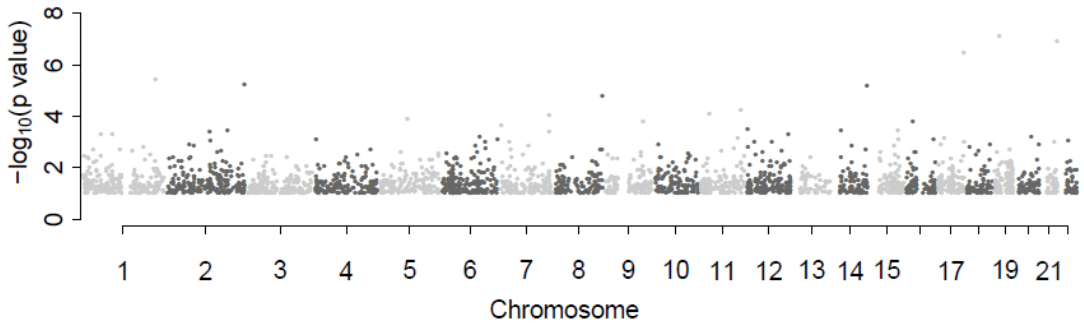


Figure 33 Manhattan Plot showing results of PD GWA testing between 206 patients and 172 controls from Southern Spain.

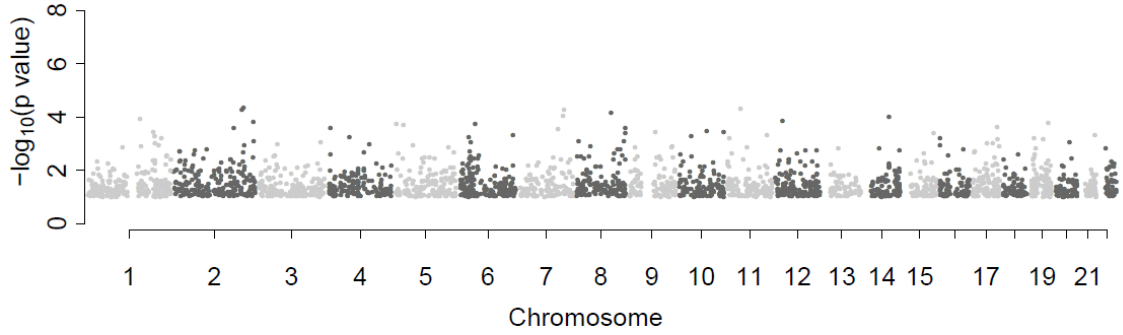


Figure 34. Manhattan Plot showing results of PD GWA with age at onset testing between 206 patients and 172 controls from Southern Spain.

Although we did not show any association signal with PD at this stringent p-value, we managed to marginally replicate association on the basis of a nominal uncorrected p-value < 0.05 with the following loci previously identified in a large-scale PD GWAS meta-analysis: *ACMSD/TMEM163, MAPT, STK39, MIR4697* and *SREBF/RAI1* **(Table 12)** (Nalls et al., 2011).

Table 12. Results of the replication association analyses

Nearest gene (s)	SNP	CHR	BP	A1	A2	MAF	OR	SE	L95	U95	STAT	p	OR	U95a	L95a
ACMSD/TMEM163	rs6430538	2	135539967	C	T	0.4112	1.533	0.1865	1.064	2.21	2.291	0.02195	1.14	1.10	1.18
MAPT	rs17649553	17	43994648	T	C	0.3073	0.6318	0.2055	0.4224	0.9452	2.234	0.02547	0.77	0.74	0.80
STK39	rs1955337	2	169129145	T	G	0.1789	2.346	0.4031	1.065	5.169	2.115	0.03442	1.20	1.15	1.26
MIR4697	rs329648	11	133765367	T	C	0.4399	1.443	0.1736	1.027	2.028	2.113	0.03458	1.09	1.06	1.13
SREBF/RAI1	rs11868035	17	17715101	A	G	0.2917	0.6581	0.2094	0.4365	0.992	1.998	0.0457	0.93	0.90	0.96
SNCA	rs356181	4	90626139	C	T	0.4505	1.303	0.1633	0.9463	1.795	1.622	0.1049	0.90	0.87	0.93
RIT2	rs12456492	18	40673380	G	A	0.3216	1.349	0.1894	0.9306	1.955	1.58	0.1141	1.10	1.06	1.14
SIPA1L2	rs10797576	1	232664611	T	C	0.1589	2.111	0.5017	0.7898	5.644	1.49	0.1363	1.13	1.08	1.19
RAB7L1/NUCKS1	rs823118	1	205723572	C	T	0.3838	1.285	0.1848	0.8947	1.846	1.358	0.1745	0.88	0.86	0.91
CCDC62	rs11060180	12	123303586	G	A	0.3945	0.8088	0.1683	0.5815	1.125	1.26	0.2075	0.90	0.87	0.93
TMEM175/GAK/DGKQ	rs34311866	4	951947	C	T	0.2055	1.356	0.2974	0.7569	2.429	1.024	0.306	1.27	1.22	1.32
SNCA	rs3910105	4	90682571	C	T	0.4413	0.8579	0.1632	0.623	1.181	0.9392	0.3476	0.89	0.86	0.91
GPNMB	rs199347	7	23293746	C	T	0.4661	0.8747	0.1551	0.6454	1.185	0.8631	0.3881	0.89	0.86	0.92
FAM47E/SCARB2	rs6812193	4	77198986	T	C	0.3789	1.153	0.1794	0.8113	.639	0.7943	0.427	0.89	0.86	0.93
MCCC1	rs12637471	3	182762437	A	G	0.1758	0.7834	0.3182	0.4199	1.462	0.7672	0.443	0.84	0.81	0.87
DDRGK1	rs55785911	20	3153503	A	G	0.373	0.8852	0.1766	0.6262	1.251	0.6905	0.4899	0.91	0.88	0.94
VPS13C	rs2414739	15	61994134	G	A	0.2656	0.8594	0.2269	0.5509	1.341	0.6681	0.5041	0.89	0.86	0.93
LRRK2	rs7294619	12	40614434	T	C	0.1462	0.8173	0.3631	0.4012	1.665	0.5558	0.5783	0.86	0.82	0.90
BCKDK/STX1B	rs14235	16	31121793	A	G	0.4232	0.9306	0.1643	0.6745	1.284	0.4377	0.6616	1.09	1.05	1.13
FGF20	rs591323	8	16697091	A	G	0.2546	0.9082	0.2761	0.5287	1.56	0.3488	0.7273	0.92	0.88	0.95
BST1	rs11724635	4	15737101	C	A	0.4792	0.9513	0.1622	0.6923	1.307	0.3076	0.7584	0.89	0.86	0.91
GCH1	rs11158026	14	55348869	T	C	0.2865	1.04	0.1997	0.7028	1.538	0.1946	0.8457	0.88	0.85	0.92
HLA_DBQ1	rs115462410	6	32666660	T	C	0.09245	1.116	0.7542	0.2546	4.895	0.1459	0.884	1.25	1.17	1.34
TMEM175	rs34884217	4	944210	G	T	0.09635	0.9088	0.6654	0.2467	3.348	0.1437	0.8857	0.74	0.68	0.80
GBA	rs35749011	1	155205634	C	T	0.005222	NA	NA	NA	NA	NA	NA	0.56	0.50	0.63
GBA/SYT11	rs71628662	1	155359992	C	T	0.005208	NA	NA	NA	NA	NA	NA	0.56	0.50	0.63
NOTCH	rs8192591	6	32185796	T	C	0.009115	NA	NA	NA	NA	NA	NA	1.11	1.02	1.21
INPP5F	rs10886515	10	121710488	T	C	0.0248	NA	NA	NA	NA	NA	NA	1.10	1.06	1.14

Risk estimates are based on the dosage of A1 (minor allele). MAF refers to the minor allele.
*Values referring to the PD metanalisis *(Nalls MA et al., 2014)*

✓ ***Genetic Risk Score***

Our cumulative risk attributable to all known GWAS loci associated with PD showed significantly different GRS in this cohort (p-value < 4 x 10^{-7}). Individuals in the highest quintile of genetic risk were found to be 3.6 times more likely to develop PD than those in the lowest quintile **(Table 13), (Figure 35).** No significant association was found between GRS and AAO of disease (p=0.52).

	Risk quintile				
	First	**Second**	**Third**	**Fourth**	**Fifth**
Odds ratio	1	1.61	1.83	3.44	3.65
CI (95%)		(0.85-3.08)	(0.96-3.52)	(1.79-6.76)	(1.89-7.21)
p-value	4.0E-07				
AUC	0.63				

Table 13. Genetic risk profile in the Southern Spanish cohort based on SNPs in Table 12. AUC: Area under the curve. CI: Confidence interval.

The area under the curve (AUC), calculated based on a logistic regression of disease status vs. GRS was 0.638 **(Figure 35).**

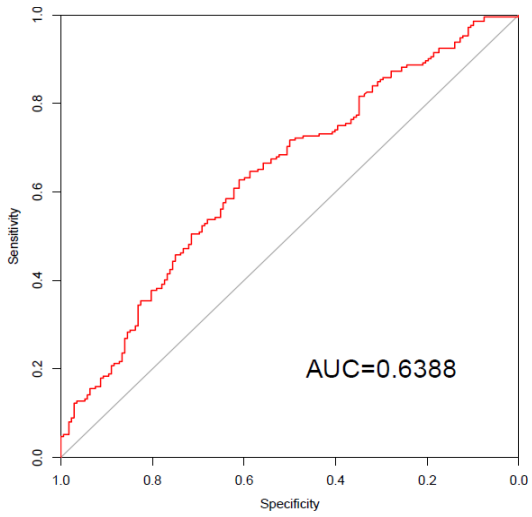


Figure 35. Cumulative Risk Score Prediction of PD. Receiver operator curve analyses. AUC: Predictive area under the curve.

- ✓ ***Genome Complex Trait Analysis***

Using GCTA, we estimated total heritability explained by the genotyped SNPs on the NeuroX array to be 13.7 % ± 40% (0-92% at 95% CI). Our NeuroX array is more focused on a lower number of exonic variants compared to earlier analyses, which may contribute to the lower estimate reported here (Keller et al., 2012). The lack of precision in this estimate likely reflects the low power of the current study, and thus the results of this heritability analysis should be viewed with caution.

- ✓ ***Identity by state comparisons***

In a genome-wide assessment of differential IBS between cases and controls, we found no evidence of excess of homozygosity in all cases vs. all controls. However, we found evidence of runs of homozygosity in two regions previously associated with PD: one intersecting the *HLA-DQB1* gene in six patients and one control; and another intersecting the *GBA-SYT11* gene in one PD case. A run of at least 98.5% homozygosity from chr6:32,333,827-32,680,928 (~347 kb) was consistently reported across subjects showing homozygosity in the *HLA-DQB1* locus. This region directly overlaps the previously reported GWAS hit (Nalls et al., 2014). Our single case with a run of homozygosity in *GBA-SYT11* exhibited a 99.6% homozygous run of 11233 kb (chr1:149,906,413-161,139,738).

✓ ***Rare variant tests***

Single-variant tests could not identify any prospective novel rare variant associated with PD **(Figure 36)**.

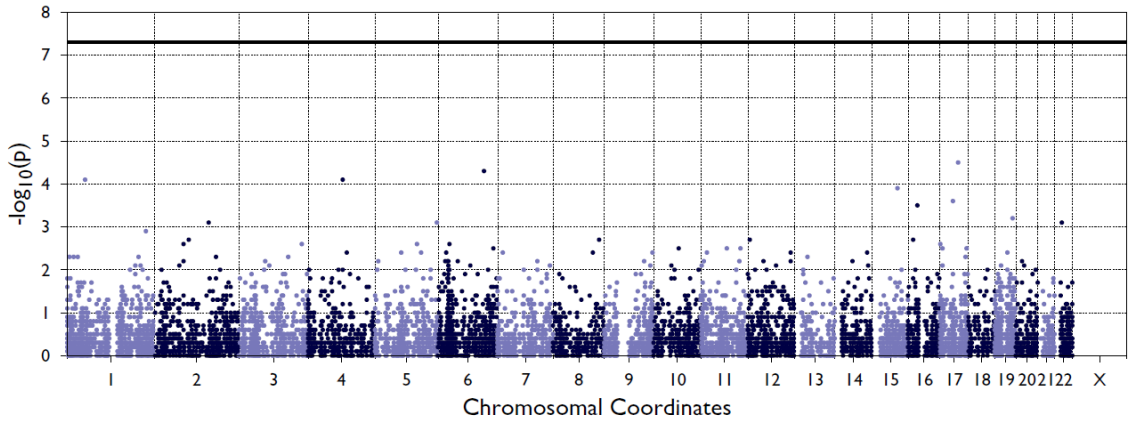


Figure 36. Single rare variant test against disease phenotype

When focusing on the cumulative effects of multiple genetic rare variants by grouping them by a gene level we also detect no significant association with PD **(Figure 37, Figure 38, Figure 39)**.

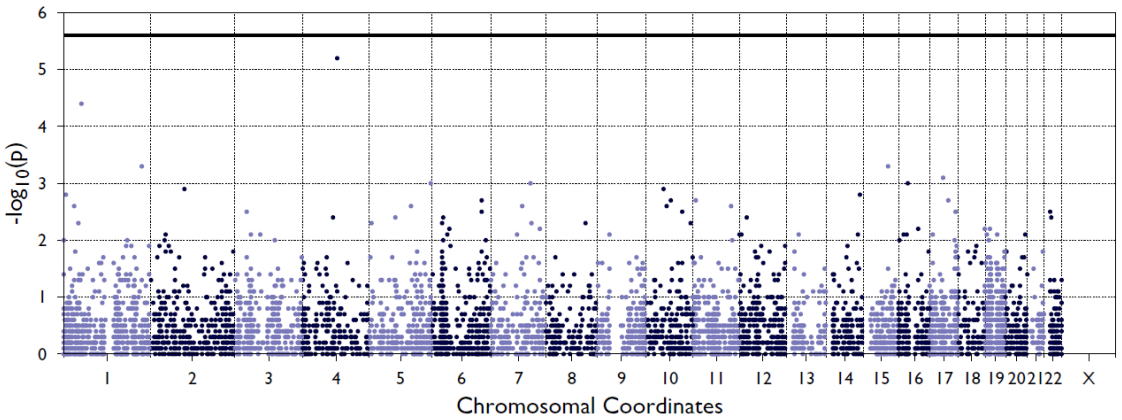


Figure 37. Additive effects of multiple genetic rare variants by Burden test at a by-gene level. Our closest hit not surpassing the GWA cut-off was in the *ADHB1* gene at Chr.4.

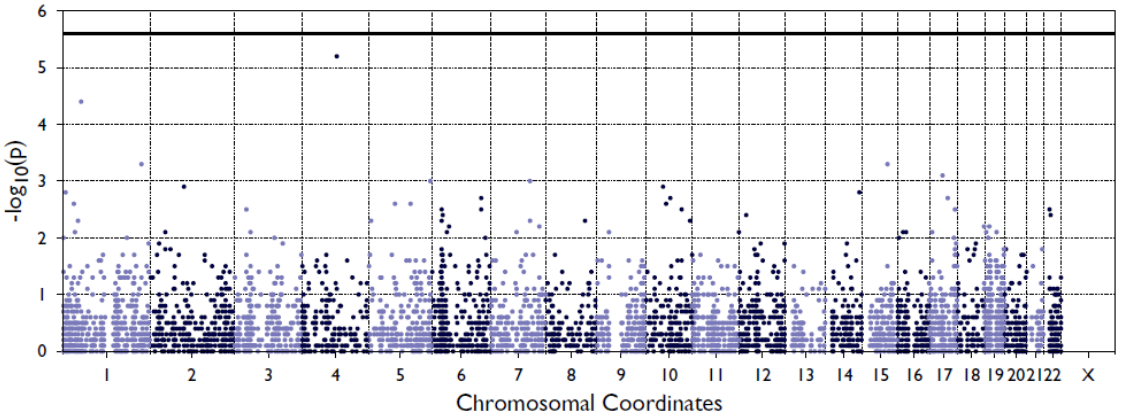


Figure 38. Additive effects of multiple non-synonymous rare variants by Burden test at a by gene level.

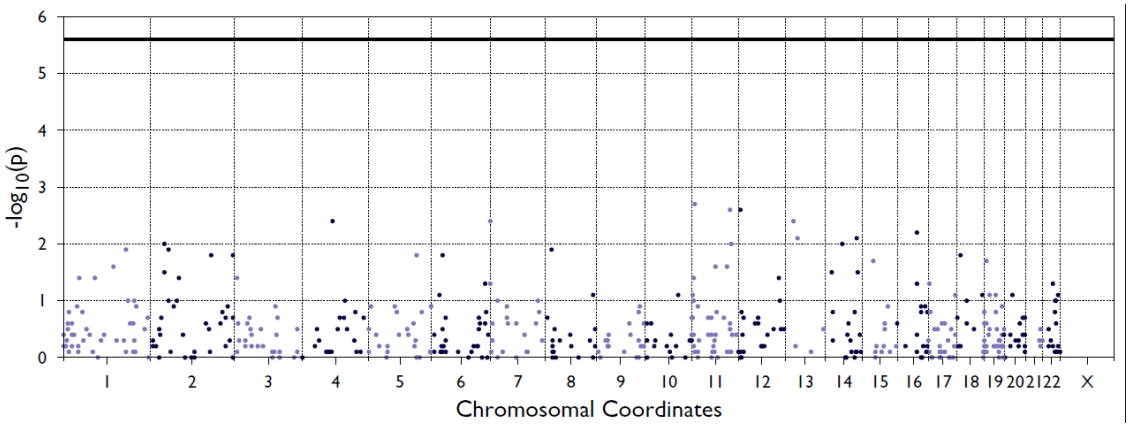


Figure 39. Additive effects of multiple synonymous rare variants by Burden test at a by gene level.

However, we explored rare variants in PD known genes present in cases but absent in our controls **(Table 14).** The *GBA* N370S risk variant was found in 8 cases (7 heterozygous and 1 homozygous) and the *LRRK2* G2019S pathogenic mutation was identified in 7 patients, all in heterozygosis **(Figure 40).**

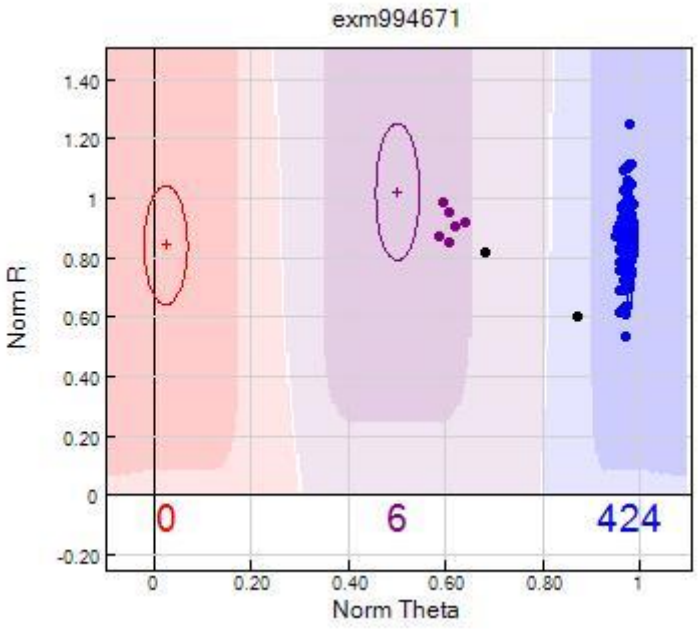


Figure 40. Cluster Plot showing *LRRK2* G2019S pathogenic mutation genotypes (*Generated from Genome Studio).* Purple dots: Heterozygous carriers for the risk allele A. Blue dots: Homozygous carriers for the non-risk allele G.

Variants of unknown pathogenicity were also detected in *PINK1* (Valente et al., 2004), *PARK2* (Bonifati et al., 2003), *GIGYF2* (Lautier et al., 2008), *DNAJC13* (Vilarino-Guell et al., 2014), *SYNJ1* (Krebs et al., 2013), and *FBXO7* (Shojaee et al., 2008).

Table 14. Genetic rare variants identified in PD known genes in cases but not in controls

CHR	Gene	BP	NeuroX	SNP	Variant	Z	R/A	Cases	Frequency (ExAc)	SIFT	Polyphen
1	*PINK1*	20971158	exm27115	rs139226733	p.Met318Leu	Het	A/T	1	0.001618	D	B
1	*PINK1*	20975021	exm27131	rs45515602	p.Ala383Thr	Het	G/A	2	0.0001961	B	T
1	*GBA*	155205634	exml06217	rs76763715	p.Asn370Ser	Het	T/C	7	0.00363	B	D
						Hom		1			
2	*GIGYF2*	233612427	exm275808	rs147623346	p.Met48lle	Het	G/T	2	0.0001499	D	U
2	*GIGYF2*	233655834	exm275883	rs148277228	p.Asp371Glu	Het	T/A	1	0.0007366	T	U
2	*GIGYF2*	233656142	exm275892	rs34845648	p.Pro445His	Het	C/T	1	0.0001132	D	U
3	*DNAJC13*	132213932	exm351306	rs35658317	IVS5-1G>A	Het	G/A	1	0.0003347	-	-
3	*DNAJC13*	132242012	exm351379	rs61731474	p.Asn1952Thr	Het	A/C	1	0.0001349	B	T
3	*DNAJC13*	132244562	exm351399	rs138693725	p.Ala2057Ser	Het	G/T	1	0.001334	B	T
6	*PARK2*	184045771	exm593521	rs9456735	p.Met192Leu	Het	T/G	1	0.0003299	T	PDG
12	*LRRK2*	40629436	exm994436	rs33995463	p.Leu119Pro	Het	T/C	2	0.00202	D	PDG
12	*LRRK2*	40677813	exm994520	rs35173587	p.Arg793Met	Het	G/T	1	0.005747	D	PDG
12	*LRRK2*	40697802	exm994561	rs143710836	p.Ala1215Thr	Het	G/A	1	0.0002101	T	B
12	*LRRK2*	40734202	exm994671	rs34637584	p.Gly2019Ser	Het	G/A	7	0.0006294	PD	D
21	*SYNJ1*	34004111	exm1566662	rs115648918	p.Val1345lle	Het	C/T	3	0.0001713	T	B
21	*SYNJ1*	34012088	exm1566685	rs145937537	p.Thr1236Met	Het	G/A	1	0.00216	T	B
21	*SYNJ1*	34029195	exm1566728	rs114053718	p.lle905Thr	Het	A/G	1	0	D	PDG
21	*SYNJ1*	34058146	exm1566794	rs114942253	p.Ala383Thr	Het	C/T	2	0.01499	B	B
22	*FBX07*	32875122	exm1602819	rs143041875	p.Ser93Ala	Het	T/G	3	0.0001498	D	B
22	*PLA2G6*	38528888	exm1608133	rs11570680	p.Ala343Thr	Hom	C/T	1	0.02574	T	B

B:Benign, D:Damaging/Deleterious, PD: possibly damaging, T: tolerated, U:Unkn own;
Frequency ExAc: Frequency of the rare variants reported for European (Non Finish) population. R: Reference allele; A: Alternate allele
** Putative PD linked genes

✓ ***Structural Genomic Variation analysis***

In addition to providing SNP variation data, the genotyping assay used generates metrics that allow detection of CNV. We evaluated both the log R ratio and the B allele frequency plots across the genome in all samples and identified a *PARK2* heterozygous deletion (Chr6: 162,206,852-162,206,882) in a PD case **(Figure 41)**.

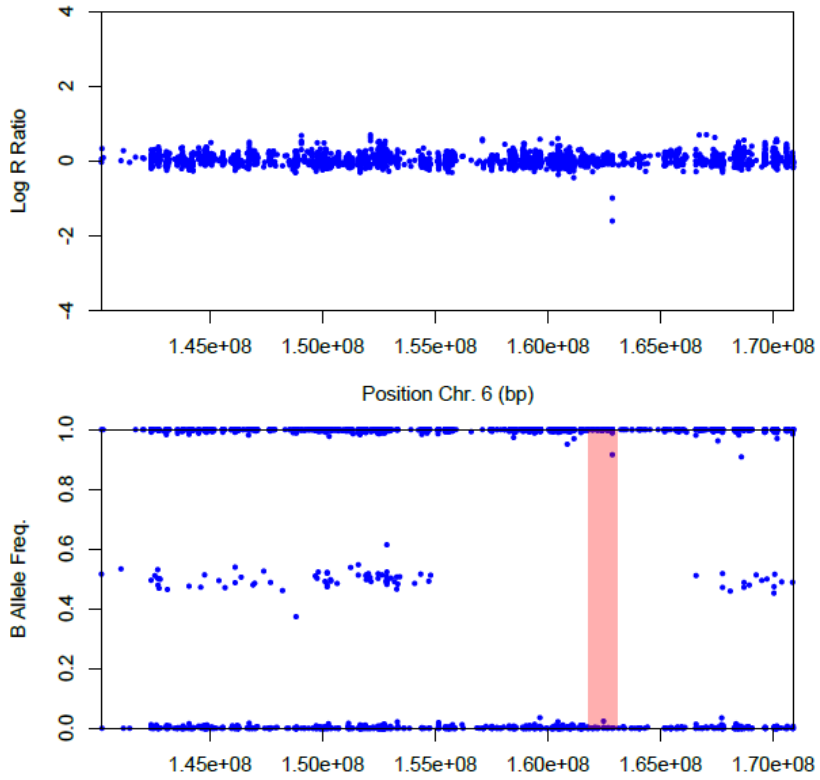


Figure 41. Genomic deletion in *PARK2*. Data from Genome Studio revealing genomic deletion in *PARK2* (Chr6: 162,206,852-162,206,882). The deletion is identified by a drop in the log R ratio (indicating a decrease in a CNV) and a lack of heterozygous genotype calls in the area, as seen in the B allele frequency plot.

VI. General Discussion

In this thesis, we have investigated the genetic basis of PD in the Southern Spanish population. Collectively, this work aimed to study in depth the role played by genetics in the aetiology of such disease in this particular cohort. We have made a considerable effort to characterise a poorly studied population in the context of PD genetics, dissecting which genetic risk variants and pathogenic mutations contribute significantly to the development of PD in Andalusia.

Previous to this work, there had been a very limited number of genetic studies on PD in the Southern Spain population (Gao et al., 2009 and Gómez-Garre et al., 2014). First, this work unravels the genetic architecture of PD in FPD and EOPD cases, as well as explores the influence of *GBA* and *LRRK2* mutations on LOPD. Second, it provides insight on the susceptibility variants predisposing to PD, underlining the importance of such risk factors in the aetiology of PD.

As part of our objectives to genetically characterize PD in this population, we used the efficiency of NGS to investigate the frequency of potentially pathogenic mutations of 8 known PD genes in EOPD and FPD cases. Our approach produced highly accurate sequence data, and we identified several genetic variants linked to the disease.

Not surprisingly, we found that p.G2019S is a common *LRRK2* mutation amongst PD patients from Granada, with a frequency of 3.7 %. Although p.G2019S is responsible of 0.5%–4% of idiopathic PD cases amongst Caucasians, its prevalence has been found much higher amongst Ashkenazi Jews and North African Arabs (Healy et al., 2008 and Lesage et al., 2006).

However, the frequency of p.G2019S-carriers in PD cases from Granada suggests that there is not a strong influence from North Africa to our genetic landscape, as previously reported in a neighbouring province (Gao et al., 2009).

Additionally, the *LRRK2* mutation p.R1441G found in our PD cohort with a frequency of 11.1% in the familial group and 1 % in the LOPD group was originally identified in both AD familial (46%) and idiopathic (2.5%) cases of PD, in the Basque region of northern Spain (Gorostidi et al., 2009 and Paisán-Ruiz et al., 2013). The p.R1441G mutation is also identified at lower frequencies in patients from other Spanish provinces (between 0.7% in the East and 2.2% in the North), but it is very rare outside Northern Spain. In addition, we identified several *LRRK2* variants of unknown significance or not considered as disease causing variants.

The most prevalent genetic risk factor in PD identified up to date is carrying heterozygous loss-of-function variants in *GBA1* (Sidransky et al., 2009). Interestingly, our PD cohort yielded 17 carriers (12.7%) of previously described pathogenic mutations. The frequency rate of *GBA* mutations varies considerably depending on the population ethnicity, with a remarkably high frequency in individuals of Ashkenazi Jewish descent. Our results show a significant higher frequency of *GBA* mutations in Southern Spain population in comparison to another study carried out in the Spanish population which found *GBA* mutations with a frequency of 9.8 % (Setó-Salvia et al., 2012). Previous studies in other European populations reported lower frequencies too; 6.4 % in Greeks (Kalinderi et al., 2009), 4.2 % in British (Neumann et al., 2009), and 8.3 % in Portuguese population (Bras et al., 2009).

The most common *GBA* variant found was N370S, encountered in 7 patients (5.2%). However, this is believed to be a rare risk factor for PD in North African Berber population (Nishioka et al., 2010). D409H, our second most prevalent *GBA* mutation, was detected in 4 patients. Additionally, we found the E326K mutation in 3 patients, 2 of which carried simultaneously the L444P variant (mutation with unknown phase).

The present study ascertains that mutations in *GBA* contribute substantially to FPD and SPD in Andalusian population. As we only screened selected *LRRK2* and *GBA* exons (mutation hotspots) in LOPD cases, some mutant alleles may have been missed.

Of particular interest is *PARK2*, which is the most frequently mutated gene in autosomal-recessive EOPD, with mutations found in 10%–20% of early-onset familial cases (Periquet et al., 2003). However, their prevalence and involvement in the modulation of PD risk have a wide variation depending on the studied population and the age of the subjects under study (Bardien et al., 2009; Bras et al., 2008 and Schlitter et al., 2006). Our *PARK2* mutational spectrum included the homozygous deletion p.N52fs responsible for a recessive EOPD and an FPD case with a frequency of 5.4 %. The heterozygous deletion p.N52fs was found to cause FPD in 2.7 % of our cohort. Moreover, MLPA revealed heterozygous deletions in exons 3 and 4 of *PARK2*, which were also present in the affected brother. Furthermore, we found the previously reported homozygous variant p.G309D in *PINK1* in 1 FPD case (Valente et al., 2004).

However, we found *SNCA* and *GCH1* deleterious mutations to be rare causes of the disease in EOPD and FPD cases from Southern Spain.

Mutations in *VPS35* have been described as responsible of autosomal-dominant PD in late-onset familial cases. One of our most interesting findings is the presence of a novel mutation (p.R32S) which has not been reported in public single nucleotide polymorphism databases including ExAc, EVS, and 1000 Genome project. It was found in 1 EOPD case without a positive familial history for PD. Although it has been predicted as damaging by the *in silico* analyses, we cannot assume that this mutation may be causative, because we could not screen it in matched controls and we could not test segregation of the mutation in other members of his family.

Secondly, we performed a traditional case-control association study by assessing 64 SNPs in cases and controls from this population. Although we are aware that candidate gene studies where the association of only a few markers to disease often leads to false positive results (Ioannidis et al., 2005), we were very stringent in our analysis.

Our case-control study provided strong support for the susceptibility role of *SNCA* genetic risk variants in individuals affected with PD from Southern Spain. We found an association between rs2736990 and rs356204, both in the intron 4 of *SNCA,* and PD pathogenesis. Moreover, when considering dependent effects from *SNCA* SNPs in LD, we also found an increasing risk for PD in the TGGT haplotype carriers. Similarly, previous studies showed that the rs2736990-G allele increases the risk for PD in Caucasian and Japanese populations (Edwards et al., 2010, Simon-Sánchez et al., 2009, Miyake et al., 2012).

In concordance with the literature, the *SNCA* rs356204-T allele was also linked to PD (Westerlund et al., 2008). Although this non-coding SNP only showed a trend toward significance in our study, it has been associated with PD in two previous case-control studies conducted in Norway (Myhreet al., 2008), USA (Mata et al., 2010), and in a large multicentre case-control study (Elbaz et al., 2011).

We could not establish any other association between the remaining SNPs and PD since they did not surpassed our stringent adjustment. We are confident that the signals we detected as significant in an unadjusted model might represent a true association with PD. As these loci are PD confirmed risk factors and our cohort under study is homogeneous, the adjusted threshold could have caused an underestimation of our results. In fact, haplotype analyses combining dependent effects of variants in LD, revealed an association with PD at the *GBA* locus, which could not be detected when focusing on single independent effects. No differences were found when we examined the proportion of the two *GBA* risk haplotypes between *GBA* mutation carriers and non-carriers, indicating that the haplotypes conferring PD risk are independent to the occurrence of carrying GBA mutations.

We went a step forward, and we carried out what to our knowledge is the first GWAS for PD in a Spanish cohort, and the second in a Southern European population (Kara et al., 2014). We thought it was of paramount importance to determine whether risk factors described by other authors were globally applicable in our population in an unbiased manner. Although we were aware that the sample size itself was a limitation, it comprised most PD cases from the province of Granada.

Therefore, one of the strengths of this study was that no potential problems of population stratification were expected as the sample comes from a relatively homogeneous ancestry background.

Our results suggested that there was no detectable high risk variant for PD present in our population, consistent with results of other GWAS with small sample size (Hernández et al., 2012, Fung et al., 2006). However, we marginally replicated association of five previously reported PD-related loci (Nalls et al., 2014) at nominal uncorrected p-value < 0.05, even though these associations do not pass multiple corrections at n=30 (0.05/30 ≈ 0.002). Our limited replication at the stringent p-value of a GWAS (p value < 5×10^{-8}) is likely due at least in part to insufficient statistical power to detect the relatively low effect of these alleles in our smaller cohort. For example, assuming an alpha of 0.002, disease prevalence of 4.4%, allele frequency of 0.2, and relative risk of 1.3, our power in this study would be 0.065. The presence of population-specific differences in the Southern Spanish sample and PD subphenotypes with distinct genetic etiologies might also be responsible, among other factors, of this lack of association.

This present study ascertains excesses of homozygosity in specific genomic regions overrepresented in cases, suggesting that variants in *HLA-DQB1* and *GBA-SYT11* contribute to idiopathic PD in our population. As commented above, these sites have been linked to idiopathic PD in the past (Sidransky et al., 2009, Hamza et al., 2010), although further work characterizing these loci in PD patients remains.

Our genetic risk profiling for PD demonstrated between-quintile odds ratios of a similar magnitude reported in other previous studies (Hernández et al., 2012, Nalls et al., 2011, International Parkinson's Disease Consortium, 2011, Kara et al., 2014), showing that the risk profiles found in other populations of European descent were applicable to the Andalusian population.

Consistent with these earlier studies, the area under the curve (AUC), calculated based on a logistic regression of disease status vs. GRS was 0.638. This suggest that the currently known risk loci are not sufficient to predict disease status and thus it is of limited clinical utility when used alone. When used in conjunction with age, gender, olfaction, and family history of PD, however, the GRS has been shown to contribute to disease status prediction (Nalls et al., 2015c).

To date, limited progress has been made in understanding how genetic variation affects PD phenotype. No GWAS has identified and independently replicated common variants associated with AAO on a genome-wide scale. Our study, in agreement with the literature, did not reveal any significant variant to be associated with this PD feature. However, recent studies have found that increasing genetic risk scores are related to earlier age at onset (Nalls et al., 2015b, Lill et al., 2015) suggesting that early onset forms of the illness are not exclusively caused by highly penetrant Mendelian mutations, but can also be influenced by an accumulation of common polygenic alleles with relatively low size effects (Escott-Price et al., 2015). We showed no association between GRS and AAO.

We know that common genetic variation plays a substantial role in PD, but an important proportion of the genetic risk might result from rare alleles.

Given the size of our cohort, it is likely that we were unable to find any novel prospective rare variant simply due to their low frequency. If a disease-causing rare variant is present in 0.5% of cases, we would expect to see it only one case in our population, severely limiting statistical power. Indeed, no rare variant or summation of rare variants within genes showed a significant association with PD status.

However, when examining known PD gene loci, we identified the *LRRK2* G2019S pathogenic mutation in 7 cases and the *GBA* N370S risk variant in 8 cases, replicating previous findings reported in the first objective of this thesis.

Structural genomic variation analysis revealed that an early-onset PD case (0.48 % of patients) carried a heterozygous CNV mutation in *PARK2*. CNVs constitute a particular challenge in GWAs and we assume that there may be additional CNVs conferring risk for the disease poorly covered or undetectable by the array. That could explain the lower frequency of heterozygous exonic rearrangements in *PARK2* in our study in comparison with other studies (Huttenlocher et al., 2015; Kay et al., 2010). Moreover, we failed to replicate the frameshift deletion p.N52fs and the exon rearrangements detected in *PARK2* by MLPA, as detailed above.

Although our estimates of phenotypic variance provided imprecise results due to the limited sample size, there is compelling evidence that a large proportion of heritability in PD has yet to be discovered (Keller et al., 2012). We also note that reported heritability is likely biased to be high because of the enrichment of neurodegenerative disease-associated loci on the NeuroX chip (Nalls et al., 2015a). We propose that there will be a considerable number of variants that impart risk for disease outside the limits of what can be reliably detected by our genotyping platform.

Missing heritability is the center of much of the current debates, and possible explanations include lack of power to detect common low-risk variants, rare variants, gene-gene interactions, structural variants such as deletions or duplications, and inversions not detected by the current technology, as well as gene-environmental interactions (epigenetics).

It is hoped that over the next years, insights will be gained into the pathogenic relevance of missing genetic variability. Processes such as methylation, phosphorylation, acetylation and generation of micro-RNAs (miRNA) allow modulation of gene expression and translation in response to environmental stimuli. Epigenetic research is at present an underdeveloped field, but it may filling in the missing heritability gap mentioned.

Finally, this work questions the possibility of implicating the process of genetic testing in the daily clinical practice. It should be pointed out that genetic testing has to be used with caution and several aspects should be taken into consideration before its broader implementation.

Genetic testing is a procedure of large cost that can be carried out only in few laboratories with first-line technology. Moreover, we should keep in mind that only 6 genes have been unequivocally linked to Mendelian forms of PD. In the current scenario, the genetic testing might lead to an etiological diagnosis in a minority of PD cases (< 5-10 %). Moreover, a positive genetic test does not always mean that the carrier of the mutation will surely develop the disease, as many disease causing mutations show reduced penetrance.

The factors governing the penetrance of the mutation and its clinical expressivity (i.e onset age and disease severity) are still poorly understood. It is therefore impossible to predict accurately if and when the disease will develop in an asymptomatic mutation carrier.

The screening itself is technically complex –mutations in several genes might cause PD and in each of these genes, many different mutations, including novel variants, might be found.

It is often difficult to interpret whether a gene variant is a disease-causing or a benign, neutral variant. This is especially relevant in the current era of NGS technologies (gene panels, diagnostic exome sequencing).

On the other hand, genetic testing can have a great personal and socioeconomic impact, but unfortunately without any disease-modulating or neuroprotective agents being available in the market. Even if a definitely pathogenic mutation is detected in a PD case, currently this will not influence the therapeutic choices.

However, reaching an etiological diagnosis might still be beneficial for both patients and physicians, particularly in cases with difficult and atypical presentations, or for the patients in early stages of disease, and those with early-onset. Reaching the diagnosis sooner might avoid additional, invasive and expensive tests, and genetic results might inform family and influence life-planning decisions (especially in cases with dominant, deleterious highly-penetrant mutations).

Genetic testing should be always supported by genetic counselling, and it is recommended only in specific circumstances including cases of early onset PD, positive family history and in specific risk populations of PD. Of course, susceptibility genetic testing is strongly discouraged. If mutations are not identified in the currently known genes and with the available technology, it does not mean that there are no mutations in these or in other genes; the result of the test might therefore be regarded as inconclusive in most PD cases. These uncertainties might increase the patients concern about the cause of the disease.

Great steps forward have been achieved in the field of PD research, however, the route to PD therapy is still long and difficult. In the years to follow, it is anticipated that new advances in technology and improved informatics systems will provide more information regarding the genetic background of the disease and potential predisposing factors. Hopefully, understanding the molecular mechanisms implicated in PD pathogenesis will allow the development of targeted therapies.

VII. Conclusions/Conclusiones

Despite the potential genetic background of Southern Spain due to the secular exchange with other populations of the Mediterranean coast, to date there have been few studies in relation to diseases of possible genetic cause. This thesis has focused on the analysis of genetic alterations of PD in that population. Our findings may have important clinical implications for the preclinical and differential diagnosis of this disease, and have allowed us to reach the following conclusions:

1. We performed the first GWAS in a Spanish population and the second in a Southern European population, marginally replicating the association of 5 reported PD-related loci (*ACMSD/TMEM163, MAPT, STK39, MIR4697, SREBF/RAI1*) at a p value < 0.05.

2. We identified pathogenic mutations in 22.4 % of our PD population. *GBA* mutations E326K, T369M, N370S, D409H and L444P appear to be considerably frequent. In concordance with previous studies, *LRRK2* genetic variants G2019S and R1441G are common causes of PD in Andalusian individuals.

3. We found the novel mutation R32S in the gene *VPS35.* The mutant amino acid was predicted to be damaging to the structural effect, stability and function of the vacuolar protein sorting-associated protein 35. However, further functional and genetic studies should be conducted to evaluate the possible pathogenicity of this novel variant.

4. MLPA analysis revealed the presence of a *PARK2* heterozygous deletion of exon 3 and 4 in an early-onset FPD case carrying the heterozygous frameshift deletion p.N52fs in the exon 2 of *PARK2.*

5. We found *SNCA* and *GCH1* mutations to be rare causes of the disease in early-onset PD and familial PD cases from Southern Spain. However, our preliminary genotyping results support the notion that common SNPs in the *SNCA* and *GBA* loci are risk factors for sporadic/idiopathic PD.

6. Our genetic risk profile is consistent with previous studies, showing that individuals with the highest burden of genetic risk were found to be 3.6 times more likely to develop PD than those in the lowest. Our area under the curve (AUC) analysis suggests that the currently known risk loci are not sufficient to predict disease status.

7. We revealed an excess of homozygosity in cases, in the *HLA-DBQ1* and *GBA-SYT11* loci, strengthening evidence for the role of these loci in the etiology of PD.

8. We failed to identify any novel rare risk variant (MAF < 1 %) associated with PD in our population. However, we identified variants of unknown pathogenicity, which were present in our cases and absent in our controls, in PD-related genes as *PINK1, PARK2, GIGYF2, DNAJC13, SYN1 and FBXO7.*

A pesar de la potencial riqueza genética del sur de España, debido al intercambio secular con otras poblaciones de la ribera mediterránea, se han realizado hasta la fecha pocos estudios en relación a enfermedades de posible causa genética. Esta tesis se ha centrado en el análisis de las alteraciones genéticas de la EP en la citada población. Nuestros resultados pueden tener importantes implicaciones clínicas en el diagnóstico preclínico y diferencial de esta enfermedad, y nos han permitido llegar a las siguientes conclusiones:

1. El presente estudio es el primer genotipado a gran escala realizado en España y el segundo en la población del Sur de Europa. Identificamos 5 loci previamente descritos como asociados a la EP (ACMSD/TMEM163, MAPT, STK39, MIR4697, SREBF/RAI1) a un p valor < 0.05.

2. Identificamos mutaciones patogénicas en un 22,4 % de los casos sometidos a screening genético. Las mutaciones E326K, T369M, N370S, D409H y L444P en el gen *GBA* aparecen con una frecuencia más alta en nuestra población en comparación con otras poblaciones europeas. Coincidiendo con estudios previos, las mutaciones genéticas G2019S y R1441G en el gen *LRRK2* son causas comunes de EP en la población estudiada.

3. Detectamos una nueva mutación en el gen *VPS35,* la R32S. Los análisis de simulación bioinformática predijeron que el cambio amino acídico de Arginina por Serina es posiblemente dañino para la estructura, estabilidad y funcionamiento de la proteína codificada por este gen. Estudios genéticos y funcionales adicionales son necesarios para dilucidar el posible grado de patogenicidad de esta variante.

4. El análisis de MLPA reveló la deleción completa en heterocigosis de los exones 3 y 4 del gen *PARK2* en un caso de EP familiar portador de la deleción puntual p.N52fs en el exon 2 del mismo gen*.*

5. Mutaciones en los genes *SNCA* y *GCH1* parecen ser causas poco frecuentes en los casos de EP de inicio temprano y/o familiar en la población estudiada. Sin embargo, nuestros resultados de genotipado sustentan la hipótesis de que SNPs comunes en los loci *SNCA* y *GBA* son factores de riesgo para la EP idiopática.

6. Nuestro perfil de riesgo genético concuerda con estudios previos, mostrando que aquellos individuos con la mayor carga genética de riesgo presentan una susceptibilidad 3,6 veces superior de desarrollar EP que aquellos individuos portadores de un menor número de alelos de riesgo. Nuestro análisis del área encerrada bajo la curva (AUC) sugiere que los loci de riesgo identificados hasta el momento, no son suficientes para predecir la EP.

7. Identificamos un exceso de homocigosis en casos respecto a controles en los genes *HLA-DBQ1* y *GBA-SYT11*, fortaleciendo la evidencia del papel de estos dos loci en la etiología de la EP.

8. No identificamos ninguna nueva variante de riesgo rara (MAF < 1%) asociada a la EP en nuestra población. Sin embargo, detectamos variantes de patogenicidad desconocida, presentes en los casos y ausentes en los controles, en los siguientes genes asociados a la EP: *PINK1, PARK2, GIGYF2, DNAJC13, SYN1 y FBXO7.*

Agradecimientos/Acknowlegments

Tengo que reconocer que durante el tiempo transcurrido en la realización de esta tesis, he atravesado momentos difíciles, a veces con incertidumbre y a veces con frustración. Esta etapa me ha llevado a entender que el científico debe ser una persona perseverante, con ilusión, disciplina y capacidad de trabajo incansable para superar las dificultades.

Ahora, en el tramo final, con la lucidez que da el paso del tiempo para analizar y valorar cada uno de los momentos vividos, me doy cuenta de lo afortunada que he sido. Estoy inmensamente agradecida a las personas que han hecho este camino más fácil.

En primer lugar, a mi director de tesis el Dr. Francisco Vives, por acogerme en el departamento, depositar toda su confianza en mí y hacerme crecer en el mundo académico. Paco, contigo empezó todo. Gracias por tu sabiduría, experiencia y comprensión.

A mi directora, la Dra. Raquel Durán, agradezco infinitamente tu paciencia y humildad. De ti he aprendido la capacidad de organización y el orden. Me contagiaste tu pasión por la genética y me pusiste las alas para volar hacia la investigación internacional.

A los neurólogos Paco Barrero, Paco Escamilla, Paco Pérez Errázquin y Mª José Gómez, por darme la visión clínica de la Enfermedad de Parkinson. Gracias por vuestro entusiasmo y por permitirme pasar innumerables horas en vuestras consultas.

A los pacientes de Parkinson incluidos en este trabajo, gracias por creer en mí y en mi investigación.

A todos los miembros de mi tribunal de Tesis, por aceptar formar parte de este trabajo.

A la Universidad de Granada, el Ministerio de Educación y todas las fuentes nacionales e internacionales que han financiado esta tesis.

A todos mis compañeros del Instituto de Neurociencias, especialmente a Blanca Gutiérrez y Margarita Rivera. Os agradezco vuestro consejo y asesoramiento genético, las muestras control de esta tesis y vuestra disponibilidad para efectuar las revisiones.

A mis veteranos colegas de trinchera Bea, Ana, Gloria, Serena y Migue, y a Clara, el nuevo fichaje de la ciencia, porque día tras día habéis dejado de ser compañeros de pasillo para convertiros en amigos. Gracias por vuestro humor y los buenos ratos que hemos pasado juntos.

A Juanmi, por guiarme en mis primeros pasos en el laboratorio, a Elisa por alegrarme con su sonrisa cada mañana, y a Chari, la secretaria más eficiente que he conocido.

A todos los compañeros del Departamento de Fisiología, mención especial a la profesora Rosa Montes, por acogerme con calidez y por impulsarme al mundo académico con esa fuerza de misil que solo ella tiene.

Volviendo la mirada al ámbito personal, gratitud a mis amigas farmacéuticas, en especial a Ana, Vicky (mi gemela de tesis), Mamen y Mei, por sus inyectables de energía e ilusión, a mis compañeras de piso por su eterna positividad y a mi grupo "Precarios por la Ciencia". A Lucía y Sonia. Gracias por tanto y por todo.

I am very grateful that I was giving the opportunity to perform an important part of this thesis in two very prestigious laboratories of neurogenetics in the world.

A) Institute of Neurology, University College London (UCL)

I am particularly grateful to Dr. Niccolò Mencacci, great scientist and person. Thanks for your mentorship, for teaching me that much about genetics and for allowing me to take part in your dystonia projects. To Dr. Alan Pittman for your useful advice and discussion, and for supervising this work during my short- stay at ION. Many thanks to Dr. John Hardy, likely the most brilliant scientist I will ever meet. Thank you very much for having me in your lab.

In particular, I would like to thank my labmates for their friendship. Thank you guys for such a great experience.

B) LNG, National Institute on Aging, (NIH)

Thank you to Dr. Andrew Singleton and Dr. Mike Nalls for having me as a fellow, and for their guidance and advice for my career progression.

To Sampath and Ryan for their help to bring the Spanish GWAS to successful publication. To Nikki and Charlotte for all the support in the background.

To Monia and Monica. Thanks for your scientific advice and the exome-sequencing teaching classes. And last but not least, thanks to all my NIH labmates, particularly to Isabella for being such a fabulous flatmate.

A las personas de mi entorno más cercano, perdonar que no os nombre a todos pero sabed que os tengo presentes.

Y no, no me he olvidado de los más importantes: mi **FAMILIA**, que ha sobrevivido con gran elegancia y humor esta tesis. Por vuestro apoyo incondicional, por transmitirme vuestra positividad y por aguantarme en todo momento. Estando cerca o estando lejos, siempre he sentido y tenido muy presente vuestro cariño. Sin duda, habéis sido el motor de esta travesía.

GRACIAS A TODOS

THANK YOU ALL

References

A

Abou-Sleiman, P.M., Healy, D.G., Quinn, N., Lees, A.J., Wood, N.W., 2003. The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann. Neurol. 54, 283–286.

Aiken, C.T., Kaake, R.M., Wang, X., Huang, L., 2011. Oxidative stress-mediated regulation of proteasome complexes. Mol. Cell Proteomics 10, R110.006924.

Alegre-Abarrategui, J., Ansorge, O., Esiri, M., Wade-Martins, R., 2008. LRRK2 is a component of granular alpha-synuclein pathology in the brainstem of Parkinson's disease. Neuropathol. Appl. Neurobiol. 34, 272–283.

Adams, S.M., Bosch, E., Balaresque, P.L., Ballereau, S.J., Lee, A.C., Arroyo, E., López-Parra, A.M., Aler, M., Grifo, M.S.G., Brion, M., Carracedo, A., Lavinha, J., Martínez-Jarreta, B., Quintana-Murci, L., Picornell, A., Ramon, M., Skorecki, K., Behar, D.M., Calafell, F., Jobling, M.A., 2008. The Genetic Legacy of Religious Diversity and Intolerance: Paternal Lineages of Christians, Jews, and Muslims in the Iberian Peninsula. Am. J. Hum. Genet. 83, 725–736.

Annesi, G., Savettieri, G., Pugliese, P., D'Amelio, M., Tarantino, P., Ragonese, P., La Bella, V., Piccoli, T., Civitelli, D., Annesi, F., Fierro, B., Piccoli, F., Arabia, G., Caracciolo, M., Cirò Candiano, I.C., Quattrone, A., 2005. DJ-1 mutations and parkinsonism-dementia-amyotrophic lateral sclerosis complex. Ann. Neurol. 58, 803–807.

Appel-Cresswell, S., Vilarino-Guell, C., Encarnacion, M., Sherman, H., Yu, I., Shah, B., Weir, D., Thompson, C., Szu-Tu, C., Trinh, J., Aasly, J.O., Rajput, A., Rajput, A.H., Jon Stoessl, A., Farrer, M.J., 2013. Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. Mov. Disord. 28, 811–813.

Athanassiadou, A., Voutsinas, G., Psiouri, L., Leroy, E., Polymeropoulos, M.H., Ilias, A., Maniatis, G.M., Papapetropoulos, T., 1999. Genetic analysis of families with Parkinson disease that carry the Ala53Thr mutation in the gene encoding alpha-synuclein. Am. J. Hum. Genet. 65, 555–558.

B

Bach, J.-P., Riedel, O., Klotsche, J., Spottke, A., Dodel, R., Wittchen, H.-U., 2012. Impact of complications and comorbidities on treatment costs and health-related quality of life of patients with Parkinson's disease. J. Neurol. Sci. 314, 41–47.

Baker, M., Litvan, I., Houlden, H., Adamson, J., Dickson, D., Perez-Tur, J., Hardy, J., Lynch, T., Bigio, E., Hutton, M., 1999. Association of an extended haplotype in the tau gene with progressive supranuclear palsy. Hum. Mol. Genet. 8, 711–715.

Baldereschi, M., Di Carlo, A., Rocca, W.A., Vanni, P., Maggi, S., Perissinotto, E., Grigoletto, F., Amaducci, L., Inzitari, D., 2000. Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging. Neurology 55, 1358–1363.

Balding, D.J., 2006. A tutorial on statistical methods for population association studies. Nat. Rev. Genet. 7, 781–791.

Balsinde, J., Balboa, M.A., 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A2 in activated cells. Cell. Signal. 17, 1052–1062.

Bandrés-Ciga, S., Mencacci, N.E., Durán, R., Barrero, F.J., Escamilla-Sevilla, F., Morgan, S., Hehir, J., Vives, F., Hardy, J., Pittman, A.M., 2016. Analysis of the genetic variability in Parkinson's disease from Southern Spain. Neurobiol. Aging 37, 210.e1–5.

Bardien, S., Keyser, R., Yako, Y., Lombard, D., Carr, J., 2009. Molecular analysis of the parkin gene in South African patients diagnosed with Parkinson's disease. Parkinsonism Relat. Disord. 15, 116–121.

Baudier, J., Cole, R.D., 1987. Phosphorylation of tau proteins to a state like that in Alzheimer's brain is catalyzed by a calcium/calmodulin-dependent kinase and modulated by phospholipids. J. Biol. Chem. 262, 17577–17583.

Beilina, A., Rudenko, I.N., Kaganovich, A., Civiero, L., Chau, H., Kalia, S.K., Kalia, L.V., Lobbestael, E., Chia, R., Ndukwe, K., Ding, J., Nalls, M.A., International Parkinson's Disease Genomics Consortium, North American Brain Expression Consortium, Olszewski, M., Hauser, D.N., Kumaran, R., Lozano, A.M., Baekelandt, V., Greene, L.E., Taymans, J.-M., Greggio, E., Cookson, M.R., 2014. Unbiased screen for interactors of leucine-rich repeat kinase 2 supports a common pathway for sporadic and familial Parkinson disease. Proc. Natl. Acad. Sci. U.S.A. 111, 2626–2631.

Bendor, J.T., Logan, T.P., Edwards, R.H., 2013. The function of α-synuclein. Neuron 79, 1044–1066.

Bertolin, G., Ferrando-Miguel, R., Jacoupy, M., Traver, S., Grenier, K., Greene, A.W., Dauphin, A., Waharte, F., Bayot, A., Salamero, J., Lombès, A., Bulteau, A.-L., Fon, E.A., Brice, A., Corti, O., 2013. The TOMM machinery is a molecular switch in PINK1 and PARK2/PARKIN-dependent mitochondrial clearance. Autophagy 9, 1801–1817.

Bland, R.D., Clarke, T.L., Harden, L.B., 1976. Rapid infusion of sodium bicarbonate and albumin into high-risk premature infants soon after birth: a controlled, prospective trial. Am. J. Obstet. Gynecol. 124, 263–267.

Blesa, J., Trigo-Damas, I., Quiroga-Varela, A., Jackson-Lewis, V.R., 2015. Oxidative stress and Parkinson's disease. Front Neuroanat 9, 91.

Bonetti, M., Ferraris, A., Petracca, M., Bentivoglio, A.R., Dallapiccola, B., Valente, E.M., 2009. GIGYF2 variants are not associated with Parkinson's disease in Italy. Mov. Disord. 24, 1867–1868; author reply 1868–1869.

Bonifati, V., Rizzu, P., Squitieri, F., Krieger, E., Vanacore, N., van Swieten, J.C., Brice, A., van Duijn, C.M., Oostra, B., Meco, G., Heutink, P., 2003. DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurol. Sci. 24, 159–160.

Bonifati, V., Rohé, C.F., Breedveld, G.J., Fabrizio, E., De Mari, M., Tassorelli, C., Tavella, A., Marconi, R., Nicholl, D.J., Chien, H.F., Fincati, E., Abbruzzese, G., Marini, P., De Gaetano, A., Horstink, M.W., Maat-Kievit, J.A., Sampaio, C., Antonini, A., Stocchi, F., Montagna, P., Toni, V., Guidi, M., Dalla Libera, A., Tinazzi, M., De Pandis, F., Fabbrini, G., Goldwurm, S., de Klein, A., Barbosa, E., Lopiano, L., Martignoni, E., Lamberti, P., Vanacore, N., Meco, G., Oostra, B.A., Italian Parkinson Genetics Network, 2005. Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. Neurology 65, 87–95.

Bostantjopoulou, S., Katsarou, Z., Karakasis, C., Peitsidou, E., Milioni, D., Rossopoulos, N., 2013. Evaluation of non-motor symptoms in Parkinson's Disease: An underestimated necessity. Hippokratia 17, 214–219.

Braak, H., Del Tredici, K., Rüb, U., de Vos, R.A.I., Jansen Steur, E.N.H., Braak, E., 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197–211.

Braidy, N., Gai, W.-P., Xu, Y.H., Sachdev, P., Guillemin, G.J., Jiang, X.-M., Ballard, J.W.O., Horan, M.P., Fang, Z.M., Chong, B.H., Chan, D.K.Y., 2014. Alpha-synuclein transmission and mitochondrial toxicity in primary human foetal enteric neurons in vitro. Neurotox Res 25, 170–182.

Bras, J., Paisan-Ruiz, C., Guerreiro, R., Ribeiro, M.H., Morgadinho, A., Januario, C., Sidransky, E., Oliveira, C., Singleton, A., 2009a. Complete screening for glucocerebrosidase mutations in Parkinson disease patients from Portugal. Neurobiol. Aging 30, 1515–1517.

Bras, J., Simón-Sánchez, J., Federoff, M., Morgadinho, A., Januario, C., Ribeiro, M., Cunha, L., Oliveira, C., Singleton, A.B., 2009b. Lack of replication of association between GIGYF2 variants and Parkinson disease. Hum. Mol. Genet. 18, 341–346.

Bras, J., Verloes, A., Schneider, S.A., Mole, S.E., Guerreiro, R.J., 2012. Mutation of the parkinsonism gene ATP13A2 causes neuronal ceroid-lipofuscinosis. Hum. Mol. Genet. 21, 2646–2650.

Bras, J., Guerreiro, R., Ribeiro, M., Morgadinho, A., Januario, C., Dias, M., Calado, A., Semedo, C., Oliveira, C., Hardy, J., Singleton, A., 2008. Analysis of Parkinson disease patients from Portugal for mutations in SNCA, PRKN, PINK1 and LRRK2. BMC Neurol. 8, 1.

Brieger, K., Schiavone, S., Miller, F.J., Krause, K.-H., 2012. Reactive oxygen species: from health to disease. Swiss Med Wkly 142, w13659.

Brüggemann, N., Hagenah, J., Reetz, K., Schmidt, A., Kasten, M., Buchmann, I., Eckerle, S., Bähre, M., Münchau, A., Djarmati, A., van der Vegt, J., Siebner, H., Binkofski, F., Ramirez, A., Behrens, M.I., Klein, C., 2010. Recessively inherited parkinsonism: effect of ATP13A2 mutations on the clinical and neuroimaging phenotype. Arch. Neurol. 67, 1357–1363.

Brüggemann, N., Spiegler, J., Hellenbroich, Y., Opladen, T., Schneider, S.A., Stephani, U., Boor, R., Gillessen-Kaesbach, G., Sperner, J., Klein, C., 2012. Beneficial prenatal levodopa therapy in autosomal recessive guanosine triphosphate cyclohydrolase 1 deficiency. Arch. Neurol. 69, 1071–1075.

Brundin, P., Li, J.-Y., Holton, J.L., Lindvall, O., Revesz, T., 2008. Research in motion: the enigma of Parkinson's disease pathology spread. Nat. Rev. Neurosci. 9, 741–745.

Burchell, V.S., Nelson, D.E., Sanchez-Martinez, A., Delgado-Camprubi, M., Ivatt, R.M., Pogson, J.H., Randle, S.J., Wray, S., Lewis, P.A., Houlden, H., Abramov, A.Y., Hardy, J., Wood, N.W., Whitworth, A.J., Laman, H., Plun-Favreau, H., 2013. The Parkinson's disease-linked proteins Fbxo7 and Parkin interact to mediate mitophagy. Nat. Neurosci. 16, 1257–1265.

C

Canet-Avilés, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A., Cookson, M.R., 2004. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc. Natl. Acad. Sci. U.S.A. 101, 9103–9108.

Cao, L., Zhang, T., Zheng, L., Wang, Y., Wang, G., Zhang, J., Fei, Q.Z., Cui, P.J., Wang, X.J., Ma, J.F., Xiao, Q., Chen, S.D., 2010. The GIGYF2 variants are not associated with Parkinson's disease in the mainland Chinese population. Parkinsonism Relat. Disord. 16, 294–297.

Champy, P., Höglinger, G.U., Féger, J., Gleye, C., Hocquemiller, R., Laurens, A., Guérineau, V., Laprévote, O., Medja, F., Lombès, A., Michel, P.P., Lannuzel, A., Hirsch, E.C., Ruberg, M., 2004. Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical parkinsonism in Guadeloupe. J. Neurochem. 88, 63–69.

Charlesworth, G., Gandhi, S., Bras, J.M., Barker, R.A., Burn, D.J., Chinnery, P.F., Gentleman, S.M., Guerreiro, R., Hardy, J., Holton, J.L., Lees, A., Morrison, K., Sheerin, U.-M., Williams, N., Morris, H., Revesz, T., Wood, N.W., 2012. Tau acts as an independent genetic risk factor in pathologically proven PD. Neurobiol. Aging 33, 838.e7–11.

Chartier-Harlin, M.-C., Dachsel, J.C., Vilariño-Güell, C., Lincoln, S.J., Leprêtre, F., Hulihan, M.M., Kachergus, J., Milnerwood, A.J., Tapia, L., Song, M.-S., Le Rhun, E., Mutez, E., Larvor, L., Duflot, A., Vanbesien-Mailliot, C., Kreisler, A., Ross, O.A., Nishioka, K., Soto-Ortolaza, A.I., Cobb, S.A., Melrose, H.L., Behrouz, B., Keeling, B.H., Bacon, J.A., Hentati, E., Williams, L., Yanagiya, A., Sonenberg, N., Lockhart, P.J., Zubair, A.C., Uitti, R.J., Aasly, J.O., Krygowska-Wajs, A., Opala, G., Wszolek, Z.K., Frigerio, R., Maraganore, D.M., Gosal, D., Lynch, T., Hutchinson, M., Bentivoglio, A.R., Valente, E.M., Nichols, W.C., Pankratz, N., Foroud, T., Gibson, R.A., Hentati, F., Dickson, D.W., Destée, A., Farrer, M.J., 2011b. Translation initiator EIF4G1 mutations in familial Parkinson disease. Am. J. Hum. Genet. 89, 398–406.

Chartier-Harlin, M.-C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., Waucquier, N., Defebvre, L., Amouyel, P., Farrer, M., Destée, A., 2004. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 364, 1167–1169.

Choi, J.M., Woo, M.S., Ma, H.-I., Kang, S.Y., Sung, Y.-H., Yong, S.W., Chung, S.J., Kim, J.-S., Shin, H., Lyoo, C.H., Lee, P.H., Baik, J.S., Kim, S.-J., Park, M.Y., Sohn, Y.H., Kim, J.-H., Kim, J.W., Lee, M.S., Lee, M.C., Kim, D.-H., Kim, Y.J., 2008. Analysis of PARK genes in a Korean cohort of early-onset Parkinson disease. Neurogenetics 9, 263–269.

Choubey, V., Safiulina, D., Vaarmann, A., Cagalinec, M., Wareski, P., Kuum, M., Zharkovsky, A., Kaasik, A., 2011. Mutant A53T alpha-synuclein induces neuronal death by increasing mitochondrial autophagy. J. Biol. Chem. 286, 10814–10824.

Clark, L.N., Ross, B.M., Wang, Y., Mejia-Santana, H., Harris, J., Louis, E.D., Cote, L.J., Andrews, H., Fahn, S., Waters, C., Ford, B., Frucht, S., Ottman, R., Marder, K., 2007a. Mutations in the glucocerebrosidase gene are associated with early-onset Parkinson disease. Neurology 69, 1270–1277.

Crews, L., Spencer, B., Desplats, P., Patrick, C., Paulino, A., Rockenstein, E., Hansen, L., Adame, A., Galasko, D., Masliah, E., 2010. Selective molecular alterations in the autophagy pathway in patients with Lewy body disease and in models of alpha-synucleinopathy. PLoS ONE 5, e9313.

Cullen, V., Lindfors, M., Ng, J., Paetau, A., Swinton, E., Kolodziej, P., Boston, H., Saftig, P., Woulfe, J., Feany, M.B., Myllykangas, L., Schlossmacher, M.G., Tyynelä, J., 2009. Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo. Mol Brain 2, 5.

Cullen, V., Sardi, S.P., Ng, J., Xu, Y.-H., Sun, Y., Tomlinson, J.J., Kolodziej, P., Kahn, I., Saftig, P., Woulfe, J., Rochet, J.-C., Glicksman, M.A., Cheng, S.H., Grabowski, G.A., Shihabuddin, L.S., Schlossmacher, M.G., 2011. Acid β-glucosidase mutants linked to Gaucher disease, Parkinson disease, and Lewy body dementia alter α-synuclein processing. Ann. Neurol. 69, 940–953.

D

Dasuri, K., Zhang, L., Keller, J.N., 2013. Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis. Free Radic. Biol. Med. 62, 170–185.

Deas, E., Wood, N.W., Plun-Favreau, H., 2011. Mitophagy and Parkinson's disease: the PINK1-parkin link. Biochim. Biophys. Acta 1813, 623–633.

Dehay, B., Martinez-Vicente, M., Caldwell, G.A., Caldwell, K.A., Yue, Z., Cookson, M.R., Klein, C., Vila, M., Bezard, E., 2013. Lysosomal impairment in Parkinson's disease. Mov. Disord. 28, 725–732.

Dehay, B., Ramirez, A., Martinez-Vicente, M., Perier, C., Canron, M.-H., Doudnikoff, E., Vital, A., Vila, M., Klein, C., Bezard, E., 2012. Loss of P-type ATPase ATP13A2/PARK9 function induces general lysosomal deficiency and leads to Parkinson disease neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. 109, 9611–9616.

Dehay, B., Vila, M., Bezard, E., Brundin, P., Kordower, J.H., 2016. Alpha-synuclein propagation: New insights from animal models. Mov. Disord. 31, 161–168.

De Lau, L.M.L., Breteler, M.M.B., 2006. Epidemiology of Parkinson's disease. Lancet Neurol 5, 525–535.

De Lau, L.M.L., Giesbergen, P.C.L.M., de Rijk, M.C., Hofman, A., Koudstaal, P.J., Breteler, M.M.B., 2004. Incidence of parkinsonism and Parkinson disease in a general population: the Rotterdam Study. Neurology 63, 1240–1244.

Deng, H., Gao, K., Jankovic, J., 2013. The VPS35 gene and Parkinson's disease. Mov. Disord. 28, 569–575.

Deng, H.-X., Shi, Y., Yang, Y., Ahmeti, K.B., Miller, N., Huang, C., Cheng, L., Zhai, H., Deng, S., Nuytemans, K., Corbett, N.J., Kim, M.J., Deng, H., Tang, B., Yang, Z., Xu, Y., Chan, P., Huang, B., Gao, X.-P., Song, Z., Liu, Z., Fecto, F., Siddique, N., Foroud, T., Jankovic, J., Ghetti, B., Nicholson, D.A., Krainc, D., Melen, O., Vance, J.M., Pericak-Vance, M.A., Ma, Y.-C., Rajput, A.H., Siddique, T., 2016. Identification of TMEM230 mutations in familial Parkinson's disease. Nat. Genet. 48, 733–739.

De Rijk, M.C., Tzourio, C., Breteler, M.M., Dartigues, J.F., Amaducci, L., Lopez-Pousa, S., Manubens-Bertran, J.M., Alpérovitch, A., Rocca, W.A., 1997. Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. J. Neurol. Neurosurg. Psychiatr. 62, 10–15.

De Rosa, A., Criscuolo, C., Mancini, P., De Martino, M., Giordano, I.A., Pappatà, S., Filla, A., De Michele, G., 2009. Genetic screening for LRRK2 gene G2019S mutation in Parkinson's disease patients from Southern Italy. Parkinsonism Relat. Disord. 15, 242–244.

Devi, L., Raghavendran, V., Prabhu, B.M., Avadhani, N.G., Anandatheerthavarada, H.K., 2008. Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. J. Biol. Chem. 283, 9089–9100.

Di Fonzo, A., Dekker, M.C.J., Montagna, P., Baruzzi, A., Yonova, E.H., Correia Guedes, L., Szczerbinska, A., Zhao, T., Dubbel-Hulsman, L.O.M., Wouters, C.H., de Graaff, E., Oyen, W.J.G., Simons, E.J., Breedveld, G.J., Oostra, B.A., Horstink, M.W., Bonifati, V., 2009. FBXO7 mutations cause autosomal recessive, early-onset parkinsonian-pyramidal syndrome. Neurology 72, 240–245.

Di Fonzo, A., Fabrizio, E., Thomas, A., Fincati, E., Marconi, R., Tinazzi, M., Breedveld, G.J., Simons, E.J., Chien, H.F., Ferreira, J.J., Horstink, M.W., Abbruzzese, G., Borroni, B., Cossu, G., Dalla Libera, A., Fabbrini, G., Guidi, M., De Mari, M., Lopiano, L., Martignoni, E., Marini, P., Onofrj, M., Padovani, A., Stocchi, F., Toni, V., Sampaio, C., Barbosa, E.R., Meco, G., Italian Parkinson Genetics Network, Oostra, B.A., Bonifati, V., 2009. GIGYF2 mutations are not a frequent cause of familial Parkinson's disease. Parkinsonism Relat. Disord. 15, 703–705.

Di Fonzo, A., Wu-Chou, Y.-H., Lu, C.-S., van Doeselaar, M., Simons, E.J., Rohé, C.F., Chang, H.-C., Chen, R.-S., Weng, Y.-H., Vanacore, N., Breedveld, G.J., Oostra, B.A., Bonifati, V., 2006. A common missense variant in the LRRK2 gene, Gly2385Arg, associated with Parkinson's disease risk in Taiwan. Neurogenetics 7, 133–138.

Do, C.B., Tung, J.Y., Dorfman, E., Kiefer, A.K., Drabant, E.M., Francke, U., Mountain, J.L., Goldman, S.M., Tanner, C.M., Langston, J.W., Wojcicki, A., Eriksson, N., 2011. Web-based genome-wide association study identifies two novel loci and a substantial genetic component for Parkinson's disease. PLoS Genet. 7, e1002141.

Duran, R., Barrero, F.J., Morales, B., Luna, J.D., Ramirez, M., Vives, F., 2011. Oxidative stress and aminopeptidases in Parkinson's disease patients with and without treatment. Neurodegener Dis 8, 109–116.

Duran, R., Mencacci, N.E., Angeli, A.V., Shoai, M., Deas, E., Houlden, H., Mehta, A., Hughes, D., Cox, T.M., Deegan, P., Schapira, A.H., Lees, A.J., Limousin, P., Jarman, P.R., Bhatia, K.P., Wood, N.W., Hardy, J., Foltynie, T., 2013. The glucocerobrosidase E326K variant predisposes to Parkinson's disease, but does not cause Gaucher's disease. Mov. Disord. 28, 232–236.

Duvoisin, R.C., Eldridge, R., Williams, A., Nutt, J., Calne, D., 1981. Twin study of Parkinson disease. Neurology 31, 77–80.

E

Edvardson, S., Cinnamon, Y., Ta-Shma, A., Shaag, A., Yim, Y.-I., Zenvirt, S., Jalas, C., Lesage, S., Brice, A., Taraboulos, A., Kaestner, K.H., Greene, L.E., Elpeleg, O., 2012. A deleterious mutation in DNAJC6 encoding the neuronal-specific clathrin-uncoating co-chaperone auxilin, is associated with juvenile parkinsonism. PLoS ONE 7, e36458.

Edwards, T.L., Scott, W.K., Almonte, C., Burt, A., Powell, E.H., Beecham, G.W., Wang, L., Züchner, S., Konidari, I., Wang, G., Singer, C., Nahab, F., Scott, B., Stajich, J.M., Pericak-Vance, M., Haines, J., Vance, J.M., Martin, E.R., 2010. Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Ann. Hum. Genet. 74, 97–109.

Elbaz, A., Ross, O.A., Ioannidis, J.P.A., Soto-Ortolaza, A.I., Moisan, F., Aasly, J., Annesi, G., Bozi, M., Brighina, L., Chartier-Harlin, M.-C., Destée, A., Ferrarese, C., Ferraris, A., Gibson, J.M., Gispert, S., Hadjigeorgiou, G.M., Jasinska-Myga, B., Klein, C., Krüger, R., Lambert, J.-C., Lohmann, K., van de Loo, S., Loriot, M.-A., Lynch, T., Mellick, G.D., Mutez, E., Nilsson, C., Opala, G., Puschmann, A., Quattrone, A., Sharma, M., Silburn, P.A., Stefanis, L., Uitti, R.J., Valente, E.M., Vilariño-Güell, C., Wirdefeldt, K., Wszolek, Z.K., Xiromerisiou, G., Maraganore, D.M., Farrer, M.J., Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium, 2011. Independent and joint effects of the MAPT and SNCA genes in Parkinson disease. Ann. Neurol. 69, 778–792.

Eldridge, R., Ince, S.E., 1984. The low concordance rate for Parkinson's disease in twins: a possible explanation. Neurology 34, 1354–1356.

Escott-Price, V., International Parkinson's Disease Genomics Consortium, Nalls, M.A., Morris, H.R., Lubbe, S., Brice, A., Gasser, T., Heutink, P., Wood, N.W., Hardy, J., Singleton, A.B., Williams, N.M., IPDGC consortium members, 2015. Polygenic risk of Parkinson disease is correlated with disease age at onset. Ann. Neurol. 77, 582–591.

Esteves, A.R., Arduíno, D.M., Swerdlow, R.H., Oliveira, C.R., Cardoso, S.M., 2009. Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids. Antioxid. Redox Signal. 11, 439–448.

F

Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D.-S., Hulihan, M., Maraganore, D., Gwinn-Hardy, K., Wszolek, Z., Dickson, D., Langston, J.W., 2004. Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. Ann. Neurol. 55, 174–179.

Fauvet, B., Fares, M.-B., Samuel, F., Dikiy, I., Tandon, A., Eliezer, D., Lashuel, H.A., 2012. Characterization of semisynthetic and naturally Nα-acetylated α-synuclein in vitro and in intact cells: implications for aggregation and cellular properties of α-synuclein. J. Biol. Chem. 287, 28243–28262.

Ferese, R., Modugno, N., Campopiano, R., Santilli, M., Zampatti, S., Giardina, E., Nardone, A., Postorivo, D., Fornai, F., Novelli, G., Romoli, E., Ruggieri, S., Gambardella, S., 2015. Four Copies of SNCA Responsible for Autosomal Dominant Parkinson's Disease in Two Italian Siblings. Parkinsons Dis 2015, 546462.

Fitzgerald, J.C., Plun-Favreau, H., 2008. Emerging pathways in genetic Parkinson's disease: autosomal-recessive genes in Parkinson's disease--a common pathway? FEBS J. 275, 5758–5766.

Follett, J., Norwood, S.J., Hamilton, N.A., Mohan, M., Kovtun, O., Tay, S., Zhe, Y., Wood, S.A., Mellick, G.D., Silburn, P.A., Collins, B.M., Bugarcic, A., Teasdale, R.D., 2014. The Vps35 D620N mutation linked to Parkinson's disease disrupts the cargo sorting function of retromer. Traffic 15, 230–244.

Forman, M.S., Trojanowski, J.Q., Lee, V.M.-Y., 2004. Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. Nat. Med. 10, 1055–1063.

Foroud, T., Uniacke, S.K., Liu, L., Pankratz, N., Rudolph, A., Halter, C., Shults, C., Marder, K., Conneally, P.M., Nichols, W.C., Parkinson Study Group, 2003. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 60, 796–801.

Fortin, D.L., Troyer, M.D., Nakamura, K., Kubo, S., Anthony, M.D., Edwards, R.H., 2004. Lipid rafts mediate the synaptic localization of alpha-synuclein. J. Neurosci. 24, 6715–6723.

Franco, R., Ferré, S., Agnati, L., Torvinen, M., Ginés, S., Hillion, J., Casadó, V., Lledó, P., Zoli, M., Lluis, C., Fuxe, K., 2000. Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. Neuropsychopharmacology 23, S50–59.

Fuchs, J., Nilsson, C., Kachergus, J., Munz, M., Larsson, E.-M., Schüle, B., Langston, J.W., Middleton, F.A., Ross, O.A., Hulihan, M., Gasser, T., Farrer, M.J., 2007. Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication. Neurology 68, 916–922.

Fujioka, S., Sundal, C., Strongosky, A.J., Castanedes, M.C., Rademakers, R., Ross, O.A., Vilariño-Güell, C., Farrer, M.J., Wszolek, Z.K., Dickson, D.W., 2013. Sequence variants in eukaryotic translation initiation factor 4-gamma (eIF4G1) are associated with Lewy body dementia. Acta Neuropathol. 125, 425–438.

Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K., Iwatsubo, T., 2002. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat. Cell Biol. 4, 160–164.

Funayama, M., Hasegawa, K., Kowa, H., Saito, M., Tsuji, S., Obata, F., 2002. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann. Neurol. 51, 296–301.

Fung, H.-C., Scholz, S., Matarin, M., Simón-Sánchez, J., Hernandez, D., Britton, A., Gibbs, J.R., Langefeld, C., Stiegert, M.L., Schymick, J., Okun, M.S., Mandel, R.J., Fernandez, H.H., Foote, K.D., Rodríguez, R.L., Peckham, E., De Vrieze, F.W., Gwinn-Hardy, K., Hardy, J.A., Singleton, A., 2006a. Genome-wide genotyping in Parkinson's disease and neurologically normal controls: first stage analysis and public release of data. Lancet Neurol 5, 911–916.

Fuxe, K., Marcellino, D., Borroto-Escuela, D.O., Guescini, M., Fernández-Dueñas, V., Tanganelli, S., Rivera, A., Ciruela, F., Agnati, L.F., 2010. Adenosine-dopamine interactions in the pathophysiology and treatment of CNS disorders. CNS Neurosci Ther 16, e18–42.

G

Gandhi, S., Abramov, A.Y., 2012. Mechanism of oxidative stress in neurodegeneration. Oxid Med Cell Longev 2012, 428010.

Gan-Or, Z., Giladi, N., Orr-Urtreger, A., 2009. Differential phenotype in Parkinson's disease patients with severe versus mild GBA mutations. Brain 132, e125.

Gao, L., Gómez-Garre, P., Díaz-Corrales, F.J., Carrillo, F., Carballo, M., Palomino, A., Díaz-Martín, J., Mejías, R., Vime, P.J., López-Barneo, J., Mir, P., 2009. Prevalence and clinical features of LRRK2 mutations in patients with Parkinson's disease in southern Spain. Eur. J. Neurol. 16, 957–960.

Gasser, T., 2015. Usefulness of Genetic Testing in PD and PD Trials: A Balanced Review. J Parkinsons Dis 5, 209–215.

Gasser, T., Müller-Myhsok, B., Wszolek, Z.K., Oehlmann, R., Calne, D.B., Bonifati, V., Bereznai, B., Fabrizio, E., Vieregge, P., Horstmann, R.D., 1998. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat. Genet. 18, 262–265.

Gautier, C.A., Kitada, T., Shen, J., 2008. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 105, 11364–11369.

Gelb, D.J., Oliver, E., Gilman, S., 1999. Diagnostic criteria for Parkinson disease. Arch. Neurol. 56, 33–39.

Gibb, W.R., Lees, A.J., 1988. A comparison of clinical and pathological features of young- and old-onset Parkinson's disease. Neurology 38, 1402–1406.

Gibrat, C., Bousquet, M., Saint-Pierre, M., Lévesque, D., Calon, F., Rouillard, C., Cicchetti, F., 2010. Cystamine prevents MPTP-induced toxicity in young adult mice via the up-regulation of the brain-derived neurotrophic factor. Prog. Neuropsychopharmacol. Biol. Psychiatry 34, 193–203.

Goedert, M., Jakes, R., Spillantini, M.G., Hasegawa, M., Smith, M.J., Crowther, R.A., 1996. Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. Nature 383, 550–553.

Goker-Alpan, O., Lopez, G., Vithayathil, J., Davis, J., Hallett, M., Sidransky, E., 2008. The spectrum of parkinsonian manifestations associated with glucocerebrosidase mutations. Arch. Neurol. 65, 1353–1357.

Gómez-Garre, P., Jesús, S., Carrillo, F., Cáceres-Redondo, M.T., Huertas-Fernández, I., Bernal-Bernal, I., Bonilla-Toribio, M., Vargas-González, L., Carballo, M., Mir, P., 2014. Systematic mutational analysis of FBXO7 in a Parkinson's disease population from southern Spain. Neurobiol. Aging 35, 727.e5–7.

Gómez-Suaga, P., Fdez, E., Blanca Ramírez, M., Hilfiker, S., 2012. A Link between Autophagy and the Pathophysiology of LRRK2 in Parkinson's Disease. Parkinsons Dis 2012, 324521.

Gorostidi, A., Ruiz-Martínez, J., Lopez de Munain, A., Alzualde, A., Martí Massó, J.F., 2009. LRRK2 G2019S and R1441G mutations associated with Parkinson's disease are common in the Basque Country, but relative prevalence is determined by ethnicity. Neurogenetics 10, 157–159.

Greggio, E., 2012. Role of LRRK2 kinase activity in the pathogenesis of Parkinson's disease. Biochem. Soc. Trans. 40, 1058–1062.

Greggio, E., Taymans, J.-M., Zhen, E.Y., Ryder, J., Vancraenenbroeck, R., Beilina, A., Sun, P., Deng, J., Jaffe, H., Baekelandt, V., Merchant, K., Cookson, M.R., 2009. The Parkinson's disease kinase LRRK2 autophosphorylates its GTPase domain at multiple sites. Biochem. Biophys. Res. Commun. 389, 449–454.

Grünewald, A., Arns, B., Seibler, P., Rakovic, A., Münchau, A., Ramirez, A., Sue, C.M., Klein, C., 2012. ATP13A2 mutations impair mitochondrial function in fibroblasts from patients with Kufor-Rakeb syndrome. Neurobiol. Aging 33, 1843.e1–7.

Grünewald, A., Voges, L., Rakovic, A., Kasten, M., Vandebona, H., Hemmelmann, C., Lohmann, K., Orolicki, S., Ramirez, A., Schapira, A.H.V., Pramstaller, P.P., Sue, C.M., Klein, C., 2010. Mutant Parkin impairs mitochondrial function and morphology in human fibroblasts. PLoS ONE 5, e12962.

J

Jaberi, E., Rohani, M., Shahidi, G.A., Nafissi, S., Arefian, E., Soleimani, M., Moghadam, A., Arzenani, M.K., Keramatian, F., Klotzle, B., Fan, J.-B., Turk, C., Steemers, F., Elahi, E., 2016. Mutation in ADORA1 identified as likely cause of early-onset parkinsonism and cognitive dysfunction. Mov. Disord. 31, 1004–1011.

H

Haavik, J., 1997. L-DOPA is a substrate for tyrosine hydroxylase. J. Neurochem. 69, 1720–1728.

Haft, C.R., de la Luz Sierra, M., Bafford, R., Lesniak, M.A., Barr, V.A., Taylor, S.I., 2000. Human orthologs of yeast vacuolar protein sorting proteins Vps26, 29, and 35: assembly into multimeric complexes. Mol. Biol. Cell 11, 4105–4116.

Hagenah, J., Saunders-Pullman, R., Hedrich, K., Kabakci, K., Habermann, K., Wiegers, K., Mohrmann, K., Lohnau, T., Raymond, D., Vieregge, P., Nygaard, T., Ozelius, L.J., Bressman, S.B., Klein, C., 2005. High mutation rate in dopa-responsive dystonia: detection with comprehensive GCHI screening. Neurology 64, 908–911.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59, 1609–1623.

Halperin, A., Elstein, D., Zimran, A., 2006. Increased incidence of Parkinson disease among relatives of patients with Gaucher disease. Blood Cells Mol. Dis. 36, 426–428.Hamza, T.H., Zabetian, C.P., Tenesa, A., Laederach, A., Montimurro, J., Yearout, D., Kay, D.M., Doheny, K.F., Paschall, J., Pugh, E., Kusel, V.I., Collura, R., Roberts, J., Griffith, A., Samii, A., Scott, W.K., Nutt, J., Factor, S.A., Payami, H., 2010. Common genetic variation in the HLA region is associated with late-onset sporadic Parkinson's disease. Nat. Genet. 42, 781–785.

Hardy, J., Lewis, P., Revesz, T., Lees, A., Paisan-Ruiz, C., 2009. The genetics of Parkinson's syndromes: a critical review. Curr. Opin. Genet. Dev. 19, 254–265.

Harman, D., 1972. The biologic clock: the mitochondria? J Am Geriatr Soc 20, 145–147.

Haugarvoll, K., Wszolek, Z.K., 2009. Clinical features of LRRK2 parkinsonism. Parkinsonism Relat. Disord. 15 Suppl 3, S205–208.

Hauser, D.N., Hastings, T.G., 2013. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. Neurobiol. Dis. 51, 35–42.

Healy, D.G., Abou-Sleiman, P.M., Casas, J.P., Ahmadi, K.R., Lynch, T., Gandhi, S., Muqit, M.M.K., Foltynie, T., Barker, R., Bhatia, K.P., Quinn, N.P., Lees, A.J., Gibson, J.M., Holton, J.L., Revesz, T., Goldstein, D.B., Wood, N.W., 2006. UCHL-1 is not a Parkinson's disease susceptibility gene. Ann. Neurol. 59, 627–633.

Healy, D.G., Falchi, M., O'Sullivan, S.S., Bonifati, V., Durr, A., Bressman, S., Brice, A., Aasly, J., Zabetian, C.P., Goldwurm, S., Ferreira, J.J., Tolosa, E., Kay, D.M., Klein, C., Williams, D.R., Marras, C., Lang, A.E., Wszolek, Z.K., Berciano, J., Schapira, A.H.V., Lynch, T., Bhatia, K.P., Gasser, T., Lees, A.J., Wood, N.W., International LRRK2 Consortium, 2008. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. Lancet Neurol. 7, 583–590.

Healy, D.G., Abou-Sleiman, P.M., Gibson, J.M., Ross, O.A., Jain, S., Gandhi, S., Gosal, D., Muqit, M.M.K., Wood, N.W., Lynch, T., 2004. PINK1 (PARK6) associated Parkinson disease in Ireland. Neurology 63, 1486–1488.

Hedrich, K., Eskelson, C., Wilmot, B., Marder, K., Harris, J., Garrels, J., Meija-Santana, H., Vieregge, P., Jacobs, H., Bressman, S.B., Lang, A.E., Kann, M., Abbruzzese, G., Martinelli, P., Schwinger, E., Ozelius, L.J., Pramstaller, P.P., Klein, C., Kramer, P., 2004. Distribution, type, and origin of Parkin mutations: review and case studies. Mov. Disord. 19, 1146–1157.

Hedrich, K., Kann, M., Lanthaler, A.J., Dalski, A., Eskelson, C., Landt, O., Schwinger, E., Vieregge, P., Lang, A.E., Breakefield, X.O., Ozelius, L.J., Pramstaller, P.P., Klein, C., 2001. The importance of gene dosage studies: mutational analysis of the parkin gene in early-onset parkinsonism. Hum. Mol. Genet. 10, 1649–1656.

Heo, J.Y., Park, J.H., Kim, S.J., Seo, K.S., Han, J.S., Lee, S.H., Kim, J.M., Park, J.I., Park, S.K., Lim, K., Hwang, B.D., Shong, M., Kweon, G.R., 2012. DJ-1 null dopaminergic neuronal cells exhibit defects in mitochondrial function and structure: involvement of mitochondrial complex I assembly. PLoS ONE 7, e32629.

Hernandez, D.G., Nalls, M.A., Ylikotila, P., Keller, M., Hardy, J.A., Majamaa, K., Singleton, A.B., 2012. Genome wide assessment of young onset Parkinson's disease from Finland. PLoS ONE 7, e41859.

Hernandez, D.G., Reed, X., Singleton, A.B., 2016. Genetics in Parkinson disease: Mendelian versus non-Mendelian inheritance. J. Neurochem.

Hicks, A.A., Pétursson, H., Jónsson, T., Stefánsson, H., Jóhannsdóttir, H.S., Sainz, J., Frigge, M.L., Kong, A., Gulcher, J.R., Stefánsson, K., Sveinbjörnsdóttir, S., 2002. A susceptibility gene for late-onset idiopathic Parkinson's disease. Ann. Neurol. 52, 549–555.

Hirsch, E.C., Hunot, S., 2009. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol 8, 382–397.

Hodara, R., Norris, E.H., Giasson, B.I., Mishizen-Eberz, A.J., Lynch, D.R., Lee, V.M.-Y., Ischiropoulos, H., 2004. Functional consequences of alpha-synuclein tyrosine nitration: diminished binding to lipid vesicles and increased fibril formation. J. Biol. Chem. 279, 47746–47753.

Holmans, P., Moskvina, V., Jones, L., Sharma, M., International Parkinson's Disease Genomics Consortium, Vedernikov, A., Buchel, F., Saad, M., Sadd, M., Bras, J.M., Bettella, F., Nicolaou, N., Simón-Sánchez, J., Mittag, F., Gibbs, J.R., Schulte, C., Durr, A., Guerreiro, R., Hernandez, D., Brice, A., Stefánsson, H., Majamaa, K., Gasser, T., Heutink, P., Wood, N.W., Martinez, M., Singleton, A.B., Nalls, M.A., Hardy, J., Morris, H.R., Williams, N.M., 2013. A pathway-based analysis provides additional support for an immune-related genetic susceptibility to Parkinson's disease. Hum. Mol. Genet. 22, 1039–1049.

Houlden, H., Singleton, A.B., 2012. The genetics and neuropathology of Parkinson's disease. Acta Neuropathol. 124, 325–338.

Hruska, K.S., LaMarca, M.E., Scott, C.R., Sidransky, E., 2008. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum. Mutat. 29, 567–583.

Hughes, A.J., Daniel, S.E., Kilford, L., Lees, A.J., 1992. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J. Neurol. Neurosurg. Psychiatr. 55, 181–184.

Huttenlocher, J., Stefansson, H., Steinberg, S., Helgadottir, H.T., Sveinbjörnsdóttir, S., Riess, O., Bauer, P., Stefansson, K., 2015. Heterozygote carriers for CNVs in PARK2 are at increased risk of Parkinson's disease. Hum. Mol. Genet. 24, 5637–5643.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van

Swieten, J., Mann, D., Lynch, T., Heutink, P., 1998. Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702–705.

Hyun, D.-H., Gray, D.A., Halliwell, B., Jenner, P., 2004. Interference with ubiquitination causes oxidative damage and increased protein nitration: implications for neurodegenerative diseases. J. Neurochem. 90, 422–430.

I

Ibáñez, P., Bonnet, A.-M., Débarges, B., Lohmann, E., Tison, F., Pollak, P., Agid, Y., Dürr, A., Brice, A., 2004. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet 364, 1169–1171.

Ibáñez, P., Lesage, S., Janin, S., Lohmann, E., Durif, F., Destée, A., Bonnet, A.-M., Brefel-Courbon, C., Heath, S., Zelenika, D., Agid, Y., Dürr, A., Brice, A., French Parkinson's Disease Genetics Study Group, 2009. Alpha-synuclein gene rearrangements in dominantly inherited parkinsonism: frequency, phenotype, and mechanisms. Arch. Neurol. 66, 102–108.

Ibáñez, P., Lesage, S., Lohmann, E., Thobois, S., De Michele, G., Borg, M., Agid, Y., Dürr, A., Brice, A., French Parkinson's Disease Genetics Study Group, 2006. Mutational analysis of the PINK1 gene in early-onset parkinsonism in Europe and North Africa. Brain 129, 686–694.

Ichinose, H., Ohye, T., Takahashi, E., Seki, N., Hori, T., Segawa, M., Nomura, Y., Endo, K., Tanaka, H., Tsuji, S., 1994. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat. Genet. 8, 236–242.

Ikeuchi, T., Kakita, A., Shiga, A., Kasuga, K., Kaneko, H., Tan, C.-F., Idezuka, J., Wakabayashi, K., Onodera, O., Iwatsubo, T., Nishizawa, M., Takahashi, H., Ishikawa, A., 2008. Patients homozygous and heterozygous for SNCA duplication in a family with parkinsonism and dementia. Arch. Neurol. 65, 514–519.

International HapMap Consortium, 2003. The International HapMap Project. Nature 426, 789–796.

International Parkinson Disease Genomics Consortium, Nalls, M.A., Plagnol, V., Hernandez, D.G., Sharma, M., Sheerin, U.-M., Saad, M., Simón-Sánchez, J., Schulte, C., Lesage, S., Sveinbjörnsdóttir, S., Stefánsson, K., Martinez, M., Hardy, J., Heutink, P., Brice, A., Gasser, T., Singleton, A.B., Wood, N.W., 2011. Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. Lancet 377, 641–649.

International Parkinson's Disease Genomics Consortium (IPDGC), Wellcome Trust Case Control Consortium 2 (WTCCC2), 2011. A two-stage meta-analysis identifies several new loci for Parkinson's disease. PLoS Genet. 7, e1002142.

Iravani, M.M., Sadeghian, M., Leung, C.C.M., Jenner, P., Rose, S., 2012. Lipopolysaccharide-induced nigral inflammation leads to increased IL-1β tissue content and expression of astrocytic glial cell line-derived neurotrophic factor. Neurosci. Lett. 510, 138–142.

J

Jenner, P., Olanow, C.W., 2006. The pathogenesis of cell death in Parkinson's disease. Neurology 66, S24–36.

K

Kalda, A., Yu, L., Oztas, E., Chen, J.-F., 2006. Novel neuroprotection by caffeine and adenosine A(2A) receptor antagonists in animal models of Parkinson's disease. J. Neurol. Sci. 248, 9–15.

Kalinderi, K., Bostantjopoulou, S., Fidani, L., 2016. The genetic background of Parkinson's disease: current progress and future prospects. Acta Neurol. Scand.

Kalinderi, K., Bostantjopoulou, S., Paisan-Ruiz, C., Katsarou, Z., Hardy, J., Fidani, L., 2009. Complete screening for glucocerebrosidase mutations in Parkinson disease patients from Greece. Neurosci. Lett. 452, 87–89.

Kalinderi, K., Fidani, L., Bostantjopoulou, S., Katsarou, Z., Kotsis, A., 2007. The G2019S LRRK2 mutation is uncommon amongst Greek patients with sporadic Parkinson's disease. Eur. J. Neurol. 14, 1088–1090.

Kara, E., Xiromerisiou, G., Spanaki, C., Bozi, M., Koutsis, G., Panas, M., Dardiotis, E., Ralli, S., Bras, J., Letson, C., Edsall, C., Pliner, H., Arepalli, S., Kalinderi, K., Fidani, L., Bostantjopoulou, S., Keller, M.F., Wood, N.W., Hardy, J., Houlden, H., Stefanis, L., Plaitakis, A., Hernandez, D., Hadjigeorgiou, G.M., Nalls, M.A., Singleton, A.B., 2014. Assessment of Parkinson's disease risk loci in Greece. Neurobiol. Aging 35, 442.e9–442.e16.

Kay, D.M., Stevens, C.F., Hamza, T.H., Montimurro, J.S., Zabetian, C.P., Factor, S.A., Samii, A., Griffith, A., Roberts, J.W., Molho, E.S., Higgins, D.S., Gancher, S., Moses, L., Zareparsi, S., Poorkaj, P., Bird, T., Nutt, J., Schellenberg, G.D., Payami, H., 2010. A comprehensive analysis of deletions, multiplications, and copy number variations in PARK2. Neurology 75, 1189–1194.

Keller, M.F., Saad, M., Bras, J., Bettella, F., Nicolaou, N., Simón-Sánchez, J., Mittag, F., Büchel, F., Sharma, M., Gibbs, J.R., Schulte, C., Moskvina, V., Durr, A., Holmans, P., Kilarski, L.L., Guerreiro, R., Hernandez, D.G., Brice, A., Ylikotila, P., Stefánsson, H., Majamaa, K., Morris, H.R., Williams, N., Gasser, T., Heutink, P., Wood, N.W., Hardy, J., Martinez, M., Singleton, A.B., Nalls, M.A., International Parkinson's Disease Genomics Consortium (IPDGC), Wellcome Trust Case Control Consortium 2 (WTCCC2), 2012. Using genome-wide complex trait analysis to quantify "missing heritability" in Parkinson's disease. Hum. Mol. Genet. 21, 4996–5009.

Khan, N.L., Graham, E., Critchley, P., Schrag, A.E., Wood, N.W., Lees, A.J., Bhatia, K.P., Quinn, N., 2003. Parkin disease: a phenotypic study of a large case series. Brain 126, 1279–1292.

Ki, C.-S., Stavrou, E.F., Davanos, N., Lee, W.Y., Chung, E.J., Kim, J.-Y., Athanassiadou, A., 2007. The Ala53Thr mutation in the alpha-synuclein gene in a Korean family with Parkinson disease. Clin. Genet. 71, 471–473.

Kiely, A.P., Asi, Y.T., Kara, E., Limousin, P., Ling, H., Lewis, P., Proukakis, C., Quinn, N., Lees, A.J., Hardy, J., Revesz, T., Houlden, H., Holton, J.L., 2013. α-Synucleinopathy associated with G51D SNCA mutation: a link between Parkinson's disease and multiple system atrophy? Acta Neuropathol. 125, 753–769.

Kim, C., Lee, S.-J., 2008. Controlling the mass action of alpha-synuclein in Parkinson's disease. J. Neurochem. 107, 303–316.

Kim, G.H., Kim, J.E., Rhie, S.J., Yoon, S., 2015. The Role of Oxidative Stress in Neurodegenerative Diseases. Exp Neurobiol 24, 325–340.

Kinghorn, K.J., Castillo-Quan, J.I., Bartolome, F., Angelova, P.R., Li, L., Pope, S., Cochemé, H.M., Khan, S., Asghari, S., Bhatia, K.P., Hardy, J., Abramov, A.Y., Partridge, L., 2015. Loss of PLA2G6 leads to elevated mitochondrial lipid peroxidation and mitochondrial dysfunction. Brain 138, 1801–1816.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., Shimizu, N., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392, 605–608.

Klein, C., Djarmati, A., Hedrich, K., Schäfer, N., Scaglione, C., Marchese, R., Kock, N., Schüle, B., Hiller, A., Lohnau, T., Winkler, S., Wiegers, K., Hering, R., Bauer, P., Riess, O., Abbruzzese, G., Martinelli, P., Pramstaller, P.P., 2005. PINK1, Parkin, and DJ-1 mutations in Italian patients with early-onset parkinsonism. Eur. J. Hum. Genet. 13, 1086–1093.

Kong, S.M.Y., Chan, B.K.K., Park, J.-S., Hill, K.J., Aitken, J.B., Cottle, L., Farghaian, H., Cole, A.R., Lay, P.A., Sue, C.M., Cooper, A.A., 2014. Parkinson's disease-linked human PARK9/ATP13A2 maintains zinc homeostasis and promotes α-Synuclein externalization via exosomes. Hum. Mol. Genet. 23, 2816–2833.

Köroğlu, Ç., Baysal, L., Cetinkaya, M., Karasoy, H., Tolun, A., 2013. DNAJC6 is responsible for juvenile parkinsonism with phenotypic variability. Parkinsonism Relat. Disord. 19, 320–324.

Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H., Yoshihara, H., Hirokawa, T., Endo, T., Fon, E.A., Trempe, J.-F., Saeki, Y., Tanaka, K., Matsuda, N., 2014. Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature 510, 162–166.

Krebs, C.E., Karkheiran, S., Powell, J.C., Cao, M., Makarov, V., Darvish, H., Di Paolo, G., Walker, R.H., Shahidi, G.A., Buxbaum, J.D., De Camilli, P., Yue, Z., Paisán-Ruiz, C., 2013. The Sac1 domain of SYNJ1 identified mutated in a family with early-onset progressive Parkinsonism with generalized seizures. Hum. Mutat. 34, 1200–1207.

Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J.T., Schöls, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. 18, 106–108.

Krüger, R., Vieira-Saecker, A.M., Kuhn, W., Berg, D., Müller, T., Kühnl, N., Fuchs, G.A., Storch, A., Hungs, M., Woitalla, D., Przuntek, H., Epplen, J.T., Schöls, L., Riess, O., 1999. Increased susceptibility to sporadic Parkinson's disease by a certain combined alpha-synuclein/apolipoprotein E genotype. Ann. Neurol. 45, 611–617.

Kumar, P., Henikoff, S., Ng, P.C., 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat. Protoc. 4, 1073–1081.

L

Laman, H., 2006. Fbxo7 gets proactive with cyclin D/cdk6. Cell Cycle 5, 279–282.

Langston, J.W., Ballard, P.A., 1983. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N. Engl. J. Med. 309, 310.

Langston, J.W., Ballard, P., Tetrud, J.W., Irwin, I., 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979–980.

Lavedan, C., Dehejia, A., Pike, B., Dutra, A., Leroy, E., Ide, S.E., Root, H., Rubenstein, J., Boyer, R.L., Chandrasekharappa, S., Makalowska, I., Nussbaum, R.L., Polymeropoulos, M.H., 1998. Contig map of the Parkinson's disease region on 4q21-q23. DNA Res. 5, 19–23.

Lees, A.J., Hardy, J., Revesz, T., 2009. Parkinson's disease. Lancet 373, 2055–2066.

Lee, S., Emond, M.J., Bamshad, M.J., Barnes, K.C., Rieder, M.J., Nickerson, D.A., NHLBI GO Exome Sequencing Project—ESP Lung Project Team, Christiani, D.C., Wurfel, M.M., Lin, X., 2012. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. Am. J. Hum. Genet. 91, 224–237.

Lesage, S., Dürr, A., Tazir, M., Lohmann, E., Leutenegger, A.-L., Janin, S., Pollak, P., Brice, A., French Parkinson's Disease Genetics Study Group, 2006. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N. Engl. J. Med. 354, 422–423.

Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honoré, A., Rozas, N., Pieri, L., Madiona, K., Dürr, A., Melki, R., Verny, C., Brice, A., French Parkinson's Disease Genetics Study Group, 2013. G51D α-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. Ann. Neurol. 73, 459–471.

Lesage, S., Condroyer, C., Klebe, S., Honoré, A., Tison, F., Brefel-Courbon, C., Dürr, A., Brice, A., French Parkinson's Disease Genetics Study Group, 2012. Identification of VPS35 mutations replicated in French families with Parkinson disease. Neurology 78, 1449–1450.

Lesage, S., Drouet, V., Majounie, E., Deramecourt, V., Jacoupy, M., Nicolas, A., Cormier-Dequaire, F., Hassoun, S.M., Pujol, C., Ciura, S., Erpapazoglou, Z., Usenko, T., Maurage, C.-A., Sahbatou, M., Liebau, S., Ding, J., Bilgic, B., Emre, M., Erginel-Unaltuna, N., Guven, G., Tison, F., Tranchant, C., Vidailhet, M., Corvol, J.-C., Krack, P., Leutenegger, A.-L., Nalls, M.A., Hernandez, D.G., Heutink, P., Gibbs, J.R., Hardy, J., Wood, N.W., Gasser, T., Durr, A., Deleuze, J.-F., Tazir, M., Destée, A., Lohmann, E., Kabashi, E., Singleton, A., Corti, O., Brice, A., French Parkinson's Disease Genetics Study (PDG), International Parkinson's Disease Genomics Consortium (IPDGC), 2016. Loss of VPS13C Function in Autosomal-Recessive Parkinsonism Causes Mitochondrial Dysfunction and Increases PINK1/Parkin-Dependent Mitophagy. Am. J. Hum. Genet. 98, 500–513.

Lesage, S., Magali, P., Lohmann, E., Lacomblez, L., Teive, H., Janin, S., Cousin, P.-Y., Dürr, A., Brice, A., French Parkinson Disease Genetics Study Group, 2007. Deletion of the parkin and PACRG gene promoter in early-onset parkinsonism. Hum. Mutat. 28, 27–32.

Lesage, S., Patin, E., Condroyer, C., Leutenegger, A.-L., Lohmann, E., Giladi, N., Bar-Shira, A., Belarbi, S., Hecham, N., Pollak, P., Ouvrard-Hernandez, A.-M., Bardien, S., Carr, J., Benhassine, T., Tomiyama, H., Pirkevi, C., Hamadouche, T., Cazeneuve, C., Basak, A.N., Hattori, N., Dürr, A., Tazir, M., Orr-Urtreger, A., Quintana-Murci, L., Brice, A., French Parkinson's Disease Genetics Study Group, 2010. Parkinson's disease-related LRRK2 G2019S mutation results from independent mutational events in humans. Hum. Mol. Genet. 19, 1998–2004.

Li, L., Funayama, M., Tomiyama, H., Li, Y., Yoshino, H., Sasaki, R., Kokubo, Y., Kuzuhara, S., Mizuno, Y., Hattori, N., 2010. No evidence for pathogenic role of GIGYF2 mutation in Parkinson disease in Japanese patients. Neurosci. Lett. 479, 245–248.

Lill, C.M., Hansen, J., Olsen, J.H., Binder, H., Ritz, B., Bertram, L., 2015. Impact of Parkinson's disease risk loci on age at onset. Mov. Disord. 30, 847–850.

Lill, C.M., Roehr, J.T., McQueen, M.B., Kavvoura, F.K., Bagade, S., Schjeide, B.-M.M., Schjeide, L.M., Meissner, E., Zauft, U., Allen, N.C., Liu, T., Schilling, M., Anderson, K.J., Beecham, G., Berg, D., Biernacka, J.M., Brice, A., DeStefano, A.L., Do, C.B., Eriksson, N., Factor, S.A., Farrer, M.J., Foroud, T., Gasser, T., Hamza, T., Hardy, J.A., Heutink, P., Hill-Burns, E.M., Klein, C., Latourelle, J.C., Maraganore, D.M., Martin, E.R., Martinez, M., Myers, R.H., Nalls, M.A., Pankratz, N., Payami, H., Satake, W., Scott, W.K., Sharma, M., Singleton, A.B., Stefansson, K., Toda, T., Tung, J.Y., Vance, J., Wood, N.W., Zabetian, C.P., 23andMe Genetic Epidemiology of Parkinson's Disease Consortium, International Parkinson's Disease Genomics Consortium, Parkinson's Disease GWAS Consortium, Wellcome Trust Case Control Consortium 2), Young, P., Tanzi, R.E., Khoury, M.J., Zipp, F., Lehrach, H., Ioannidis, J.P.A., Bertram, L., 2012. Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: The PDGene database. PLoS Genet. 8, e1002548.

Lindersson, E., Beedholm, R., Højrup, P., Moos, T., Gai, W., Hendil, K.B., Jensen, P.H., 2004. Proteasomal inhibition by alpha-synuclein filaments and oligomers. J. Biol. Chem. 279, 12924–12934.

Li, Y., Tomiyama, H., Sato, K., Hatano, Y., Yoshino, H., Atsumi, M., Kitaguchi, M., Sasaki, S., Kawaguchi, S., Miyajima, H., Toda, T., Mizuno, Y., Hattori, N., 2005. Clinicogenetic study of PINK1 mutations in autosomal recessive early-onset parkinsonism. Neurology 64, 1955–1957.Lücking, C.B., Dürr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B.S., Meco, G., Denèfle, P., Wood, N.W., Agid, Y., Brice, A., French Parkinson's Disease Genetics Study Group, European Consortium on Genetic Susceptibility in Parkinson's Disease, 2000. Association between early-onset Parkinson's disease and mutations in the parkin gene. N. Engl. J. Med. 342, 1560–1567.

Luk, K.C., Kehm, V.M., Zhang, B., O'Brien, P., Trojanowski, J.Q., Lee, V.M.Y., 2012. Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice. J. Exp. Med. 209, 975–986.

Lwin, A., Orvisky, E., Goker-Alpan, O., LaMarca, M.E., Sidransky, E., 2004. Glucocerebrosidase mutations in subjects with parkinsonism. Mol. Genet. Metab. 81, 70–73.

M

Malik, I., Turk, J., Mancuso, D.J., Montier, L., Wohltmann, M., Wozniak, D.F., Schmidt, R.E., Gross, R.W., Kotzbauer, P.T., 2008. Disrupted membrane homeostasis and accumulation of ubiquitinated proteins in a mouse model of infantile neuroaxonal dystrophy caused by PLA2G6 mutations. Am. J. Pathol. 172, 406–416.

Maraganore, D.M., de Andrade, M., Lesnick, T.G., Strain, K.J., Farrer, M.J., Rocca, W.A., Pant, P.V.K., Frazer, K.A., Cox, D.R., Ballinger, D.G., 2005. High-resolution whole-genome association study of Parkinson disease. Am. J. Hum. Genet. 77, 685–693.

Maraganore, D.M., Lesnick, T.G., Elbaz, A., Chartier-Harlin, M.-C., Gasser, T., Krüger, R., Hattori, N., Mellick, G.D., Quattrone, A., Satoh, J.-I., Toda, T., Wang, J., Ioannidis, J.P.A., de Andrade, M., Rocca, W.A., Toda, T., UCHL1 Global Genetics Consortium, 2004. UCHL1 is a Parkinson's disease susceptibility gene. Ann. Neurol. 55, 512–521.

Marongiu, R., Brancati, F., Antonini, A., Ialongo, T., Ceccarini, C., Scarciolla, O., Capalbo, A., Benti, R., Pezzoli, G., Dallapiccola, B., Goldwurm, S., Valente, E.M., 2007. Whole gene deletion and splicing mutations expand the PINK1 genotypic spectrum. Hum. Mutat. 28, 98.

Martins, L.M., Morrison, A., Klupsch, K., Fedele, V., Moisoi, N., Teismann, P., Abuin, A., Grau, E., Geppert, M., Livi, G.P., Creasy, C.L., Martin, A., Hargreaves, I., Heales, S.J., Okada, H., Brandner, S., Schulz, J.B., Mak, T., Downward, J., 2004. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. Mol. Cell. Biol. 24, 9848–9862.

Mata, I.F., Kachergus, J.M., Taylor, J.P., Lincoln, S., Aasly, J., Lynch, T., Hulihan, M.M., Cobb, S.A., Wu, R.-M., Lu, C.-S., Lahoz, C., Wszolek, Z.K., Farrer, M.J., 2005a. Lrrk2 pathogenic substitutions in Parkinson's disease. Neurogenetics 6, 171–177.

Mata, I.F., Shi, M., Agarwal, P., Chung, K.A., Edwards, K.L., Factor, S.A., Galasko, D.R., Ginghina, C., Griffith, A., Higgins, D.S., Kay, D.M., Kim, H., Leverenz, J.B., Quinn, J.F., Roberts, J.W., Samii, A., Snapinn, K.W., Tsuang, D.W., Yearout, D., Zhang, J., Payami, H., Zabetian, C.P., 2010. SNCA variant associated with Parkinson disease and plasma alpha-synuclein level. Arch. Neurol. 67, 1350–1356.

Mata, I.F., Taylor, J.P., Kachergus, J., Hulihan, M., Huerta, C., Lahoz, C., Blazquez, M., Guisasola, L.M., Salvador, C., Ribacoba, R., Martinez, C., Farrer, M., Alvarez, V., 2005b. LRRK2 R1441G in Spanish patients with Parkinson's disease. Neurosci. Lett. 382, 309–311

Matarin, M., Simon-Sanchez, J., Fung, H.-C., Scholz, S., Gibbs, J.R., Hernandez, D.G., Crews, C., Britton, A., De Vrieze, F.W., Brott, T.G., Brown, R.D., Worrall, B.B., Silliman, S., Case, L.D., Hardy, J.A., Rich, S.S., Meschia, J.F., Singleton, A.B., 2008. Structural genomic variation in ischemic stroke. Neurogenetics 9, 101–108.

Mathew, C.G., 2008. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. Nat. Rev. Genet. 9, 9–14.

Mazzulli, J.R., Xu, Y.-H., Sun, Y., Knight, A.L., McLean, P.J., Caldwell, G.A., Sidransky, E., Grabowski, G.A., Krainc, D., 2011. Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell 146, 37–52.

McCarthy, M.I., Abecasis, G.R., Cardon, L.R., Goldstein, D.B., Little, J., Ioannidis, J.P.A., Hirschhorn, J.N., 2008. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat. Rev. Genet. 9, 356–369.

McGeer, P.L., Itagaki, S., Boyes, B.E., McGeer, E.G., 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38, 1285–1291.

McNaught, K.S.P., Belizaire, R., Isacson, O., Jenner, P., Olanow, C.W., 2003. Altered proteasomal function in sporadic Parkinson's disease. Exp. Neurol. 179, 38–46.

Meeus, B., Nuytemans, K., Crosiers, D., Engelborghs, S., Pals, P., Pickut, B., Peeters, K., Mattheijssens, M., Corsmit, E., Cras, P., De Deyn, P.P., Theuns, J., Van Broeckhoven, C., 2011. GIGYF2 has no major role in Parkinson genetic etiology in a Belgian population. Neurobiol. Aging 32, 308–312.

Mekler, L.B., 1975. On the problem of oncogene of tumour viruses. Acta Virol. 19, 501–508.

Metzker, M.L., 2010. Sequencing technologies - the next generation. Nat. Rev. Genet. 11, 31–46.

Mencacci, N.E., Isaias, I.U., Reich, M.M., Ganos, C., Plagnol, V., Polke, J.M., Bras, J., Hersheson, J., Stamelou, M., Pittman, A.M., Noyce, A.J., Mok, K.Y., Opladen, T., Kunstmann, E., Hodecker, S., Münchau, A., Volkmann, J., Samnick, S., Sidle, K., Nanji, T., Sweeney, M.G., Houlden, H., Batla, A., Zecchinelli, A.L., Pezzoli, G., Marotta, G., Lees, A., Alegria, P., Krack, P., Cormier-Dequaire, F., Lesage, S., Brice, A., Heutink, P., Gasser, T., Lubbe, S.J., Morris, H.R., Taba, P., Koks, S., Majounie, E., Raphael Gibbs, J., Singleton, A., Hardy, J., Klebe, S., Bhatia, K.P., Wood, N.W., International Parkinson's Disease Genomics Consortium and UCL-exomes consortium, 2014. Parkinson's disease in GTP cyclohydrolase 1 mutation carriers. Brain 137, 2480–2492.

Mhyre, T.R., Boyd, J.T., Hamill, R.W., Maguire-Zeiss, K.A., 2012. Parkinson's disease. Subcell. Biochem. 65, 389–455.

Michel, P.P., Hirsch, E.C., Hunot, S., 2016. Understanding Dopaminergic Cell Death Pathways in Parkinson Disease. Neuron 90, 675–691.

Mitsumoto, A., Nakagawa, Y., 2001. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic. Res. 35, 885–893.

Miyake, Y., Tanaka, K., Fukushima, W., Kiyohara, C., Sasaki, S., Tsuboi, Y., Yamada, T., Oeda, T., Shimada, H., Kawamura, N., Sakae, N., Fukuyama, H., Hirota, Y., Nagai, M., Fukuoka Kinki Parkinson's Disease Study Group, 2012. SNCA polymorphisms, smoking, and sporadic Parkinson's disease in Japanese. Parkinsonism Relat. Disord. 18, 557–561.

Moskvina, V., Harold, D., Russo, G., Vedernikov, A., Sharma, M., Saad, M., Holmans, P., Bras, J.M., Bettella, F., Keller, M.F., Nicolaou, N., Simón-Sánchez, J., Gibbs, J.R., Schulte, C., Durr, A., Guerreiro, R., Hernandez, D., Brice, A., Stefánsson, H., Majamaa, K., Gasser, T., Heutink, P., Wood, N., Martinez, M., Singleton, A.B., Nalls, M.A., Hardy, J., Owen, M.J., O'Donovan, M.C., Williams, J., Morris, H.R., Williams, N.M., IPDGC and GERAD Investigators, 2013. Analysis of genome-wide association studies of Alzheimer disease and of Parkinson disease to determine if these 2 diseases share a common genetic risk. JAMA Neurol 70, 1268–1276.

Moore, A.H., Bigbee, M.J., Boynton, G.E., Wakeham, C.M., Rosenheim, H.M., Staral, C.J., Morrissey, J.L., Hund, A.K., 2010. Non-Steroidal Anti-Inflammatory Drugs in Alzheimer's Disease and Parkinson's Disease: Reconsidering the Role of Neuroinflammation. Pharmaceuticals (Basel) 3, 1812–1841. doi:10.3390/ph3061812

Mubaidin, A., Roberts, E., Hampshire, D., Dehyyat, M., Shurbaji, A., Mubaidien, M., Jamil, A., Al-Din, A., Kurdi, A., Woods, C.G., 2003. Karak syndrome: a novel degenerative disorder of the basal ganglia and cerebellum. J. Med. Genet. 40, 543–546.

Myers, A.J., Kaleem, M., Marlowe, L., Pittman, A.M., Lees, A.J., Fung, H.C., Duckworth, J., Leung, D., Gibson, A., Morris, C.M., de Silva, R., Hardy, J., 2005. The H1c haplotype at the MAPT locus is associated with Alzheimer's disease. Hum. Mol. Genet. 14, 2399–2404.

Myhre, R., Toft, M., Kachergus, J., Hulihan, M.M., Aasly, J.O., Klungland, H., Farrer, M.J., 2008. Multiple alpha-synuclein gene polymorphisms are associated with Parkinson's disease in a Norwegian population. Acta Neurol. Scand. 118, 320–327.

N

Nagakubo, D., Taira, T., Kitaura, H., Ikeda, M., Tamai, K., Iguchi-Ariga, S.M., Ariga, H., 1997. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem. Biophys. Res. Commun. 231, 509–513.

Najim al-Din, A.S., Wriekat, A., Mubaidin, A., Dasouki, M., Hiari, M., 1994. Pallido-pyramidal degeneration, supranuclear upgaze paresis and dementia: Kufor-Rakeb syndrome. Acta Neurol. Scand. 89, 347–352.

Nakamura, K., Nemani, V.M., Azarbal, F., Skibinski, G., Levy, J.M., Egami, K., Munishkina, L., Zhang, J., Gardner, B., Wakabayashi, J., Sesaki, H., Cheng, Y., Finkbeiner, S., Nussbaum, R.L., Masliah, E., Edwards, R.H., 2011. Direct membrane association drives mitochondrial fission by the Parkinson disease-associated protein alpha-synuclein. J. Biol. Chem. 286, 20710–20726.

Nalls, M.A., Bras, J., Hernandez, D.G., Keller, M.F., Majounie, E., Renton, A.E., Saad, M., Jansen, I., Guerreiro, R., Lubbe, S., Plagnol, V., Gibbs, J.R., Schulte, C., Pankratz, N., Sutherland, M., Bertram, L., Lill, C.M., DeStefano, A.L., Faroud, T., Eriksson, N., Tung, J.Y., Edsall, C., Nichols, N., Brooks, J., Arepalli, S., Pliner, H., Letson, C., Heutink, P., Martinez, M., Gasser, T., Traynor, B.J., Wood, N., Hardy, J., Singleton, A.B., International Parkinson's Disease Genomics Consortium (IPDGC), Parkinson's Disease meta-analysis consortium, 2015a. NeuroX, a fast and efficient genotyping platform for investigation of neurodegenerative diseases. Neurobiol. Aging 36, 1605.e7–12.

Nalls, M.A., Escott-Price, V., Williams, N.M., Lubbe, S., Keller, M.F., Morris, H.R., Singleton, A.B., International Parkinson's Disease Genomics Consortium (IPDGC), 2015b. Genetic risk and age in Parkinson's disease: Continuum not stratum. Mov. Disord. 30, 850–854.

Nalls, M.A., McLean, C.Y., Rick, J., Eberly, S., Hutten, S.J., Gwinn, K., Sutherland, M., Martinez, M., Heutink, P., Williams, N.M., Hardy, J., Gasser, T., Brice, A., Price, T.R., Nicolas, A., Keller, M.F., Molony, C., Gibbs, J.R., Chen-Plotkin, A., Suh, E., Letson, C., Fiandaca, M.S., Mapstone, M., Federoff, H.J., Noyce, A.J., Morris, H., Van Deerlin, V.M., Weintraub, D., Zabetian, C., Hernandez, D.G., Lesage, S., Mullins, M., Conley, E.D., Northover, C.A.M., Frasier, M., Marek, K., Day-Williams, A.G., Stone, D.J., Ioannidis, J.P.A., Singleton, A.B., Parkinson's Disease Biomarkers Program and Parkinson's Progression Marker Initiative investigators, 2015c. Diagnosis of Parkinson's disease on the basis of clinical and genetic classification: a population-based modelling study. Lancet Neurol 14, 1002–1009.

Nalls, M.A., Pankratz, N., Lill, C.M., Do, C.B., Hernandez, D.G., Saad, M., DeStefano, A.L., Kara, E., Bras, J., Sharma, M., Schulte, C., Keller, M.F., Arepalli, S., Letson, C., Edsall, C., Stefansson, H., Liu, X., Pliner, H., Lee, J.H., Cheng, R., International Parkinson's Disease Genomics Consortium (IPDGC), Parkinson's Study Group (PSG) Parkinson's Research: The Organized GENetics Initiative (PROGENI), 23andMe, GenePD, NeuroGenetics Research Consortium (NGRC), Hussman Institute of Human Genomics (HIHG), Ashkenazi Jewish Dataset Investigator, Cohorts for Health and Aging Research in Genetic Epidemiology (CHARGE), North American Brain Expression Consortium (NABEC), United Kingdom Brain Expression Consortium (UKBEC), Greek Parkinson's Disease Consortium, Alzheimer Genetic Analysis Group, Ikram, M.A., Ioannidis, J.P.A., Hadjigeorgiou, G.M., Bis, J.C., Martinez, M., Perlmutter, J.S., Goate, A., Marder, K., Fiske, B., Sutherland, M., Xiromerisiou, G., Myers, R.H., Clark, L.N., Stefansson, K., Hardy, J.A., Heutink, P., Chen, H., Wood, N.W., Houlden, H., Payami, H., Brice, A., Scott, W.K., Gasser, T., Bertram, L., Eriksson, N., Foroud, T., Singleton, A.B., 2014. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat. Genet. 46, 989–993.

Nardocci, N., Zorzi, G., Blau, N., Fernandez Alvarez, E., Sesta, M., Angelini, L., Pannacci, M., Invernizzi, F., Garavaglia, B., 2003. Neonatal dopa-responsive extrapyramidal syndrome in twins with recessive GTPCH deficiency. Neurology 60, 335–337.

Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.-F., Gautier, C.A., Shen, J., Cookson, M.R., Youle, R.J., 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298.

Narendra, D., Walker, J.E., Youle, R., 2012. Mitochondrial quality control mediated by PINK1 and Parkin: links to parkinsonism. Cold Spring Harb Perspect Biol 4.

Neumann, J., Bras, J., Deas, E., O'Sullivan, S.S., Parkkinen, L., Lachmann, R.H., Li, A., Holton, J., Guerreiro, R., Paudel, R., Segarane, B., Singleton, A., Lees, A., Hardy, J., Houlden, H., Revesz, T., Wood, N.W., 2009. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain 132, 1783–1794.

Neudorfer, O., Giladi, N., Elstein, D., Abrahamov, A., Turezkite, T., Aghai, E., Reches, A., Bembi, B., Zimran, A., 1996. Occurrence of Parkinson's syndrome in type I Gaucher disease. QJM 89, 691–694.

Nichols, N., Bras, J.M., Hernandez, D.G., Jansen, I.E., Lesage, S., Lubbe, S., Singleton, A.B., International Parkinson's Disease Genomics Consortium, 2015. EIF4G1 mutations do not cause Parkinson's disease. Neurobiol. Aging 36, 2444.e1–4.

Nichols, W.C., Kissell, D.K., Pankratz, N., Pauciulo, M.W., Elsaesser, V.E., Clark, K.A., Halter, C.A., Rudolph, A., Wojcieszek, J., Pfeiffer, R.F., Foroud, T., Parkinson Study Group-PROGENI Investigators, 2009. Variation in GIGYF2 is not associated with Parkinson disease. Neurology 72, 1886–1892.

Nikam, S., Nikam, P., Ahaley, S.K., Sontakke, A.V., 2009. Oxidative stress in Parkinson's disease. Indian J Clin Biochem 24, 98–101.

Nishioka, K., Hayashi, S., Farrer, M.J., Singleton, A.B., Yoshino, H., Imai, H., Kitami, T., Sato, K., Kuroda, R., Tomiyama, H., Mizoguchi, K., Murata, M., Toda, T., Imoto, I., Inazawa, J., Mizuno, Y., Hattori, N., 2006. Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease. Ann. Neurol. 59, 298–309.

Nishioka, K., Vilariño-Güell, C., Cobb, S.A., Kachergus, J.M., Ross, O.A., Wider, C., Gibson, R.A., Hentati, F., Farrer, M.J., 2010. Glucocerebrosidase mutations are not a common risk factor for Parkinson disease in North Africa. Neurosci. Lett. 477, 57–60.

Nothwehr, S.F., Ha, S.A., Bruinsma, P., 2000. Sorting of yeast membrane proteins into an endosome-to-Golgi pathway involves direct interaction of their cytosolic domains with Vps35p. J. Cell Biol. 151, 297–310.

Noyce, A.J., Bestwick, J.P., Silveira-Moriyama, L., Hawkes, C.H., Giovannoni, G., Lees, A.J., Schrag, A., 2012. Meta-analysis of early nonmotor features and risk factors for Parkinson disease. Ann. Neurol. 72, 893–901.

Noyce, A.J., Lees, A.J., Schrag, A.-E., 2016. The prediagnostic phase of Parkinson's disease. J. Neurol. Neurosurg. Psychiatr. 87, 871–878.

Nuytemans, K., Bademci, G., Inchausti, V., Dressen, A., Kinnamon, D.D., Mehta, A., Wang, L., Züchner, S., Beecham, G.W., Martin, E.R., Scott, W.K., Vance, J.M., 2013. Whole exome sequencing of rare variants in EIF4G1 and VPS35 in Parkinson disease. Neurology 80, 982–989.

Nuytemans, K., Theuns, J., Cruts, M., Van Broeckhoven, C., 2010. Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. Hum. Mutat. 31, 763–780.

Nyholt, D.R., 2004. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am. J. Hum. Genet. 74, 765–769.

O

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P.J., Haass, C., 2000. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. J. Biol. Chem. 275, 390–397.

Olgiati, S., De Rosa, A., Quadri, M., Criscuolo, C., Breedveld, G.J., Picillo, M., Pappatà, S., Quarantelli, M., Barone, P., De Michele, G., Bonifati, V., 2014. PARK20 caused by SYNJ1 homozygous Arg258Gln mutation in a new Italian family. Neurogenetics 15, 183–188.

Ono, K., Hirohata, M., Yamada, M., 2007. Anti-fibrillogenic and fibril-destabilizing activity of nicotine in vitro: implications for the prevention and therapeutics of Lewy body diseases. Exp. Neurol. 205, 414–424.

Orsi, A., Razi, M., Dooley, H.C., Robinson, D., Weston, A.E., Collinson, L.M., Tooze, S.A., 2012. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. Mol. Biol. Cell 23, 1860–1873.

Ott, J., Wang, J., Leal, S.M., 2015. Genetic linkage analysis in the age of whole-genome sequencing. Nat. Rev. Genet. 16, 275–284.

P

Paisan-Ruiz, C., Bhatia, K.P., Li, A., Hernandez, D., Davis, M., Wood, N.W., Hardy, J., Houlden, H., Singleton, A., Schneider, S.A., 2009. Characterization of PLA2G6 as a locus for dystonia-parkinsonism. Ann. Neurol. 65, 19–23.

Paisán-Ruíz, C., Jain, S., Evans, E.W., Gilks, W.P., Simón, J., van der Brug, M., López de Munain, A., Aparicio, S., Gil, A.M., Khan, N., Johnson, J., Martinez, J.R., Nicholl, D., Carrera, I.M., Pena, A.S., de Silva, R., Lees, A., Martí-Massó, J.F., Pérez-Tur, J., Wood, N.W., Singleton, A.B., 2004. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44, 595–600.

Paisán-Ruíz, C., Jain, S., Evans, E.W., Gilks, W.P., Simón, J., van der Brug, M., López de Munain, A., Aparicio, S., Gil, A.M., Khan, N., Johnson, J., Martinez, J.R., Nicholl, D., Carrera, I.M., Pena, A.S., de Silva, R., Lees, A., Martí-Massó, J.F., Pérez-Tur, J., Wood, N.W., Singleton, A.B., 2004. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44, 595–600.

Paisán-Ruiz, C., Lewis, P.A., Singleton, A.B., 2013. LRRK2: cause, risk, and mechanism. J. Park. Dis. 3, 85–103.

Pankratz, N., Beecham, G.W., DeStefano, A.L., Dawson, T.M., Doheny, K.F., Factor, S.A., Hamza, T.H., Hung, A.Y., Hyman, B.T., Ivinson, A.J., Krainc, D., Latourelle, J.C., Clark, L.N., Marder, K., Martin, E.R., Mayeux, R., Ross, O.A., Scherzer, C.R., Simon, D.K., Tanner, C., Vance, J.M., Wszolek, Z.K., Zabetian, C.P., Myers, R.H., Payami, H., Scott, W.K., Foroud, T., PD GWAS Consortium, 2012. Meta-analysis of Parkinson's disease: identification of a novel locus, RIT2. Ann. Neurol. 71, 370–384.

Pankratz, N., Nichols, W.C., Uniacke, S.K., Halter, C., Rudolph, A., Shults, C., Conneally, P.M., Foroud, T., Parkinson Study Group, 2003. Significant linkage of Parkinson disease to chromosome 2q36-37. Am. J. Hum. Genet. 72, 1053–1057.

Pankratz, N., Pauciulo, M.W., Elsaesser, V.E., Marek, D.K., Halter, C.A., Wojcieszek, J., Rudolph, A., Shults, C.W., Foroud, T., Nichols, W.C., Parkinson Study Group - PROGENI Investigators, 2006. Mutations in DJ-1 are rare in familial Parkinson disease. Neurosci. Lett. 408, 209–213.

Pankratz, N., Wilk, J.B., Latourelle, J.C., DeStefano, A.L., Halter, C., Pugh, E.W., Doheny, K.F., Gusella, J.F., Nichols, W.C., Foroud, T., Myers, R.H., PSG-PROGENI and GenePD Investigators, Coordinators and Molecular Genetic Laboratories, 2009. Genomewide association study for susceptibility genes contributing to familial Parkinson disease. Hum. Genet. 124, 593–605.

Papapetropoulos, S., Adi, N., Shehadeh, L., Bishopric, N., Singer, C., Argyriou, A.A., Chroni, E., 2008. Is the G2019S LRRK2 mutation common in all southern European populations? J Clin Neurosci 15, 1027–1030.

Papapetropoulos, S., Paschalis, C., Athanassiadou, A., Papadimitriou, A., Ellul, J., Polymeropoulos, M.H., Papapetropoulos, T., 2001. Clinical phenotype in patients with alpha-synuclein Parkinson's disease living in Greece in comparison with patients with sporadic Parkinson's disease. J. Neurol. Neurosurg. Psychiatr. 70, 662–665.

Parihar, M.S., Parihar, A., Fujita, M., Hashimoto, M., Ghafourifar, P., 2008. Mitochondrial association of alpha-synuclein causes oxidative stress. Cell. Mol. Life Sci. 65, 1272–1284.

Parkinson, J., 2002. An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci 14, 223–236; discussion 222.

Park, J.K., Tayebi, N., Stubblefield, B.K., LaMarca, M.E., MacKenzie, J.J., Stone, D.L., Sidransky, E., 2002. The E326K mutation and Gaucher disease: mutation or polymorphism? Clin. Genet. 61, 32–34.

Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J.-M., Chung, J., 2006. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature 441, 1157–1161.

Park, J.-S., Blair, N.F., Sue, C.M., 2015. The role of ATP13A2 in Parkinson's disease: Clinical phenotypes and molecular mechanisms. Mov. Disord. 30, 770–779.

Pasanen, P., Myllykangas, L., Siitonen, M., Raunio, A., Kaakkola, S., Lyytinen, J., Tienari, P.J., Pöyhönen, M., Paetau, A., 2014. Novel α-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. Neurobiol. Aging 35, 2180.e1–5.

Periquet, M., Latouche, M., Lohmann, E., Rawal, N., De Michele, G., Ricard, S., Teive, H., Fraix, V., Vidailhet, M., Nicholl, D., Barone, P., Wood, N.W., Raskin, S., Deleuze, J.-F., Agid, Y., Dürr, A., Brice, A., French Parkinson's Disease Genetics Study Group, European Consortium on Genetic Susceptibility in Parkinson's Disease, 2003. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 126, 1271–1278.

Perry, V.H., 2012. Innate inflammation in Parkinson's disease. Cold Spring Harb Perspect Med 2, a009373.

Picillo, M., Ranieri, A., Orefice, G., Bonifati, V., Barone, P., 2014. Clinical progression of SYNJ1-related early onset atypical parkinsonism: 3-year follow up of the original Italian family. J. Neurol. 261, 823–824.

Pihlstrøm, L., Axelsson, G., Bjørnarå, K.A., Dizdar, N., Fardell, C., Forsgren, L., Holmberg, B., Larsen, J.P., Linder, J., Nissbrandt, H., Tysnes, O.-B., Ohman, E., Dietrichs, E., Toft, M., 2013. Supportive evidence for 11 loci from genome-wide association studies in Parkinson's disease. Neurobiol. Aging 34, 1708.e7–13.

Pino, O., Guilera, G., Rojo, J.E., Gómez-Benito, J., Bernardo, M., Crespo-Facorro, B., Cuesta, M.J., Franco, M., Martinez-Aran, A., Segarra, N., Tabarés-Seisdedos, R., Vieta, E., Purdon, S.E., Díez, T., Rejas, J., Spanish Working Group in Cognitive Function, 2008. Spanish version of the Screen for Cognitive Impairment in Psychiatry (SCIP-S): psychometric properties of a brief scale for cognitive evaluation in schizophrenia. Schizophr. Res. 99, 139–148.

Pittman, A., Hardy, J., 2013. Genetic analysis in neurology: the next 10 years. JAMA Neurol 70, 696–702.

Pittman, A.M., Myers, A.J., Abou-Sleiman, P., Fung, H.C., Kaleem, M., Marlowe, L., Duckworth, J., Leung, D., Williams, D., Kilford, L., Thomas, N., Morris, C.M., Dickson, D., Wood, N.W., Hardy, J., Lees, A.J., de Silva, R., 2005. Linkage disequilibrium fine mapping and haplotype association analysis of the tau gene in progressive supranuclear palsy and corticobasal degeneration. J. Med. Genet. 42, 837–846.

Plun-Favreau, H., Burchell, V.S., Holmström, K.M., Yao, Z., Deas, E., Cain, K., Fedele, V., Moisoi, N., Campanella, M., Miguel Martins, L., Wood, N.W., Gourine, A.V., Abramov, A.Y., 2012. HtrA2 deficiency causes mitochondrial uncoupling through the F_1F_0-ATP synthase and consequent ATP depletion. Cell Death Dis 3, e335.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., Nussbaum, R.L., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–2047.

Priyadarshi, A., Khuder, S.A., Schaub, E.A., Shrivastava, S., 2000. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology 21, 435–440.

Proukakis, C., Dudzik, C.G., Brier, T., MacKay, D.S., Cooper, J.M., Millhauser, G.L., Houlden, H., Schapira, A.H., 2013. A novel α-synuclein missense mutation in Parkinson disease. Neurology 80, 1062–1064.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J., Sham, P.C., 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575.

Puschmann, A., Ross, O.A., Vilariño-Güell, C., Lincoln, S.J., Kachergus, J.M., Cobb, S.A., Lindquist, S.G., Nielsen, J.E., Wszolek, Z.K., Farrer, M., Widner, H., van Westen, D., Hägerström, D., Markopoulou, K., Chase, B.A., Nilsson, K., Reimer, J., Nilsson, C., 2009. A Swedish family with de novo alpha-synuclein A53T mutation: evidence for early cortical dysfunction. Parkinsonism Relat. Disord. 15, 627–632.

Q

Quadri, M., Fang, M., Picillo, M., Olgiati, S., Breedveld, G.J., Graafland, J., Wu, B., Xu, F., Erro, R., Amboni, M., Pappatà, S., Quarantelli, M., Annesi, G., Quattrone, A., Chien, H.F., Barbosa, E.R., International Parkinsonism Genetics Network, Oostra, B.A., Barone, P., Wang, J., Bonifati, V., 2013. Mutation in the SYNJ1 gene associated with autosomal recessive, early-onset Parkinsonism. Hum. Mutat. 34, 1208–1215.

Quik, M., 2004. Smoking, nicotine and Parkinson's disease. Trends Neurosci. 27, 561–568.

R

Ramirez, A., Heimbach, A., Gründemann, J., Stiller, B., Hampshire, D., Cid, L.P., Goebel, I., Mubaidin, A.F., Wriekat, A.-L., Roeper, J., Al-Din, A., Hillmer, A.M., Karsak, M., Liss, B., Woods, C.G., Behrens, M.I., Kubisch, C., 2006. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat. Genet. 38, 1184–1191.

Recasens, A., Dehay, B., Bové, J., Carballo-Carbajal, I., Dovero, S., Pérez-Villalba, A., Fernagut, P.-O., Blesa, J., Parent, A., Perier, C., Fariñas, I., Obeso, J.A., Bezard, E., Vila, M., 2014. Lewy body extracts from Parkinson disease brains trigger α-synuclein pathology and neurodegeneration in mice and monkeys. Ann. Neurol. 75, 351–362.

Reich, D.E., Lander, E.S., 2001. On the allelic spectrum of human disease. Trends Genet. 17, 502–510.

Rentzos, M., Nikolaou, C., Andreadou, E., Paraskevas, G.P., Rombos, A., Zoga, M., Tsoutsou, A., Boufidou, F., Kapaki, E., Vassilopoulos, D., 2007. Circulating interleukin-15 and RANTES chemokine in Parkinson's disease. Acta Neurol. Scand. 116, 374–379.

Requejo-Aguilar, R., Lopez-Fabuel, I., Jimenez-Blasco, D., Fernandez, E., Almeida, A., Bolaños, J.P., 2015. DJ1 represses glycolysis and cell proliferation by transcriptionally up-regulating Pink1. Biochem. J. 467, 303–310.

Risch, N., Merikangas, K., 1996. The future of genetic studies of complex human diseases. Science 273, 1516–1517.

Ross, O.A., Wu, Y.-R., Lee, M.-C., Funayama, M., Chen, M.-L., Soto, A.I., Mata, I.F., Lee-Chen, G.-J., Chen, C.M., Tang, M., Zhao, Y., Hattori, N., Farrer, M.J., Tan, E.-K., Wu, R.-M., 2008. Analysis of Lrrk2 R1628P as a risk factor for Parkinson's disease. Ann. Neurol. 64, 88–92.

Rubio, J.P., Topp, S., Warren, L., St Jean, P.L., Wegmann, D., Kessner, D., Novembre, J., Shen, J., Fraser, D., Aponte, J., Nangle, K., Cardon, L.R., Ehm, M.G., Chissoe, S.L., Whittaker, J.C., Nelson, M.R., Mooser, V.E., 2012. Deep sequencing of the LRRK2 gene in 14,002 individuals reveals evidence of purifying selection and independent origin of the p.Arg1628Pro mutation in Europe. Hum. Mutat. 33, 1087–1098.

S

Saad, M., Lesage, S., Saint-Pierre, A., Corvol, J.-C., Zelenika, D., Lambert, J.-C., Vidailhet, M., Mellick, G.D., Lohmann, E., Durif, F., Pollak, P., Damier, P., Tison, F., Silburn, P.A., Tzourio, C., Forlani, S., Loriot, M.-A., Giroud, M., Helmer, C., Portet, F., Amouyel, P., Lathrop, M., Elbaz, A., Durr, A., Martinez, M., Brice, A., French Parkinson's Disease Genetics Study Group, 2011. Genome-wide association study confirms BST1 and suggests a locus on 12q24 as the risk loci for Parkinson's disease in the European population. Hum. Mol. Genet. 20, 615–627.

Satake, W., Nakabayashi, Y., Mizuta, I., Hirota, Y., Ito, C., Kubo, M., Kawaguchi, T., Tsunoda, T., Watanabe, M., Takeda, A., Tomiyama, H., Nakashima, K., Hasegawa, K., Obata, F., Yoshikawa, T., Kawakami, H., Sakoda, S., Yamamoto, M., Hattori, N., Murata, M., Nakamura, Y., Toda, T., 2009. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat. Genet. 41, 1303–1307.

Schapira, A.H.V., 2008. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. Lancet Neurol 7, 97–109.

Schlitter, A.M., Woitalla, D., Mueller, T., Epplen, J.T., Dekomien, G., 2006. The LRRK2 gene in Parkinson's disease: mutation screening in patients from Germany. J. Neurol. Neurosurg. Psychiatr. 77, 891–892.

Schmoldt, A., Benthe, H.F., Haberland, G., 1975. Digitoxin metabolism by rat liver microsomes. Biochem. Pharmacol. 24, 1639–1641.

Schlitter, A.M., Kurz, M., Larsen, J.P., Woitalla, D., Müller, T., Epplen, J.T., Dekomien, G., 2006. Parkin gene variations in late-onset Parkinson's disease: comparison between Norwegian and German cohorts. Acta Neurol. Scand. 113, 9–13.

Schulte, C., Gasser, T., 2011. Genetic basis of Parkinson's disease: inheritance, penetrance, and expression. Appl Clin Genet 4, 67–80.

Seaman, M.N., McCaffery, J.M., Emr, S.D., 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. J. Cell Biol. 142, 665–681.

Setó-Salvia, N., Pagonabarraga, J., Houlden, H., Pascual-Sedano, B., Dols-Icardo, O., Tucci, A., Paisán-Ruiz, C., Campolongo, A., Antón-Aguirre, S., Martín, I., Muñoz, L., Bufill, E., Vilageliu, L., Grinberg, D., Cozar, M., Blesa, R., Lleó, A., Hardy, J., Kulisevsky, J., Clarimón, J., 2012. Glucocerebrosidase mutations confer a greater risk of dementia during Parkinson's disease course. Mov. Disord. 27, 393–399.

Shamoto-Nagai, M., Maruyama, W., Kato, Y., Isobe, K., Tanaka, M., Naoi, M., Osawa, T., 2003. An inhibitor of mitochondrial complex I, rotenone, inactivates proteasome by oxidative modification and induces aggregation of oxidized proteins in SH-SY5Y cells. J. Neurosci. Res. 74, 589–597.

Sharma, M., Ioannidis, J.P.A., Aasly, J.O., Annesi, G., Brice, A., Van Broeckhoven, C., Bertram, L., Bozi, M., Crosiers, D., Clarke, C., Facheris, M., Farrer, M., Garraux, G., Gispert, S., Auburger, G., Vilariño-Güell, C., Hadjigeorgiou, G.M., Hicks, A.A., Hattori, N., Jeon, B., Lesage, S., Lill, C.M., Lin, J.-J., Lynch, T., Lichtner, P., Lang, A.E., Mok, V., Jasinska-Myga, B., Mellick, G.D., Morrison, K.E., Opala, G., Pramstaller, P.P., Pichler, I., Park, S.S., Quattrone, A., Rogaeva, E., Ross, O.A., Stefanis, L., Stockton, J.D., Satake, W., Silburn, P.A., Theuns, J., Tan, E.-K., Toda, T., Tomiyama, H., Uitti, R.J., Wirdefeldt, K., Wszolek, Z., Xiromerisiou, G., Yueh, K.-C., Zhao, Y., Gasser, T., Maraganore, D., Krüger, R., GEO-PD Consortium, 2012. Large-scale replication and heterogeneity in Parkinson disease genetic loci. Neurology 79, 659–667.

Shendelman, S., Jonason, A., Martinat, C., Leete, T., Abeliovich, A., 2004. DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. PLoS Biol. 2, e362.

Sherer, T.B., Betarbet, R., Testa, C.M., Seo, B.B., Richardson, J.R., Kim, J.H., Miller, G.W., Yagi, T., Matsuno-Yagi, A., Greenamyre, J.T., 2003. Mechanism of toxicity in rotenone models of Parkinson's disease. J. Neurosci. 23, 10756–10764.

Shimura, H., Hattori, N., Kubo, S. i, Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., Suzuki, T., 2000. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. 25, 302–305.

Shojaee, S., Sina, F., Banihosseini, S.S., Kazemi, M.H., Kalhor, R., Shahidi, G.-A., Fakhrai-Rad, H., Ronaghi, M., Elahi, E., 2008. Genome-wide linkage analysis of a Parkinsonian-pyramidal syndrome pedigree by 500 K SNP arrays. Am. J. Hum. Genet. 82, 1375–1384.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., Marsden, C.D., 1994. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann. Neurol. 36, 348–355.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., Barbosa, E.R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., Chen, C.-M., Clark, L.N., Condroyer, C., De Marco, E.V., Dürr, A., Eblan, M.J., Fahn, S., Farrer, M.J., Fung, H.-C., Gan-Or, Z., Gasser, T., Gershoni-Baruch, R., Giladi, N., Griffith, A., Gurevich, T., Januario, C., Kropp, P., Lang, A.E., Lee-Chen, G.-J., Lesage, S., Marder, K., Mata, I.F., Mirelman, A., Mitsui, J., Mizuta, I., Nicoletti, G., Oliveira, C., Ottman, R., Orr-Urtreger, A., Pereira, L.V., Quattrone, A., Rogaeva, E., Rolfs, A., Rosenbaum, H., Rozenberg, R., Samii, A., Samaddar, T., Schulte, C., Sharma, M., Singleton, A., Spitz, M., Tan, E.-K., Tayebi, N., Toda, T., Troiano, A.R., Tsuji, S., Wittstock, M., Wolfsberg, T.G., Wu, Y.-R., Zabetian, C.P., Zhao, Y., Ziegler, S.G., 2009. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med. 361, 1651–1661.

Simón-Sánchez, J., Schulte, C., Bras, J.M., Sharma, M., Gibbs, J.R., Berg, D., Paisan-Ruiz, C., Lichtner, P., Scholz, S.W., Hernandez, D.G., Krüger, R., Federoff, M., Klein, C., Goate, A., Perlmutter, J., Bonin, M., Nalls, M.A., Illig, T., Gieger, C., Houlden, H., Steffens, M., Okun, M.S., Racette, B.A., Cookson, M.R., Foote, K.D., Fernandez, H.H., Traynor, B.J., Schreiber, S., Arepalli, S., Zonozi, R., Gwinn, K., van der Brug, M., Lopez, G., Chanock, S.J., Schatzkin, A., Park, Y., Hollenbeck, A., Gao, J., Huang, X., Wood, N.W., Lorenz, D., Deuschl, G., Chen, H., Riess, O., Hardy, J.A., Singleton, A.B., Gasser, T., 2009. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat. Genet. 41, 1308–1312.

Simón-Sánchez, J., Singleton, A., 2008. Genome-wide association studies in neurological disorders. Lancet Neurol 7, 1067–1072.

Simón-Sánchez, J., van Hilten, J.J., van de Warrenburg, B., Post, B., Berendse, H.W., Arepalli, S., Hernandez, D.G., de Bie, R.M.A., Velseboer, D., Scheffer, H., Bloem, B., van Dijk, K.D., Rivadeneira, F., Hofman, A., Uitterlinden, A.G., Rizzu, P., Bochdanovits, Z., Singleton, A.B., Heutink, P., 2011. Genome-wide association study confirms extant PD risk loci among the Dutch. Eur. J. Hum. Genet. 19, 655–661.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., Barbosa, E.R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., Chen, C.-M., Clark, L.N., Condroyer, C., De Marco, E.V., Dürr, A., Eblan, M.J., Fahn, S., Farrer, M.J., Fung, H.-C., Gan-Or, Z., Gasser, T., Gershoni-Baruch, R., Giladi, N., Griffith, A., Gurevich, T., Januario, C., Kropp, P., Lang, A.E., Lee-Chen, G.-J., Lesage, S., Marder, K., Mata, I.F., Mirelman, A., Mitsui, J., Mizuta, I., Nicoletti, G., Oliveira, C., Ottman, R., Orr-Urtreger, A., Pereira, L.V., Quattrone, A., Rogaeva, E., Rolfs, A., Rosenbaum, H., Rozenberg, R., Samii, A., Samaddar, T., Schulte, C., Sharma, M., Singleton, A., Spitz, M., Tan, E.-K., Tayebi, N., Toda, T., Troiano, A.R., Tsuji, S., Wittstock, M.,

Wolfsberg, T.G., Wu, Y.-R., Zabetian, C.P., Zhao, Y., Ziegler, S.G., 2009. Multicenter Analysis of Glucocerebrosidase Mutations in Parkinson's Disease. N. Engl. J. Med. 361, 1651–1661.

Singleton, A.B., Farrer, M.J., Bonifati, V., 2013. The genetics of Parkinson's disease: progress and therapeutic implications. Mov. Disord. Off. J. Mov. Disord. Soc. 28, 14–23.

Sina, F., Shojaee, S., Elahi, E., Paisán-Ruiz, C., 2009. R632W mutation in PLA2G6 segregates with dystonia-parkinsonism in a consanguineous Iranian family. Eur. J. Neurol. 16, 101–104.

Singleton, A.B., Farrer, M.J., Bonifati, V., 2013. The genetics of Parkinson's disease: progress and therapeutic implications. Mov. Disord. 28, 14–23.

Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J., Gwinn-Hardy, K., 2003. alpha-Synuclein locus triplication causes Parkinson's disease. Science 302, 841.

Singleton, A.B., Hardy, J., Traynor, B.J., Houlden, H., 2010. Towards a complete resolution of the genetic architecture of disease. Trends Genet. 26, 438–442.

Singleton, A., Hardy, J., 2016. The Evolution of Genetics: Alzheimer's and Parkinson's Diseases. Neuron 90, 1154–1163.

Singleton, A., Hardy, J., 2011. A generalizable hypothesis for the genetic architecture of disease: pleomorphic risk loci. Hum. Mol. Genet. 20, R158–162.

Skipper, L., Wilkes, K., Toft, M., Baker, M., Lincoln, S., Hulihan, M., Ross, O.A., Hutton, M., Aasly, J., Farrer, M., 2004. Linkage disequilibrium and association of MAPT H1 in Parkinson disease. Am. J. Hum. Genet. 75, 669–677.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., Goedert, M., 1997. Alpha-synuclein in Lewy bodies. Nature 388, 839–840.

Spira, P.J., Sharpe, D.M., Halliday, G., Cavanagh, J., Nicholson, G.A., 2001. Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr alpha-synuclein mutation. Ann. Neurol. 49, 313–319.

Stott, S.R.W., Barker, R.A., 2014. Time course of dopamine neuron loss and glial response in the 6-OHDA striatal mouse model of Parkinson's disease. Eur. J. Neurosci. 39, 1042–1056.

Strauss, K.M., Martins, L.M., Plun-Favreau, H., Marx, F.P., Kautzmann, S., Berg, D., Gasser, T., Wszolek, Z., Müller, T., Bornemann, A., Wolburg, H., Downward, J., Riess, O., Schulz, J.B., Krüger, R., 2005. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Hum. Mol. Genet. 14, 2099–2111.

Sveinbjornsdottir, S., 2016. The clinical symptoms of Parkinson's disease. J. Neurochem.

T

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., Sawa, A., L Dawson, V., Dawson, T.M., Ross, C.A., 2001. Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum. Mol. Genet. 10, 919–926.

Tansey, M.G., McCoy, M.K., Frank-Cannon, T.C., 2007. Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. Exp. Neurol. 208, 1–25.

Thayanidhi, N., Helm, J.R., Nycz, D.C., Bentley, M., Liang, Y., Hay, J.C., 2010. Alpha-synuclein delays endoplasmic reticulum (ER)-to-Golgi transport in mammalian cells by antagonizing ER/Golgi SNAREs. Mol. Biol. Cell 21, 1850–1863.

Thiruchelvam, M., McCormack, A., Richfield, E.K., Baggs, R.B., Tank, A.W., Di Monte, D.A., Cory-Slechta, D.A., 2003. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur. J. Neurosci. 18, 589–600.

Tofaris, G.K., Razzaq, A., Ghetti, B., Lilley, K.S., Spillantini, M.G., 2003. Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function. J. Biol. Chem. 278, 44405–44411.

Toft, M., Haugarvoll, K., Ross, O.A., Farrer, M.J., Aasly, J.O., 2007. LRRK2 and Parkinson's disease in Norway. Acta Neurol. Scand., Suppl. 187, 72–75.

Tolosa, E., Wenning, G., Poewe, W., 2006. The diagnosis of Parkinson's disease. Lancet Neurol 5, 75–86.

Trabzuni, D., Wray, S., Vandrovcova, J., Ramasamy, A., Walker, R., Smith, C., Luk, C., Gibbs, J.R., Dillman, A., Hernandez, D.G., Arepalli, S., Singleton, A.B., Cookson, M.R., Pittman, A.M., de Silva, R., Weale, M.E., Hardy, J., Ryten, M., 2012. MAPT expression and splicing is differentially regulated by brain region: relation to genotype and implication for tauopathies. Hum. Mol. Genet. 21, 4094–4103.

Trender-Gerhard, I., Sweeney, M.G., Schwingenschuh, P., Mir, P., Edwards, M.J., Gerhard, A., Polke, J.M., Hanna, M.G., Davis, M.B., Wood, N.W., Bhatia, K.P., 2009. Autosomal-dominant GTPCH1-deficient DRD: clinical characteristics and long-term outcome of 34 patients. J. Neurol. Neurosurg. Psychiatr. 80, 839–845.

Trinh, J., Farrer, M., 2013. Advances in the genetics of Parkinson disease. Nat Rev Neurol 9, 445–454.

Troiano, A.R., Cazeneuve, C., Le Ber, I., Bonnet, A.-M., Lesage, S., Brice, A., 2008. Re: Alpha-synuclein gene duplication is present in sporadic Parkinson disease. Neurology 71, 1295; author reply 1295.

U

Uchiyama, T., Ikeuchi, T., Ouchi, Y., Sakamoto, M., Kasuga, K., Shiga, A., Suzuki, M., Ito, M., Atsumi, T., Shimizu, T., Ohashi, T., 2008. Prominent psychiatric symptoms and glucose hypometabolism in a family with a SNCA duplication. Neurology 71, 1289–1291.

Usenovic, M., Krainc, D., 2012. Lysosomal dysfunction in neurodegeneration: the role of ATP13A2/PARK9. Autophagy 8, 987–988.

V

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., Wood, N.W., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304, 1158–1160.

Valente, E.M., Bentivoglio, A.R., Dixon, P.H., Ferraris, A., Ialongo, T., Frontali, M., Albanese, A., Wood, N.W., 2001. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am. J. Hum. Genet. 68, 895–900.

Van Den Eeden, S.K., Tanner, C.M., Bernstein, A.L., Fross, R.D., Leimpeter, A., Bloch, D.A., Nelson, L.M., 2003. Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. Am. J. Epidemiol. 157, 1015–1022.

Van der Mark, M., Brouwer, M., Kromhout, H., Nijssen, P., Huss, A., Vermeulen, R., 2012. Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. Environ. Health Perspect. 120, 340–347.

Varçin, M., Bentea, E., Michotte, Y., Sarre, S., 2012. Oxidative stress in genetic mouse models of Parkinson's disease. Oxid Med Cell Longev 2012, 624925.

Venselaar, H., te Beek, T.A., Kuipers, R.K., Hekkelman, M.L., Vriend, G., 2010. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. BMC Bioinformatics 11, 548.

Vilariño-Güell, C., Rajput, A., Milnerwood, A.J., Shah, B., Szu-Tu, C., Trinh, J., Yu, I., Encarnacion, M., Munsie, L.N., Tapia, L., Gustavsson, E.K., Chou, P., Tatarnikov, I., Evans, D.M., Pishotta, F.T., Volta, M., Beccano-Kelly, D., Thompson, C., Lin, M.K., Sherman, H.E., Han, H.J., Guenther, B.L., Wasserman, W.W., Bernard, V., Ross, C.J., Appel-Cresswell, S., Stoessl, A.J., Robinson, C.A., Dickson, D.W., Ross, O.A., Wszolek, Z.K., Aasly, J.O., Wu, R.-M., Hentati, F., Gibson, R.A., McPherson, P.S., Girard, M., Rajput, M., Rajput, A.H., Farrer, M.J., 2014. DNAJC13 mutations in Parkinson disease. Hum. Mol. Genet. 23, 1794–1801.

Vilariño-Güell, C., Ross, O.A., Soto, A.I., Farrer, M.J., Haugarvoll, K., Aasly, J.O., Uitti, R.J., Wszolek, Z.K., 2009. Reported mutations in GIGYF2 are not a common cause of Parkinson's disease. Mov. Disord. 24, 619–620.

Vilariño-Güell, C., Wider, C., Ross, O.A., Dachsel, J.C., Kachergus, J.M., Lincoln, S.J., Soto-Ortolaza, A.I., Cobb, S.A., Wilhoite, G.J., Bacon, J.A., Behrouz, B., Melrose, H.L., Hentati, E., Puschmann, A., Evans, D.M., Conibear, E., Wasserman, W.W., Aasly, J.O., Burkhard, P.R., Djaldetti, R., Ghika, J., Hentati, F., Krygowska-Wajs, A., Lynch, T., Melamed, E., Rajput, A., Rajput, A.H., Solida, A., Wu, R.-M., Uitti, R.J., Wszolek, Z.K., Vingerhoets, F., Farrer, M.J., 2011. VPS35 mutations in Parkinson disease. Am. J. Hum. Genet. 89, 162–167.

Vitte, J., Traver, S., Maués De Paula, A., Lesage, S., Rovelli, G., Corti, O., Duyckaerts, C., Brice, A., 2010. Leucine-rich repeat kinase 2 is associated with the endoplasmic reticulum in dopaminergic neurons and accumulates in the core of Lewy bodies in Parkinson disease. J. Neuropathol. Exp. Neurol. 69, 959–972.

Vives-Bauza, C., Zhou, C., Huang, Y., Cui, M., de Vries, R.L.A., Kim, J., May, J., Tocilescu, M.A., Liu, W., Ko, H.S., Magrané, J., Moore, D.J., Dawson, V.L., Grailhe, R., Dawson, T.M., Li, C., Tieu, K., Przedborski,

S., 2010. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc. Natl. Acad. Sci. U.S.A. 107, 378–383.

W

Wade-Martins, R., 2012. Genetics: The MAPT locus-a genetic paradigm in disease susceptibility. Nat Rev Neurol 8, 477–478.

Walker, J.M., Lwin, A., Tayebi, N., LaMarca, M.E., Orvisky, E., Sidransky, E., 2003. Glucocerebrosidase mutation T369M appears to be another polymorphism. Clin. Genet. 63, 237–238.

Wang, Q., Liu, Y., Zhou, J., 2015. Neuroinflammation in Parkinson's disease and its potential as therapeutic target. Transl Neurodegener 4, 19.

Ward, C.D., Duvoisin, R.C., Ince, S.E., Nutt, J.D., Eldridge, R., Calne, D.B., 1983. Parkinson's disease in 65 pairs of twins and in a set of quadruplets. Neurology 33, 815–824.

West, A., Periquet, M., Lincoln, S., Lücking, C.B., Nicholl, D., Bonifati, V., Rawal, N., Gasser, T., Lohmann, E., Deleuze, J.-F., Maraganore, D., Levey, A., Wood, N., Dürr, A., Hardy, J., Brice, A., Farrer, M., French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility on Parkinson's Disease, 2002. Complex relationship between Parkin mutations and Parkinson disease. Am. J. Med. Genet. 114, 584–591.

Westerlund, M., Belin, A.C., Anvret, A., Håkansson, A., Nissbrandt, H., Lind, C., Sydow, O., Olson, L., Galter, D., 2008. Cerebellar alpha-synuclein levels are decreased in Parkinson's disease and do not correlate with SNCA polymorphisms associated with disease in a Swedish material. FASEB J. 22, 3509–3514.

Wilhelmus, M.M.M., Nijland, P.G., Drukarch, B., de Vries, H.E., van Horssen, J., 2012. Involvement and interplay of Parkin, PINK1, and DJ1 in neurodegenerative and neuroinflammatory disorders. Free Radic. Biol. Med. 53, 983–992.

Williams, D.R., Hadeed, A., al-Din, A.S.N., Wreikat, A.-L., Lees, A.J., 2005. Kufor Rakeb disease: autosomal recessive, levodopa-responsive parkinsonism with pyramidal degeneration, supranuclear gaze palsy, and dementia. Mov. Disord. 20, 1264–1271.

Winner, B., Jappelli, R., Maji, S.K., Desplats, P.A., Boyer, L., Aigner, S., Hetzer, C., Loher, T., Vilar, M., Campioni, S., Tzitzilonis, C., Soragni, A., Jessberger, S., Mira, H., Consiglio, A., Pham, E., Masliah, E., Gage, F.H., Riek, R., 2011. In vivo demonstration that alpha-synuclein oligomers are toxic. Proc. Natl. Acad. Sci. U.S.A. 108, 4194–4199.

Winslow, A.R., Chen, C.-W., Corrochano, S., Acevedo-Arozena, A., Gordon, D.E., Peden, A.A., Lichtenberg, M., Menzies, F.M., Ravikumar, B., Imarisio, S., Brown, S., O'Kane, C.J., Rubinsztein, D.C., 2010. α-Synuclein impairs macroautophagy: implications for Parkinson's disease. J. Cell Biol. 190, 1023–1037.

Wirdefeldt, K., Adami, H.-O., Cole, P., Trichopoulos, D., Mandel, J., 2011. Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur. J. Epidemiol. 26 Suppl 1, S1–58.

Wooten, G.F., Currie, L.J., Bovbjerg, V.E., Lee, J.K., Patrie, J., 2004. Are men at greater risk for Parkinson's disease than women? J. Neurol. Neurosurg. Psychiatr. 75, 637–639.

X

Xu, J., Zhong, N., Wang, H., Elias, J.E., Kim, C.Y., Woldman, I., Pifl, C., Gygi, S.P., Geula, C., Yankner, B.A., 2005. The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. Hum. Mol. Genet. 14, 1231–1241.

Y

Yang, J., Lee, S.H., Goddard, M.E., Visscher, P.M., 2011. GCTA: a tool for genome-wide complex trait analysis. Am. J. Hum. Genet. 88, 76–82. doi:10.1016/j.ajhg.2010.11.011

Yan, M.H., Wang, X., Zhu, X., 2013. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic. Biol. Med. 62, 90–101.

Yoshino, H., Tomiyama, H., Tachibana, N., Ogaki, K., Li, Y., Funayama, M., Hashimoto, T., Takashima, S., Hattori, N., 2010. Phenotypic spectrum of patients with PLA2G6 mutation and PARK14-linked parkinsonism. Neurology 75, 1356–1361.

Z

Zalloua, P.A., Platt, D.E., El Sibai, M., Khalife, J., Makhoul, N., Haber, M., Xue, Y., Izaabel, H., Bosch, E., Adams, S.M., Arroyo, E., López-Parra, A.M., Aler, M., Picornell, A., Ramon, M., Jobling, M.A., Comas, D., Bertranpetit, J., Wells, R.S., Tyler-Smith, C., Genographic Consortium, 2008. Identifying genetic traces of historical expansions: Phoenician footprints in the Mediterranean. Am. J. Hum. Genet. 83, 633–642.

Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D.G., de Yebenes, J.G., 2004. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann. Neurol. 55, 164–173.

Zhang, L., Nie, Z.Y., Liu, Y., Chen, W., Xin, S.M., Sun, X.D., Fan, J.H., Liu, Y.H., Gao, X.H., Lu, L.Q., Como, P., McDermott, M.P., Qiao, Y.L., Kieburtz, K., 2005. The prevalence of PD in a nutritionally deficient rural population in China. Acta Neurol. Scand. 112, 29–35.

Zhang, Y., Zheng, L., Zhang, T., Wang, Y., Xiao, Q., Fei, Q.-Z., Cui, P.-J., Cao, L., Chen, S.-D., 2009. GIGYF2 Asn56Ser mutation is rare in Chinese Parkinson's disease patients. Neurosci. Lett. 463, 172–175.

Zhou, W., Zhu, M., Wilson, M.A., Petsko, G.A., Fink, A.L., 2006. The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. J. Mol. Biol. 356, 1036–1048.

Zhou, Z.D., Xie, S.P., Sathiyamoorthy, S., Saw, W.T., Sing, T.Y., Ng, S.H., Chua, H.P.H., Tang, A.M.Y., Shaffra, F., Li, Z., Wang, H., Ho, P.G.H., Lai, M.K.P., Angeles, D.C., Lim, T.M., Tan, E.-K., 2015. F-box protein 7 mutations promote protein aggregation in mitochondria and inhibit mitophagy. Hum. Mol. Genet. 24, 6314–6330.

Zimprich, A., Benet-Pagès, A., Struhal, W., Graf, E., Eck, S.H., Offman, M.N., Haubenberger, D., Spielberger, S., Schulte, E.C., Lichtner, P., Rossle, S.C., Klopp, N., Wolf, E., Seppi, K., Pirker, W., Presslauer, S., Mollenhauer, B., Katzenschlager, R., Foki, T., Hotzy, C., Reinthaler, E., Harutyunyan, A., Kralovics, R., Peters, A., Zimprich, F., Brücke, T., Poewe, W., Auff, E., Trenkwalder, C., Rost, B., Ransmayr, G., Winkelmann, J., Meitinger, T., Strom, T.M., 2011. A mutation in VPS35, encoding a

subunit of the retromer complex, causes late-onset Parkinson disease. Am. J. Hum. Genet. 89, 168–175.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R.J., Calne, D.B., Stoessl, A.J., Pfeiffer, R.F., Patenge, N., Carbajal, I.C., Vieregge, P., Asmus, F., Müller-Myhsok, B., Dickson, D.W., Meitinger, T., Strom, T.M., Wszolek, Z.K., Gasser, T., 2004. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44, 601–607.

Zimprich, A., Schulte, C., Reinthaler, E., Haubenberger, D., Balzar, J., Lichtner, P., El Tawil, S., Edris, S., Foki, T., Pirker, W., Katzenschlager, R., Daniel, G., Brücke, T., Auff, E., Gasser, T., 2009. PARK11 gene (GIGYF2) variants Asn56Ser and Asn457Thr are not pathogenic for Parkinson's disease. Parkinsonism Relat. Disord. 15, 532–534.

Neurobiology of Aging xxx (2015) 1–5

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Analysis of the genetic variability in Parkinson's disease from Southern Spain

Sara Bandrés-Ciga [a], Niccolò Emmanuele Mencacci [b], Raquel Durán [a], Francisco Javier Barrero [c], Francisco Escamilla-Sevilla [d], Sarah Morgan [b], Jason Hehir [b], Francisco Vives [a], John Hardy [b], Alan M. Pittman [b,*]

[a] Department of Physiology, Institute of Neurosciences Federico Olóriz, Centro de Investigacion Biomedica (CIBM), University of Granada, Granada, Spain
[b] Department of Molecular Neuroscience, Reta Lila Weston Institute of Neurological Studies, UCL Institute of Neurology, London, UK
[c] Movement Disorders Unit, University Hospital San Cecilio, Granada, Spain
[d] Movement Disorders Unit, Department of Neurology, Instituto de Investigación Biosanitaria (IBS), University Hospital Virgen de las Nieves, Granada, Spain

ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 8 September 2015
Accepted 26 September 2015

Keywords:
Parkinson's disease
Genetics
Mutations
Southern Spain population
Next-generation sequencing

ABSTRACT

To date, a large spectrum of genetic variants has been related to familial and sporadic Parkinson's disease (PD) in diverse populations worldwide. However, very little is known about the genetic landscape of PD in Southern Spain, despite its particular genetic landscape coming from multiple historical migrations. We included 134 PD patients in this study, of which 97 individuals were diagnosed with late-onset sporadic PD (LOPD), 28 with early-onset sporadic PD (EOPD), and 9 with familial PD (FPD). Genetic analysis was performed through a next-generation sequencing panel to screen 8 PD-related genes (*LRRK2*, *SNCA*, *PARKIN*, *PINK1*, *DJ-1*, *VPS35*, *GBA*, and *GCH1)* in EOPD and FPD groups and direct Sanger sequencing of *GBA* exons 8–11 and *LRRK2* exons 31 and 41 in the LOPD group. In the EOPD and FPD groups, we identified 11 known pathogenic mutations among 15 patients (40.5 %). *GBA* (E326K, N370S, D409H, L444P) mutations were identified in 7 patients (18.9 %); *LRRK2* (p.R1441G and p.G2019S) in 3 patients (8.1 %); biallelic *PARK2* mutations (p.N52fs, p.V56E, p.C212Y) in 4 cases (10.8%) and *PINK1* homozygous p.G309D in 1 patient (2.7 %). An EOPD patient carried a single *PARK2* heterozygous mutation (p.R402C), and another had a novel heterozygous mutation in *VPS35* (p.R32S), both of unknown significance. Moreover, pathogenic mutations in *GBA* (E326K, T369M, N370S, D409H, L444P) and *LRRK2* (p.R1441G and p.G2019S) were identified in 13 patients (13.4 %) and 4 patients (4.1 %), respectively, in the LOPD group. A large number of known pathogenic mutations related to PD have been identified. In particular, *GBA* and *LRRK2* mutations appear to be considerably frequent in our population, suggesting a strong Jewish influence. Further research is needed to study the contribution of the novel found mutation p.R32S in *VPS35* to the pathogenesis of PD.

1. Introduction

In the last decade, several loci and risk variants have been identified and linked to the pathology of familial and sporadic Parkinson's disease (PD) in diverse populations worldwide (Singleton et al., 2013). Given its geographical location on the southernmost region of Spain, the population from Granada is the result of a particular genetic landscape coming from multiple historical migrations and the settlement of different civilizations. Its complex history over the last millennia has involved the long-term residence of 2 very different populations with distinct geographical origins: North African Muslims and Sephardic Jews. Southern Spain represents a potential migration network and the major cross-link between Europe and Africa. These remarkable interactions across the Mediterranean Sea and the North of Africa have contributed to a genetic enrichment and might have shaped a unique genetic profile.

The long period of coexistence between North Africa and Southern Spain during the 8 centuries of the Islamic invasion suggests a marked genetic relationship. The Jewish presence has also been widespread and long-established in Granada, and admixture analysis indicates a substantial proportion of ancestry from

* Corresponding author at: Department of Molecular Neuroscience, Reta Lila Weston Institute of Neurological Studies, UCL Institute of Neurology, London WC1N 3BG, UK. Tel.: +44 020 7679 2000; fax: +44 705 347 1832.
E-mail address: a.pittman@ucl.ac.uk (A.M. Pittman).

0197-4580/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2015.09.020

Sephardic Jews sources (Adams et al., 2008). Moreover, Southern Spain has been subject to other important influences coming from eastern Mediterranean populations such as the Greek and the Phoenician colonization (Zalloua et al., 2008). However, despite its historical background, the population from Granada has been poorly studied, and it is still unknown which genetic variants contribute significantly to the development of PD.

Our study was to assess the contribution of known genes in a cohort diagnosed with either familial PD (FPD) or early-onset sporadic PD (EOPD) from Southern Spain, predominantly Granada and its area of influence. The genes of interest have been those traditionally associated with autosomal-dominant or -recessive forms and include *LRRK2*, *SNCA* and *VPS35*, *PARKIN*, *PINK1*, and *DJ-1*, respectively. Other risk genes recently linked to the disease such as *GBA* (Sidransky et al., 2009) and *GCH1* (Mencacci et al., 2014) have been also studied. Additionally, we screened for *LRRK2* and *GBA* common pathogenic mutations in a cohort of late-onset sporadic PD (LOPD).

2. Methods

2.1. Patients

We included a group of 134 PD patients, of which 97 were diagnosed with LOPD, 28 with EOPD, and 9 unrelated FPD, all treated at the Movement Disorders Unit of the Service of Neurology in the both Hospital Clínico San Cecilio and Hospital Virgen de las Nieves of Granada (Spain). PD was diagnosed at least by 2 experienced neurologists in the field of movement disorders following the criteria of the UK PD Society Brain Bank (Gibb and Lees, 1988). EOPD defined by an age of onset $\leq$50 years. Patients who had at least 1 first-degree PD affected relative were classified as familial. The study was approved by the local ethic committee, and written informed consent was taken from each participant.

2.2. Genetic analysis

Genomic DNA was isolated from peripheral blood leucocytes or saliva as manufacturer's protocols (QIAamp DNA Blood Midi Kit, QIAGEN; Oragene Kit, DNA Genotek). EOPD and FPD patients were screened for potential mutations using next-generation sequencing (NGS). We used an Illumina's Miseq with a polymerase chain reaction (PCR) amplicon-based (TruSeq Custom amplicon) target enrichment to screen for variants across the coding exons of the 8 PD genes listed previously. Probes were designed using Illumina Truseq custom amplicon assay Design Studio v1.6 online (http://www.illumina.com/applications/designstudio.ilmn). The assay was performed according to the manufacturer's recommended protocol. Targeted exons with a coverage of less than 10 reads were subsequently screened by Sanger Sequencing. We excluded from the analysis variants with a minor allele frequency $\geq$1 % in general population according to 1000 Genome Project (http://www.1000genomes.org/).

LOPD patients were sequenced for *GBA* exons 8–11 and *LRRK2* exons 31, 41 and their flanking intronic sequences by Sanger sequencing because most pathogenic mutations are within these exons (Duran et al., 2013; Paisán-Ruiz et al., 2013). *GBA* allele names refer to the processed protein, excluding the 39-residue signal peptide.

The primers and PCR conditions we used are available on request. PCR products were bidirectional sequenced using the BigDye Terminator version 3.1 sequencing chemistry and then loaded on the ABI3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

PD cases that carried point mutations in *PARK2* and *PINK1* were screened for exon rearrangements through multiplex ligation-dependent probe amplification (MLPA) using the P051-C3 Salsa MLPA Parkinson probe set (MRC Holland, Amsterdam, the Netherlands). This set includes probes that detect exonic rearrangements in *PARK1* ([SNCA]; exons 2–7), *PARK2* ([PRKN]: exons 1–12), *PARK6* ([PINK1]; exons 1–8), *PARK7* ([DJ-1]; exons 1b, 3, 5, and 7) and *PARK9* ([ATP13A2]: exons 2 and 9), *PARK8* ([LRRK2]: exon 41). Data analysis was performed using Genemarker, version 2.6.2, software.

3. Results

3.1. Mutational screening

Demographic and clinical characteristics of the groups under study are summarized in Table 1. For the genes assessed by NGS in the FPD and EOPD group, a total of 11 known PD-related mutations were identified among 15 patients (40.5%, see Table 2). Interestingly, 1 EOPD patient carried a novel mutation in *VPS35* (p.R32S). Four different heterozygous *GBA* mutations were detected among 6 patients. Two sporadic (SP) cases with EOPD carried the mutation D409H and 1 familial case carried the L444P. The mutation N370S was found in 2 SP cases with EOPD and in 1 FPD case, and the E326K was identified in 1 SP case with EOPD. In *LRRK2*, we found 2 heterozygous pathogenic mutations in 3 patients. One familial and 1 SP case with EOPD carried the mutation p.G2019S. The mutation p.R1441G was identified in 1 FPD case. Moreover, 4 mutations were identified in *PARK2* among 5 patients. Three independent cases carried the frameshift deletion p.N52fs, 2 homozygous and 1 heterozygous. MLPA analysis revealed the presence of a *PARK2* deletion of exons 3 and 4 in the latter case. The heterozygous mutation p.R402C of uncertain significance was identified in 1 SP case with EOPD. However, no rearrangements were found in the single heterozygous *PARK2* mutation carrier. One FPD case carried the heterozygous point mutations p.V56E and p.C212Y simultaneously. The *PINK1* homozygous mutation p.G309D was identified in 1 FPD case.

The remaining 22 patients (59.5%) did not have any identifiable genetic risk variant. We found genetic variants related to the disease in 28.6 % of EOPD and 88 % of FPD cases. Four additional variants of unknown significance were detected in *LRRK2 (*p.I1371V, p.N2081D, p.M1646T, p.R1514Q) in 2 EOPD cases and 2 FPD cases, as well as 1 *DJ-1* variant (p.R98Q) in a familial PD. These variants are most likely benign as they are all reported at relatively high frequencies in the ExAc database (minor allele frequency >0.001). No mutation carriers were found for *SNCA* and *GCH1.*

In the LOPD group, we identified 6 variants in *GBA* and 2 in *LRRK2* among an overall of 17 patients (17.5%) (Table 2). The *GBA*

Table 1
Demographic and clinical characteristics of the groups under study

	SP		
	FPD	LOPD	EOPD
Age at onset (y)	46.3	65.8	49.0
Disease duration (mo)	150.8	74.0	97.8
Female:male	3:6	35:62	11:17
Disease severity (H&Y score) %			
I	26.7	29.3	11.2
II	26.7	47.6	50.4
III	26.7	19.5	30.1
IV	20.0	3.7	8.5

Key: EOPD, early-onset Parkinson's disease; FPD, familial Parkinson's disease; H&Y score, Hoehn & Yahr score; LOPD, late-onset Parkinson's disease; SP, sporadic Parkinson's disease.

Table 2
Genetic PD-related variants identified in PD patients of Southern Spain

Gene	Exon	rsID	Variant	Nucleotide change	Zygosity	Patients		
						FPD	SP	
							EOPD	LOPD
GBA	10	rs1064651	D409H	c.1342G>C	Het	—	2	2
	11	rs35095275	L444P	c.1448T>C	Het	1	—	2
	10	rs76763715	N370S	c.1226A>G	Het	1	2	4
	9	rs2230288	E326K	c.1093G>A	Het	—	1	2
	8	—	T369M	c.1223T>C	Het	—	—	2
					Hom	—	—	1
LRRK2	41	rs34637584	p.G2019S	c.6055G>A	Het	1	1	3
	31	rs33939927	p.R1441G	c.4321C>G	Het	1	—	1
PARK2	2	N/A	p.N52fsX80	c.154delA	Hom	1	1	N/A
					Het[a]	1	—	N/A
	11	rs55830907	p.R402C	c.1204C>T	Het[b]	—	1	N/A
	2	rs137853059	p.V56E	c.167T>A	Het	1	—	N/A
	6	rs137853058	p.C212Y	c.635G>A	Het	1	—	N/A
PINK1	4	rs74315355	p.G309D	c.926G>A	Hom	1	—	N/A
VPS35	2	N/A	p.R32S[c]	c.96A>T	Het	—	1	N/A

Key: EOPD, early-onset Parkinson's disease; FPD, familial Parkinson's disease; Het, Heterozygous; Hom, Homozygous; LOPD, late-onset Parkinson's disease; N/A, not applicable because it has not been studied; PD, Parkinson's disease; SP, sporadic Parkinson's disease.
[a] PARK2 heterozygous deletion of exons 3 and 4.
[b] Single heterozygous state.
[c] Novel mutation.

heterozygous mutation D409H was found in 2 patients, the heterozygous mutations L444P and E326K were identified simultaneously in 2 patients, the heterozygous mutation N370S was detected in 4 patients, and the variant T369M was found in 3 patients (2 heterozygous and 1 in the homozygous state). The remaining 80 patients (82.5 %) did not show any genetic cause. Frequencies of pathogenic mutations in the genes under study are documented for each group (Table 3).

3.2. In silico *analysis of the novel VPS35 mutation*

Because the novel variant *VPS35* p.R32S raises further questions as to the structural effect, protein stability and malfunction on the vacuolar protein sorting-associated protein 35, we performed an *in silico* analysis using the *SIFT* bioinformatics tool (Kumar et al., 2009) and the *HOPE* Web server (Venselaar et al., 2011). The mutant amino acid was predicted to be damaging (Supplementary data).

Table 3
Allele frequency of found mutations in PD patients from Southern Spain and European controls compiled from ExAc database

Gene	EOPD	FPD	LOPD	State	Controls
GBA					
D409H	0.07	—	0.02	Het	1.0×10^{-4}
L444P	—	0.11	0.02	Het	U
N370S	0.07	0.11	0.04	Het	3.0×10^{-3}
E326K + L444P	—	—	0.02	Het	U
E325K	0.03	—	—	Het	1.2×10^{-2}
T369M	—	—	0.03	Hom/Het	9.0×10^{-3}
LRRK2					
p.G2019S	0.03	0.11	0.03	Het	9.0×10^{-3}
p.R1441G	—	0.11	0.01	Het	U
PARK2					
p.N52fsX80	0.03	0.11	N/A	Hom/Het	U
p.R402C	0.04	—	N/A	Het	2.0×10^{-3}
p.V56E	—	0.11	N/A	Het	2.9×10^{-5}
p.C212Y	—	0.11	N/A	Het	1.5×10^{-5}
PINK1					
p.G309D	—	0.11	N/A	Het	U
VPS35					
p.R32S	0.04	—	N/A	Het	—

Key: EOPD, early-onset Parkinson's disease; FPD, familial Parkinson's disease; Het, Heterozygous; Hom, Homozygous; LOPD, late-onset Parkinson's disease; N/A, not applicable because it has not been studied; U, unknown.

4. Discussion

So far, there have been a very limited number of genetic studies on PD in Southern Spain population (Gao et al., 2009; Gómez-Garre et al., 2014). This is the first report aimed to study in depth the genetic contribution of PD genes in familial or EOPD cases, as well as to explore the influence of *GBA* and *LRRK2* mutations on LOPD in the Southern Spanish population. Although we are aware that the sample size is relatively small, it comprised most PD patients from the province of Granada.

As part of our effort to genetically characterize PD in this population, we used the efficiency of NGS to investigate the frequency of potentially pathogenic mutations of 8 known PD genes in EOPD and FPD cases. Our approach produced highly accurate sequence data, and we identified several genetic variants linked to the disease.

Not surprisingly, we found that p.G2019S is a common *LRRK2* mutation amongst PD patients from Granada, with a frequency of 3.7 %. Although p.G2019S is responsible of 0.5%–4% of idiopathic PD cases amongst Caucasians, its prevalence has been found much higher amongst Ashkenazi Jews and North African Arabs (Healy et al., 2008; Lesage et al., 2006). However, the frequency of p.G2019S-carriers in PD cases from Granada suggests that there is not a strong influence from North Africa to our genetic landscape, as previously reported in a neighboring province (Gao et al., 2009).

Additionally, the *LRRK2* mutation p.R1441G found in our PD cohort with a frequency of 11.1% in the familial group and 1 % in the LOPD group was originally identified in both autosomal-dominant familial (46%) and idiopathic (2.5%) cases of PD, in the Basque region of northern Spain(Gorostidi et al., 2009; Paisán-Ruíz et al., 2004). The p.R1441G mutation is also identified at lower frequencies in patients from other Spanish provinces (between 0.7% in the East and 2.2% in the North), but it is very rare outside Northern Spain. In addition, we identified several *LRRK2* variants of unknown significance or not considered as disease causing variants.

The most prevalent genetic risk factor in PD identified up to date is heterozygous loss-of-function variants in *GBA1* (Sidransky et al., 2009). Interestingly, our PD cohort yielded 17 carriers (12.7%) of previously described pathogenic mutations. The frequency rate of *GBA* mutations varies considerably depending on the population ethnicity, with a remarkably high frequency in individuals of Ashkenazi Jewish descent. Our results show a significant higher frequency of *GBA* mutations in Southern Spain population in comparison to another study carried out in the Spanish population which found *GBA* mutations with a frequency of 9.8 % (Setó-Salvia et al., 2012). Previous studies in other European populations reported lower frequencies too; 6.4 % in Greeks (Kalinderi et al., 2009), 4.2 % in British (Neumann et al., 2009), and 8.3 % in Portuguese population (Bras et al., 2009).

The most common *GBA* variant found was N370S, encountered in 7 patients (5.2%). However, this is believed to be a rare risk factor for PD in North African Berber population (Nishioka et al., 2010). D409H, our second most prevalent *GBA* mutation, was detected in 4 patients. Additionally, we found the E326K mutation in 3 patients, 2 of which carried simultaneously the L444P variant (mutation phase unknown).

The present study ascertains that mutations in *GBA* contribute substantially to FPD and SPD in Andalusian population. As we screened in LOPD cases only selected *LRRK2* and *GBA* exons (mutation hotspots), some mutant alleles may have been missed.

Of particular interest is *PARK2*, which is the most frequently mutated gene in autosomal-recessive EOPD, with mutations found in 10%–20% of early-onset familial cases (Periquet et al., 2003). However, their prevalence and involvement in the modulation of PD risk have a wide variation depending on the studied population and the age of the subjects under study (Bardien et al., 2009; Bras et al., 2008; Schlitter et al., 2006). Our *PARK2* mutational spectrum included the homozygous deletion p.N52fs responsible for a recessive EOPD and an FPD case with a frequency of 5.4 %. The heterozygous deletion p.N52fs was found to cause FPD in 2.7 % of our cohort. Moreover, MLPA revealed heterozygous deletions in exons 3 and 4 of *PARK2*, which were also present in the affected brother. Furthermore, we found the previously reported homozygous variant p.G309D in *PINK1* in 1 FPD case(Valente et al., 2004).

Mutations in *VPS35* have been described as responsible of autosomal-dominant PD in late-onset familial cases. One of our most interesting findings is the presence of a novel mutation (p.R32S) which has not been reported in public single nucleotide polymorphism databases including ExAc, EVS, and 1000 Genome project. It was found in 1 EOPD case without a positive familial history for PD. Although it has been predicted as damaging, we cannot assume this mutation may be causative because we could not screen it in matched controls and we could not test segregation of the mutation in other family members.

5. Conclusions

We identified pathogenic mutations in 22.4 % of our PD population. *GBA* mutations appear to be considerably frequent, and it might reveal a strong influence from the Jewish population. In concordance with a previous study, *LRRK2* genetic variants in Andalusian individuals are common causes of PD. Further studies should be necessary to evaluate the possible pathogenicity of the novel found mutation p.R32S in *VPS35*. We found *SNCA* and *GCH1* mutations to be rare causes of the disease in our cases. Finally, we suggest that taken as a whole, these findings have clinical implications, showing that genetic screening may aid the diagnosis of PD in this population.

Disclosure statement

The authors have no financial disclosure to report. No pharmaceutical entity has collaborated in this study, and no financial purpose exists.

Acknowledgements

The authors are grateful to the participants in this study without whom this work would not have been possible. Sara Bandrés Ciga contributed to the execution of the work, organization, writing of the first draft. Niccolò Emmanuele Mencacci contributed to the execution of the work, organization, review, and critique. Raquel Durán contributed to the review and critique. Francisco Javier Barrero contributed to the neurological and clinical assessment and patient selection. Francisco Escamilla-Sevilla contributed to the neurological and clinical assessment and patient selection. Sarah Morgan contributed to the statistical and genetic analysis. Jason Hehir contributed to the execution of the work. Francisco Vives contributed to the review and critique. John Hardy contributed to the review and critique. Alan M. Pittman contributed to the conception, organization, and review and critique. This work was supported financially by a Medical Research Council and/or Welcome Trust Strategic Award (WT089698/Z/09/Z) and a grant of the Junta de Andalucía to the CTS-438 group. Bandrés Ciga Sara held an FPU fellowship from the Spanish Ministry of Education and Science financed jointly with a short-term stay grant by CEI-Biotic and University of Granada.

Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version at http://dx.doi.org/10.1016/j.neurobiolaging.2015.09.020.

References

Adams, S.M., Bosch, E., Balaresque, P.L., Ballereau, S.J., Lee, A.C., Arroyo, E., López-Parra, A.M., Aler, M., Grifo, M.S.G., Brion, M., Carracedo, A., Lavinha, J., Martínez-Jarreta, B., Quintana-Murci, L., Picornell, A., Ramon, M., Skorecki, K., Behar, D.M., Calafell, F., Jobling, M.A., 2008. The genetic Legacy of Religious Diversity and Intolerance: Paternal Lineages of Christians, jews, and muslims in the Iberian Peninsula. Am. J. Hum. Genet. 83, 725–736.

Bardien, S., Keyser, R., Yako, Y., Lombard, D., Carr, J., 2009. Molecular analysis of the parkin gene in South African patients diagnosed with Parkinson's disease. Parkinsonism Relat. Disord. 15, 116–121.

Bras, J., Guerreiro, R., Ribeiro, M., Morgadinho, A., Januario, C., Dias, M., Calado, A., Semedo, C., Oliveira, C., Hardy, J., Singleton, A., 2008. Analysis of Parkinson's disease patients from Portugal for mutations in SNCA, PRKN, PINK1 and LRRK2. BMC Neurol. 8, 1.

Bras, J., Paisan-Ruiz, C., Guerreiro, R., Ribeiro, M.H., Morgadinho, A., Januario, C., Sidransky, E., Oliveira, C., Singleton, A., 2009. Complete screening for glucocerebrosidase mutations in Parkinson disease patients from Portugal. Neurobiol. Aging 30, 1515–1517.

Duran, R., Mencacci, N.E., Angeli, A.V., Shoai, M., Deas, E., Houlden, H., Mehta, A., Hughes, D., Cox, T.M., Deegan, P., Schapira, A.H., Lees, A.J., Limousin, P., Jarman, P.R., Bhatia, K.P., Wood, N.W., Hardy, J., Foltynie, T., 2013. The glucocerobrosidase E326K variant predisposes to Parkinson's disease, but does not cause Gaucher's disease. Mov. Disord. 28, 232–236.

Gao, L., Gómez-Garre, P., Díaz-Corrales, F.J., Carrillo, F., Carballo, M., Palomino, A., Díaz-Martín, J., Mejías, R., Vime, P.J., López-Barneo, J., Mir, P., 2009. Prevalence and clinical features of LRRK2 mutations in patients with Parkinson's disease in southern Spain. Eur. J. Neurol. 16, 957–960.

Gibb, W.R., Lees, A.J., 1988. A comparison of clinical and pathological features of young- and old-onset Parkinson's disease. Neurology 38, 1402–1406.

Gómez-Garre, P., Jesús, S., Carrillo, F., Cáceres-Redondo, M.T., Huertas-Fernández, I., Bernal-Bernal, I., Bonilla-Toribio, M., Vargas-González, L., Carballo, M., Mir, P., 2014. Systematic mutational analysis of FBXO7 in a Parkinson's disease population from southern Spain. Neurobiol. Aging 35, 727.e5–727.e7.

Gorostidi, A., Ruiz-Martínez, J., Lopez de Munain, A., Alzualde, A., Martí Massó, J.F., 2009. LRRK2 G2019S and R1441G mutations associated with Parkinson's disease are common in the Basque Country, but relative prevalence is determined by ethnicity. Neurogenetics 10, 157–159.

Healy, D.G., Falchi, M., O'Sullivan, S.S., Bonifati, V., Durr, A., Bressman, S., Brice, A., Aasly, J., Zabetian, C.P., Goldwurm, S., Ferreira, J.J., Tolosa, E., Kay, D.M., Klein, C.,

Williams, D.R., Marras, C., Lang, A.E., Wszolek, Z.K., Berciano, J., Schapira, A.H.V., Lynch, T., Bhatia, K.P., Gasser, T., Lees, A.J., Wood, N.W., International LRRK2 Consortium, 2008. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. Lancet Neurol. 7, 583–590.

Kalinderi, K., Bostantjopoulou, S., Paisan-Ruiz, C., Katsarou, Z., Hardy, J., Fidani, L., 2009. Complete screening for glucocerebrosidase mutations in Parkinson's disease patients from Greece. Neurosci. Lett. 452, 87–89.

Kumar, P., Henikoff, S., Ng, P.C., 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat. Protoc. 4, 1073–1082.

Lesage, S., Dürr, A., Tazir, M., Lohmann, E., Leutenegger, A.-L., Janin, S., Pollak, P., Brice, A., French Parkinson's Disease Genetics Study Group, 2006. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N. Engl. J. Med. 354, 422–423.

Mencacci, N.E., Isaias, I.U., Reich, M.M., Ganos, C., Plagnol, V., Polke, J.M., Bras, J., Hersheson, J., Stamelou, M., Pittman, A.M., Noyce, A.J., Mok, K.Y., Opladen, T., Kunstmann, E., Hodecker, S., Munchau, A., Volkmann, J., Samnick, S., Sidle, K., Nanji, T., Sweeney, M.G., Houlden, H., Batla, A., Zecchinelli, A.L., Pezzoli, G., Marotta, G., Lees, A., Alegria, P., Krack, P., Cormier-Dequaire, F., Lesage, S., Brice, A., Heutink, P., Gasser, T., Lubbe, S.J., Morris, H.R., Taba, P., Koks, S., Majounie, E., Raphael Gibbs, J., Singleton, A., Hardy, J., Klebe, S., Bhatia, K.P., Wood, N.W., on behalf of the International Parkinson's Disease Genomics Consortium and UCL-exomes consortium, 2014. Parkinson's disease in GTP cyclohydrolase 1 mutation carriers. Brain 137 (Pt 9), 2480–2492.

Neumann, J., Bras, J., Deas, E., O'Sullivan, S.S., Parkkinen, L., Lachmann, R.H., Li, A., Holton, J., Guerreiro, R., Paudel, R., Segarane, B., Singleton, A., Lees, A., Hardy, J., Houlden, H., Revesz, T., Wood, N.W., 2009. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain 132 (Pt 7), 1783–1794.

Nishioka, K., Vilariño-Güell, C., Cobb, S.A., Kachergus, J.M., Ross, O.A., Wider, C., Gibson, R.A., Hentati, F., Farrer, M.J., 2010. Glucocerebrosidase mutations are not a common risk factor for Parkinson disease in North Africa. Neurosci. Lett. 477, 57–60.

Paisán-Ruíz, C., Jain, S., Evans, E.W., Gilks, W.P., Simón, J., van der Brug, M., López de Munain, A., Aparicio, S., Gil, A.M., Khan, N., Johnson, J., Martinez, J.R., Nicholl, D., Carrera, I.M., Pena, A.S., de Silva, R., Lees, A., Martí-Massó, J.F., Pérez-Tur, J., Wood, N.W., Singleton, A.B., 2004. Cloning of the gene containing mutations that causes PARK8-linked Parkinson's disease. Neuron 44, 595–600.

Paisán-Ruiz, C., Lewis, P.A., Singleton, A.B., 2013. LRRK2: cause, risk, and mechanism. J. Parkinsons Dis. 3, 85–103.

Periquet, M., Latouche, M., Lohmann, E., Rawal, N., De Michele, G., Ricard, S., Teive, H., Fraix, V., Vidailhet, M., Nicholl, D., Barone, P., Wood, N.W., Raskin, S., Deleuze, J.-F., Agid, Y., Dürr, A., Brice, A., French Parkinson's Disease Genetics Study Group, European Consortium on Genetic Susceptibility in Parkinson's Disease, 2003. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 126 (Pt 6), 1271–1278.

Schlitter, A.M., Kurz, M., Larsen, J.P., Woitalla, D., Müller, T., Epplen, J.T., Dekomien, G., 2006. Parkin gene variations in late-onset Parkinson's disease: comparison between Norwegian and German cohorts. Acta Neurol. Scand. 113, 9–13.

Setó-Salvia, N., Pagonabarraga, J., Houlden, H., Pascual-Sedano, B., Dols-Icardo, O., Tucci, A., Paisán-Ruiz, C., Campolongo, A., Antón-Aguirre, S., Martín, I., Muñoz, L., Bufill, E., Vilageliu, L., Grinberg, D., Cozar, M., Blesa, R., Lleó, A., Hardy, J., Kulisevsky, J., Clarimón, J., 2012. Glucocerebrosidase mutations confer a greater risk of dementia during Parkinson's disease course. Mov Disord. 27, 393–399.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., Barbosa, E.R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., Chen, C.-M., Clark, L.N., Condroyer, C., De Marco, E.V., Dürr, A., Eblan, M.J., Fahn, S., Farrer, M.J., Fung, H.-C., Gan-Or, Z., Gasser, T., Gershoni-Baruch, R., Giladi, N., Griffith, A., Gurevich, T., Januario, C., Kropp, P., Lang, A.E., Lee-Chen, G.-J., Lesage, S., Marder, K., Mata, I.F., Mirelman, A., Mitsui, J., Mizuta, I., Nicoletti, G., Oliveira, C., Ottman, R., Orr-Urtreger, A., Pereira, L.V., Quattrone, A., Rogaeva, E., Rolfs, A., Rosenbaum, H., Rozenberg, R., Samii, A., Samaddar, T., Schulte, C., Sharma, M., Singleton, A., Spitz, M., Tan, E.-K., Tayebi, N., Toda, T., Troiano, A.R., Tsuji, S., Wittstock, M., Wolfsberg, T.G., Wu, Y.-R., Zabetian, C.P., Zhao, Y., Ziegler, S.G., 2009. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med. 361, 1651–1661.

Singleton, A.B., Farrer, M.J., Bonifati, V., 2013. The genetics of Parkinson's disease: Progress and therapeutic implications. Mov. Disord. 28, 14–23.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., Wood, N.W., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304, 1158–1160.

Venselaar, H., Te Beek, T.A., Kuipers, R.K., Hekkelman, M.L., Vriend, G., 2011. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. BMC Bioinformatics 11, 548.

Zalloua, P.A., Platt, D.E., El Sibai, M., Khalife, J., Makhoul, N., Haber, M., Xue, Y., Izaabel, H., Bosch, E., Adams, S.M., Arroyo, E., López-Parra, A.M., Aler, M., Picornell, A., Ramon, M., Jobling, M.A., Comas, D., Bertranpetit, J., Wells, R.S., Tyler-Smith, C., Genographic Consortium, 2008. Identifying genetic traces of historical expansions: Phoenician footprints in the Mediterranean. Am. J. Hum. Genet. 83, 633–642.

Neurobiology of Aging xxx (2016) 1–7

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Genome-wide assessment of Parkinson's disease in a Southern Spanish population

Sara Bandrés-Ciga [a,1], Timothy Ryan Price [b,1], Francisco Javier Barrero [c], Francisco Escamilla-Sevilla [d], Javier Pelegrina [c], Sampath Arepalli [b], Dena Hernández [b], Blanca Gutiérrez [e,f], Jorge Cervilla [e,f], Margarita Rivera [e,f], Alberto Rivera [b], Jing-hui Ding [b], Francisco Vives [a], Michael Nalls [b], Andrew Singleton [b], Raquel Durán [a,*]

[a] Department of Physiology and Institute of Neurosciences Federico Olóriz, Centro de Investigaciones Biomedicas (CIBM), University of Granada, Granada, Spain
[b] Laboratory of Neurogenetics, NIA, NIH, Bethesda, MD, USA
[c] Movement Disorders Unit, University Hospital San Cecilio, Granada, Spain
[d] Movement Disorders Unit, Department of Neurology, Instituto de Investigación Biosanitaria (IBS), University Hospital Virgen de las Nieves, Granada, Spain
[e] Department of Psychiatry and Institute of Neurosciences Federico Olóriz, Centro de Investigaciones Biomedicas (CIBM), University of Granada, Granada, Spain
[f] CIBER en Salud Mental (CIBERSAM), University of Granada, Granada, Spain

ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form 31 May 2016
Accepted 2 June 2016

Keywords:
Parkinson's disease
Genetics
GWAS
Southern Spain

ABSTRACT

Here, we set out to study the genetic architecture of Parkinson's disease (PD) through a Genome-Wide Association Study in a Southern Spanish population. About 240 PD cases and 192 controls were genotyped on the NeuroX array. We estimated genetic variation associated with PD risk and age at onset (AAO). Risk profile analyses for PD and AAO were performed using a weighted genetic risk score. Total heritability was estimated by genome-wide complex trait analysis. Rare variants were screened with single-variant and burden tests. We also screened for variation in known PD genes. Finally, we explored runs of homozygosity and structural genomic variations. We replicate PD association (uncorrected p-value < 0.05) at the following loci: *ACMSD/TMEM163, MAPT, STK39, MIR4697,* and *SREBF/RAI1*. Subjects in the highest genetic risk score quintile showed significantly increased risk of PD versus the lowest quintile (odds ratio = 3.6, p-value $< 4e^{-7}$), but no significant difference in AAO. We found evidence of runs of homozygosity in 2 PD-associated regions: one intersecting the *HLA-DQB1* gene in 6 patients and 1 control; and another intersecting the *GBA-SYT11* gene in PD case. The *GBA* N370S and the *LRRK2* G2019S variants were found in 8 and 7 cases, respectively, replicating previous work. A structural variant was found in 1 case in the *PARK2* gene locus. This current work represents a comprehensive assessment at a genome-wide level characterizing a novel population in PD genetics.

1. Introduction

Over the last several years, genetic investigation of Parkinson's disease (PD) has successfully identified many disease-causing mutations (Bonifati et al., 2003; Kitada et al., 1998; Paisán-Ruiz et al., 2004; Polymeropoulos et al., 1997; Valente et al., 2004; Vilariño-Güell et al., 2011; Zimprich et al., 2004, 2011) providing considerable insight into the molecular mechanisms underlying the etiology and pathogenesis of this multifactorial and complex disease.

Importantly, Genome Wide Association (GWA) studies (Edwards et al., 2010, Hernandez et al., 2012, Pihlstrom et al., 2013, Saad et al., 2011, Satake et al., 2009, Simón-Sánchez et al., 2009, 2011) and large scale meta-analyses (Do et al., 2011; International Parkinson's Disease Genomics Consortium and Welcome Trust Case Control Consortium 2, 2011; Lill et al., 2012; Nalls et al., 2014, International Parkinson Disease Genomics Consortium et al., 2011; Pankratz et al., 2012) have been applied to identify and replicate risk loci that fit the common disease, common variant hypothesis in PD (Reich and Lander, 2001). Despite the significant number of novel risk loci identified so far, only a small portion of the

* Corresponding author at: Department of Physiology and Institute of Neurosciences Federico Olóriz, Centro de Investigaciones Biomedicas (CIBM), University of Granada, 18016 Granada, Spain. Tel.: +34 686 63 0291.
E-mail address: rduran@ugr.es (R. Durán).
[1] These authors contributed equally to this work.

0197-4580/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2016.06.001

heritable component for PD has been explained, suggesting there is a substantial unknown genetic component to be discovered (Keller et al., 2012). The polygenic nature of the illness and the hypothetically large number of loci involved with risk variants of small effect size make analysis of the genetic contribution to disease phenotype particularly difficult.

It has been widely suggested that studies in populations with limited genetic heterogeneity are valuable for studying the genetic basis of disease (Hernandez et al., 2012). Moreover, replication studies in independent populations are absolutely necessary to test the robustness of such association reports. Andalusia, given its geographical location on the southernmost region of Spain, represents a cross-link between Europe and Africa. The interactions and interbreeding across the Mediterranean Sea and North Africa have contributed to shape a genetic profile, which to date has been poorly studied in the context of PD genetics. Motivated by these considerations, we set out to study the genetic architecture of PD in this population by performing GWAS.

The purpose of this project was to identify novel putative candidate loci associated with PD and to investigate whether single nucleotide polymorphisms (SNPs) previously identified as risk variants contribute to PD risk in the Southern Spanish population. Furthermore, we use genetic risk profiling to aggregate risk across the previously established risk loci. In the same way, we aim to understand whether this genetic risk is associated with age at disease onset. We also attempt to identify recessive founder variants by exploring runs of homozygosity enriched in cases and explore whether disease-associated rare variants are present in our cohort. Finally, we estimate PD heritability attributable to genetic variation assessed by our genotyping platform and evaluate the role of copy number variants (CNVs) as risk factors for PD in these subjects.

2. Methods

We included a group of 240 PD patients treated at the Movement Disorders Unit of the Service of Neurology in both Hospital Clínico San Cecilio and Hospital Virgen de las Nieves of Granada (Spain). PD was diagnosed at least by 2 experienced neurologists in the field of movement disorders following the criteria of the UK PD Society Brain Bank (Gelb et al., 1999). Our control group was comprised of 192 healthy individuals with no signs or symptoms of parkinsonism who were extensively assessed to rule out any sign of neurological condition. The study was approved by the local ethics committee, and written informed consent was taken from each participant.

Samples were genotyped using the NeuroX Array (Illumina), a powerful and reliable tool for the investigation of genetic factors associated with neurodegenerative disorders (Nalls et al., 2015a). It comprises standard Illumina exome content of approximately 240,000 variants densely covering previously published PD GWAS-associated loci, ancestry informative markers, markers for determination of identity by descent, X chromosome SNPs for sex determination, and over 24,000 custom content variants focusing on rare variants related to neurodegenerative diseases.

After genotyping, genotypes were clustered using Illumina Genome Studio using a previously developed cluster file for NeuroX (Nalls et al., 2015a). Stringent quality control (QC) analysis was performed as follows: samples with call rates of less than 95% and whose genetically determined sex from X chromosome heterogeneity did not match that from clinical data were excluded from the analysis. Samples exhibiting heterozygosity greater than 6 standard deviations from the population mean were excluded. Once preliminary sample-level QC was completed, SNPs with minor allele frequency (MAF) $<$ 0.01, Hardy–Weinberg Equilibrium p-value $<$ 1E-5 and missingness rates $>$ 5% were excluded. Palindromic SNPs were excluded as well. SNPs thought to be in linkage disequilibrium (LD) in a sliding window of 50 adjacent SNPs, which scrolled through the genome at a rate of 5 overlapping SNPs were also removed from the following analyses. Genetic variants passing QC numbered 33,074 SNPs, 28,393 of which remained after LD pruning. Next, samples were clustered using principal component analysis to evaluate European ancestry as compared to the HapMap3 CEU/TSI populations (International HapMap Consortium, 2003) (Figure S1). Confirmed European ancestry samples were extracted and principal components (PCs) 1 and 2 were used as covariates in all analyses. Those samples cryptically related at the level of first cousins or closer (sharing proportionally more than 12.5 % of alleles) were dropped from the following analysis. Samples passing QC numbered 206 cases and 172 controls.

For all SNPs (non-LD pruned) and samples passing QC as detailed in the previous paragraph, a logistic regression model adjusted for sex, age, and first and second genotype PCs as covariates was used to estimate risk associated with the disease. A linear regression model adjusted for the same covariates was used to explore the influence of genetic variation on the age at onset (AAO) of the disease.

Risk profile analysis for PD and AAO was performed as described in detail elsewhere (Kara et al., 2014; Nalls et al., 2015b). Briefly, a cumulative genetic risk score (GRS) was calculated incorporating 30 SNPs previously associated with PD (Nalls et al., 2014). Risk allele dosages were counted, and a GRS was generated across all loci. All SNPs were weighted by their published odds ratios (ORs), giving greater weight to alleles with higher risk estimates.

The data set was divided into quintiles based on the GRS. A regression was performed regressing disease (logistic regression) or AAO (linear regression) against quintile membership, and ORs were reported comparing the reference group (lowest risk quintile) to the remaining 4 quintiles. Risk profiling analysis was adjusted for PCs 1 and 2 to account for population substructure.

In an attempt to estimate PD heritability attributable to genome-wide assayed genetic variation, we used the genome-wide complex trait analysis package (Yang et al., 2011). This analysis has the ability to detect contribution to disease risk of variants, which do not reach genome significance because of small effect size, though not to identify the specific variants contributing to disease risk. First, a genetic relationship matrix is calculated by estimating pairwise genetic relations between the individuals in the cohort. Then, a linear mixed model is applied to estimate phenotypic variance explained by genome-wide SNPs passing QC, correcting for PCs 1 and 2, age, and gender.

For identity by state comparisons, PLINK 1.07 (Purcell et al., 2007) was used to calculate case and control specific rates of proportional allele sharing both genome-wide and within defined segments. We used a pairwise approach to estimate overall allelic similarity between each pairing of subjects. Then, case-control labels were permuted 2000 times to assess whether case and/or control pairings are more different in the actual data than those in permuted data, suggesting segregation of cases and controls into distinct populations. In these analyses, cryptically related PD individuals removed in previous steps were included to identify shared rare recessive variants.

Subsequently, we tested for association between rare variants (MAF $<$0.05) and PD using a single-variant test under an additive genetic model. This approach evaluates if there is any association between each independent variant and PD, employing a significance threshold of 5×10^{-8}. The minimum minor allele count considered for the analysis was 3, and we adjusted for the top 20 PCs, age, and sex. In addition to testing each variant individually, we evaluated cumulative effects of multiple genetic variants by

collapsing rare variants within a gene level using the SKAT-O algorithm at default settings as part of the EPACTS package (information at http://genome.sph.umich.edu/wiki/EPACTS) (Lee et al., 2012).

Moreover, we screened for variants with MAF <1% in known PD-causing genes (Table S1), checking all variants with 2 approaches: a putative dominant model (allele present only in cases and not in controls) and a putative recessive model (allele present with 2 copies only in cases and not in 2 copies in controls).

Finally, detection of structural genomic variations was performed using PennCNV-1.0.3 (Illumina Inc, San Diego, CA, USA) and visualized with R. Two metrics were assessed with this utility: B allele frequency and log R ratio. These 2 statistics allow visualization of copy number changes and are described in detail elsewhere (Matarin et al., 2008). Briefly, log R ratio gives an indirect measure of copy number of each SNP by plotting the ratio of observed to expected hybridization intensity. An R value above 1 is indicative of an increase in copy number (duplication or triplication), and an R value below 1 suggests a decrease (deletion). B allele frequency plots the proportion of times an allele is called A or B at each genotype: thus the expected ratios are 1.0 (B/B), 0.5 (A/B), and 0.0 (A/A). Significant deviations from these figures in contiguous SNPs are indicative of a CNV. Although this metric exhibits a high level of variance for individual SNPs, it does provide a measure of CNV when log R ratio values for numerous adjacent SNPs are visualized.

3. Results

Descriptive statistics of the Spanish cohort are summarized in Table 1. Visualization of the top 2 genetic PCs of our Spanish samples shows them to be similar overall to HapMap European populations (Figure S1).

Our GWA failed to detect any genetic variant significant at $p < 5e^{-8}$ (Fig. 1). Although we did not show any association signal with PD at this stringent p-value, we managed to marginally replicate association on the basis of a nominal uncorrected p-value < 0.05 with the following loci previously identified in a large-scale PD GWAS meta-analysis: *ACMSD/TMEM163, MAPT, STK39, MIR4697,* and *SREBF/RAI1* (Table 2; Figure S2), (Nalls et al., 2011). Similarly, genome-wide assessment for AAO of PD did not reveal any genetic variant to be associated at p values $< 5e^{-8}$ (Fig. 2; Figure S3).

Our cumulative risk attributable to all known GWAS loci associated with PD showed significantly different GRSs in this cohort (p-value $<4e^{-7}$). Individuals in the highest quintile of genetic risk were found to be 3.6 times more likely to develop PD than those in the lowest quintile (Table 3; Figure S4). No significant association was found between GRS and AAO of disease ($p = 0.52$, data not shown).

Using genome-wide complex trait analysis, we estimated total heritability explained by the genotyped SNPs on the NeuroX array to be 13.7 % ± 40% (0%–92% at 95% CI). Our NeuroX array is more focused on a lower number of exonic variants compared to earlier analyses, which may contribute to the lower estimate reported here (Keller et al., 2012). The lack of precision in this estimate likely reflects the low power of the present study, and thus the results of this heritability analysis should be viewed with caution.

Table 1
Demographic characteristics of the Spanish cohort

	Samples passing QC	Male/ Female	Age of study enrollment[a]	Age at onset[a]
Whole sample	378	210/168	63.14 ± 13	
PD cases	206	133/73	67.38 ± 10.51	61.88 ± 13.77
Controls	172	77/95	58.07 ± 13.99	

Key: PD, Parkinson's disease; QC, quality control.
[a] Expressed as mean age ± standard deviation.

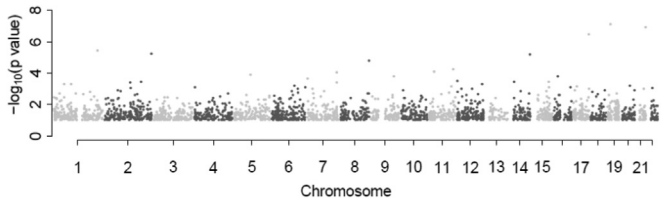


Fig. 1. Manhattan plot showing results of PD GWA testing between 206 PD patients and 172 controls from Southern Spain. Abbreviations: GWA, Genome Wide Association; PD, Parkinson's disease.

In a genome-wide assessment of differential identity by state between cases and controls, we found no evidence of excess of homozygosity in all cases versus all controls. However, we did find evidence of runs of homozygosity in 2 regions previously associated with PD: one intersecting the *HLA-DQB1* gene in 6 patients and one control; and another intersecting the *GBA-SYT11* gene in 1 PD case. A run of at least 98.5% homozygosity from chr6:32,333,827-32,680,928 (~347 kb) was consistently reported across subjects showing homozygosity in the *HLA-DQB1* locus. This region directly overlaps the previously reported GWAS hit (Nalls et al., 2014). Our single case with a run of homozygosity in *GBA-SYT11* exhibited a 99.6% homozygous run of 11,233 kb (chr1:149,906,413-161,139,738).

Single-variant tests could not identify any prospective novel rare variant associated with PD (Figure S5). When focusing on the cumulative effects of multiple genetic rare variants by grouping them by a gene level we also detect no significant association with PD (Figure S6, Figure S7, Figure S8).

However, we explored rare variants in PD known genes present in cases but absent in our controls (Table 4). The *GBA* N370S risk variant was found in 8 cases (7 heterozygous and 1 homozygous), and the *LRRK2* G2019S pathogenic mutation was identified in 7 patients, all in heterozygosis. Variants of unknown pathogenicity were also detected in *PINK1* (Valente et al., 2004), *PARK2* (Bonifati et al., 2003), *GIGYF2* (Lautier et al., 2008), *DNAJC13* (Vilariño-Güell et al., 2011), *SYNJ1* (Krebs et al., 2013), and *FBXO7* (Shojaee et al., 2008).

In addition to providing SNP variation data, the genotyping assay used generates metrics that allow detection of CNV. We evaluated both the log R ratio and the B allele frequency plots across the genome in all samples and identified a *PARK2* heterozygous deletion (Chr6: 162,206,852-162,206,882) in a PD case (Figure S9).

4. Discussion

Here, we describe what is to our knowledge the first GWAS for PD in a Spanish cohort, and the second in a Southern European population (Kara et al., 2014). Although we are aware that the sample size itself is a limitation, it comprised most PD cases from the province of Granada. Therefore, one of the strengths of this study is that no potential problems of population stratification are expected as the sample comes from a relatively homogeneous ancestry background.

Our results suggest that there is no detectable high-risk variant for PD present in our population, consistent with results of other GWASes with small sample size (Fung et al., 2006; Hernández et al., 2012). However, we have replicated association of 5 previously reported PD-related loci (Nalls et al., 2014) at nominal uncorrected

Table 2
Results of the replication association analyses

Nearest gene (s)	SNP	CHR	BP	A1	A2	MAF	OR	SE	L95	U95	STAT	*p*	OR[a]	L95[a]	U95[a]
ACMSD/TMEM163	rs6430538	2	135539967	C	T	0.4112	1.533	0.1865	1.064	2.21	2.291	0.02195	1.14	1.10	1.18
MAPT	rs17649553	17	43994648	T	C	0.3073	0.6318	0.2055	0.4224	0.9452	−2.234	0.02547	0.77	0.74	0.80
STK39	rs1955337	2	169129145	T	G	0.1789	2.346	0.4031	1.065	5.169	2.115	0.03442	1.20	1.15	1.26
MIR4697	rs329648	11	133765367	T	C	0.4399	1.443	0.1736	1.027	2.028	2.113	0.03458	1.09	1.06	1.13
SREBF/RAI1	rs11868035	17	17715101	A	G	0.2917	0.6581	0.2094	0.4365	0.992	−1.998	0.0457	0.93	0.90	0.96
SNCA	rs356181	4	90626139	C	T	0.4505	1.303	0.1633	0.9463	1.795	1.622	0.1049	0.90	0.87	0.93
RIT2	rs12456492	18	40673380	G	A	0.3216	1.349	0.1894	0.9306	1.955	1.58	0.1141	1.10	1.06	1.14
SIPA1L2	rs10797576	1	232664611	T	C	0.1589	2.111	0.5017	0.7898	5.644	1.49	0.1363	1.13	1.08	1.19
RAB7L1/NUCKS1	rs823118	1	205723572	C	T	0.3838	1.285	0.1848	0.8947	1.846	1.358	0.1745	0.88	0.86	0.91
CCDC62	rs11060180	12	123303586	G	A	0.3945	0.8088	0.1683	0.5815	1.125	−1.26	0.2075	0.90	0.87	0.93
TMEM175/GAK/DGKQ	rs34311866	4	951947	C	T	0.2055	1.356	0.2974	0.7569	2.429	1.024	0.306	1.27	1.22	1.32
SNCA	rs3910105	4	90682571	C	T	0.4413	0.8579	0.1632	0.623	1.181	−0.9392	0.3476	0.89	0.86	0.91
GPNMB	rs199347	7	23293746	C	T	0.4661	0.8747	0.1551	0.6454	1.185	−0.8631	0.3881	0.89	0.86	0.92
FAM47E/SCARB2	rs6812193	4	77198986	T	C	0.3789	1.153	0.1794	0.8113	1.639	0.7943	0.427	0.89	0.86	0.93
MCCC1	rs12637471	3	182762437	A	G	0.1758	0.7834	0.3182	0.4199	1.462	−0.7672	0.443	0.84	0.81	0.87
DDRGK1	rs55785911	20	3153503	A	G	0.373	0.8852	0.1766	0.6262	1.251	−0.6905	0.4899	0.91	0.88	0.94
VPS13C	rs2414739	15	61994134	G	A	0.2656	0.8594	0.2269	0.5509	1.341	−0.6681	0.5041	0.89	0.86	0.93
LRRK2	rs7294619	12	40614434	T	C	0.1462	0.8173	0.3631	0.4012	1.665	−0.5558	0.5783	0.86	0.82	0.90
BCKDK/STX1B	rs14235	16	31121793	A	G	0.4232	0.9306	0.1643	0.6745	1.284	−0.4377	0.6616	1.09	1.05	1.13
FGF20	rs591323	8	16697091	A	G	0.2546	0.9082	0.2761	0.5287	1.56	−0.3488	0.7273	0.92	0.88	0.95
BST1	rs11724635	4	15737101	C	A	0.4792	0.9513	0.1622	0.6923	1.307	−0.3076	0.7584	0.89	0.86	0.91
GCH1	rs11158026	14	55348869	T	C	0.2865	1.04	0.1997	0.7028	1.538	0.1946	0.8457	0.88	0.85	0.92
HLA_DBQ1	rs115462410	6	32666660	T	C	0.09245	1.116	0.7542	0.2546	4.895	0.1459	0.884	1.25	1.17	1.34
TMEM175	rs34884217	4	944210	G	T	0.09635	0.9088	0.6654	0.2467	3.348	−0.1437	0.8857	0.74	0.68	0.80
GBA	rs35749011	1	155205634	C	T	0.005222	NA	NA	NA	NA	NA	NA	0.56	0.50	0.63
GBA/SYT11	rs71628662	1	155359992	C	T	0.005208	NA	NA	NA	NA	NA	NA	0.56	0.50	0.63
NOTCH	rs8192591	6	32185796	T	C	0.009115	NA	NA	NA	NA	NA	NA	1.11	1.02	1.21
INPP5F	rs10886515	10	121710488	T	C	0.0248	NA	NA	NA	NA	NA	NA	1.10	1.06	1.14

Risk estimates are based on the dosage of A1 (minor allele).
Key: BP, base pair; CHR, chromosome; MAF, minor allele frequency; OR, odds ratio; PD, Parkinson's disease; SE, standard error; SNP, single nucleotide polymorphism; STAT, statistics.
[a] Values referring to the PD metanalisis (Nalls et al., 2014).

p-value < 0.05, though these associations do not pass multiple corrections at n = 30 (0.05/30 ≈ 0.002; Table 2). Our limited replication is likely due at least in part to insufficient statistical power to detect the relatively low effect of these alleles in our smaller cohort. For example, assuming an alpha of 0.002, disease prevalence of 4.4%, allele frequency of 0.2, and relative risk of 1.3, our power in this study would be 0.065. The presence of population-specific differences in the Southern Spanish sample and PD subphenotypes with distinct genetic etiologies might also be responsible, among other factors, of this lack of association.

This present study ascertains excesses of homozygosity in specific genomic regions overrepresented in cases, suggesting that variants in *HLA-DQB1* and *GBA-SYT11* contribute to idiopathic PD in our population. These sites have been linked to idiopathic PD in the past (Hamza et al., 2010; Sidransky et al., 2009; Duran et al., 2013), although further work characterizing these loci in patients remains.

Our genetic risk profiling for PD demonstrates between-quintile ORs of a similar magnitude reported in other previous studies (Hernandez et al., 2012; International Parkinson's Disease Consortium, 2011; Kara et al., 2014, International Parkinson Disease Genomics Consortium et al., 2011), showing that the risk profiles found in other populations of European descent are applicable to the Andalusian population. Consistent with these earlier studies, the area under the curve, calculated based on a logistic regression of disease status versus GRS was 0.638. This suggests that the currently known risk loci are not sufficient to predict disease status, and thus it is of limited clinical utility when used alone. When used in conjunction with age, gender, olfaction, and family history of PD, however, the GRS has been shown to contribute distinct information content to disease status prediction (Nalls et al., 2015c).

To date, limited progress has been made in understanding how genetic variation affects PD phenotype. No GWAS has identified and independently replicated common variants associated with AAO on a genome-wide scale. Our study, in agreement with the literature, did not reveal any significant variant to be associated with this PD feature. However, recent studies have found that increasing GRSs are related to earlier AAO (Lill et al., 2015; Nalls et al., 2015b) suggesting that early-onset forms of the illness are not exclusively caused by highly penetrant Mendelian mutations but can also be influenced by an accumulation of common polygenic alleles with relatively low effect sizes (Escott-Price et al., 2015). We show no association between GRS and AAO in this study.

We know that common genetic variation plays a substantial role in PD, but an important proportion of the genetic risk might result from rare alleles. Given the size of our cohort, it is likely that we

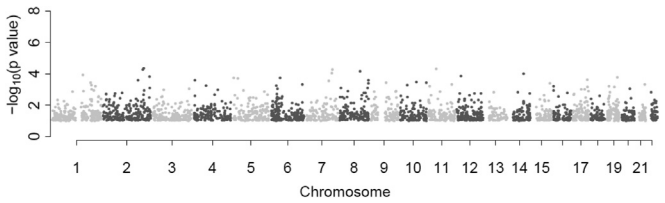


Fig. 2. Manhattan plot showing results of PD GWA with age at onset testing between 206 PD patients and 172 controls from Southern Spain. Abbreviations: GWA, Genome Wide Association; PD, Parkinson's disease.

Table 3
Genetic risk profile in the Southern Spanish cohort based on SNPs in Table 2

	Risk quintiles				
	First	Second	Third	Fourth	Fifth
Odds ratio	1	1.61	1.83	3.44	3.65
CI (95%)		(0.85–3.08)	(0.96–3.52)	(1.79–6.76)	(1.89–7.21)
p-value	4.0E-07				
AUC	0.63				

Key: AUC, area under the curve; SNP, single nucleotide polymorphism.

Table 4
Genetic rare variants (minor allele frequency [MAF] <0.01) identified in PD known genes in cases but not in controls

CHR	Gene	BP	NeuroX SNP	rs ID	Variant	Zygosity	R/A	Cases	Frequency (ExAc)	Polyphen	SIFT
1	*PINK1*	20971158	exm27115	rs139226733	p.Met318Leu	Het	A/T	1	0.001618	B	D
1	*PINK1*	20975021	exm27131	rs45515602	p.Ala383Thr	Het	G/A	2	0.0001961	B	T
1	*GBA*	155205634	exml06217	rs76763715	p.Asn370Ser	Het	T/C	7	0.00363	B	D
						Hom		1			
2	*GIGYF2**	233612427	exm275808	rs147623346	Met48lle	Het	G/T	2	0.0001499	D	U
2	*GIGYF2*	233655834	exm275883	rs 148277228	Asp371Glu	Het	T/A	1	0.0007366	T	U
2	*GIGYF2*	233656142	exm275892	rs34845648	Pro445His	Het	C/T	1	0.0001132	D	U
3	*DNAJC13**	132213932	exm351306	rs35658317	IVS5-1G>A/3942-1G>A	Het	G/A	1	0.0003347	—	—
3	*DNAJC13*	132242012	exm351379	rs61731474	p.Asn1952Thr	Het	A/C	1	0.0001349	B	T
3	*DNAJC13*	132244562	exm351399	rs 138693725	p.Ala2057Ser	Het	G/T	1	0.001334	B	T
6	*PARK2*	184045771	exm593521	rs9456735	Met192Leu	Het	T/G	1	0.0003299	T	PDG
12	*LRRK2*	40629436	exm994436	rs33995463	p.Leu119Pro	Het	T/C	2	0.00202	D	PDG
12	*LRRK2*	40677813	exm994520	rs35173587	Arg793Met	Het	G/T	1	0.005747	D	PDG
12	*LRRK2*	40697802	exm994561	rs 143710836	Ala1215Thr	Het	G/A	1	0.0002101	T	B
12	*LRRK2*	40734202	exm994671	rs34637584	p.Gly2019Ser	Het	G/A	7	0.0006294	PD	D
21	*SYNJ1*	34004111	exm1566662	rs115648918	Val1345lle	Het	C/T	3	0.0001713	T	B
21	*SYNJ1*	34012088	exm1566685	rs145937537	Thr1236Met	Het	G/A	1	0.00216	T	B
21	*SYNJ1*	34029195	exm1566728	rs114053718	lle905Thr	Het	A/G	1	0	D	PDG
21	*SYNJ1*	34058146	exm1566794	rs114942253	Ala383Thr	Het	C/T	2	0.01499	B	B
22	*FBX07*	32875122	exm1602819	rs143041875	Ser93Ala	Het	T/G	3	0.0001498	D	B
22	*PLA2G6*	38528888	exm1608133	rs11570680	Ala343Thr	Hom	C/T	1	0.02574	T	B

Frequency ExAc represents frequency of the rare variants reported for European (Non Finnish) population.
Key: A, alternate allele; B, Benign; D, Damaging/Deleterious; PDG, possibly damaging; R, reference allele; T, tolerated; U, unknown.
*Putative PD-linked genes.

were unable to find any novel prospective rare variant simply due to their rarity. If a disease-causing rare variant is present in 0.5% of cases, we would expect to see it in only 1 case in our population, severely limiting statistical power. Indeed, no rare variant or summation of rare variants within genes showed a significant association with PD status. However, when examining known PD gene loci, we identified the *LRRK2* G2019S pathogenic mutation in 7 cases and the *GBA* N370S risk variant in 8 cases, in concordance with our previous findings by sequencing (Bandrés-Ciga et al., 2016).

Structural genomic variation analysis revealed that an early-onset PD case (0.48% of patients) carried a heterozygous CNV mutation in *PARK2*. CNVs constitute a particular challenge in GWAs, and we assume there may be additional CNVs conferring risk for the disease poorly covered or undetectable by the array that could explain the lower frequency of heterozygous exonic rearrangements in *PARK2* in our study in comparison with other studies (Huttenlocher et al., 2015; Kay et al., 2010).

Although our estimates of phenotypic variance provided imprecise results due to limited sample size, there is compelling evidence that a large proportion of heritability in PD has yet to be discovered (Keller et al., 2012). We also note that reported heritability is likely biased to be high because of the enrichment of neurodegenerative disease-associated loci on the NeuroX chip (Nalls et al., 2015a). We propose there will be a considerable number of variants that impart risk for disease outside the limits of what can be reliably detected by our genotyping platform.

In conclusion, this present work represents a comprehensive assessment of a Spanish cohort at a genome-wide level, characterizing a novel population in PD genetics. We replicate the association of 5 reported PD-related loci, and our genetic risk profile is consistent with previous studies. Moreover, we reveal an excess of homozygosity in cases in the *HLA-DBQ1* and *GBA-SYT11* loci, strengthening evidence for the role of these loci in the etiology of PD. We suggest that pooling data sets of larger samples will be required for further identification and replication of PD genetic associations.

Disclosure statement

No pharmaceutical entity has collaborated in this study, and no financial purpose exists.

Acknowledgements

The authors are grateful to the participants in this study without whom this work would not have been possible. Sara Bandrés Ciga and Timothy Ryan Price contributed equally to this work. Andrew Singleton, Michael Nalls, Dena Hernández, Raquel Durán, and Francisco Vives contributed to study concept, design, revision, and critique. Sampath Arepalli and Sara Bandrés Ciga contributed for execution of the laboratory work. Timothy Ryan Price, Sara Bandrés Ciga, Alberto Rivera, and Jing-Hui Ding contributed for data analysis. Francisco Barrero-Hernández, Francisco Escamilla-Sevilla, Javier Pelegrina, Blanca Gutiérrez, Jorge Cervilla, and Margarita Rivera contributed to neurological–clinical assessment, patient and control recruitment, and sample collection. This work was supported in part by the Intramural Research Program of the National Institute on Aging, National Institutes of Health, part of the Department of Health and Human Services (project ZO1 AG000949), the following grants from the Junta de Andalucía; PI0322/2009 to the group CTS-6682; CVI-6476 and PSI2014-57643 to the CTS-438 group, and a research award from the Andalusian Society of Neurology. Sara Bandrés Ciga held an FPU fellowship FPU12-01885 and a short-term stay grant EST14/0061 from the Spanish Ministry of Education and Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.06.001.

References

Bandrés-Ciga, S., Mencacci, N.E., Durán, R., Barrero, F.J., Escamilla-Sevilla, F., Morgan, S., Hehir, J., Vives, F., Hardy, J., Pittman, A.M., 2016. Analysis of the genetic variability in Parkinson's disease from Southern Spain. Neurobiol. Aging 37, 210.e1–210.e5.

Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C.J., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J.W., Vanacore, N., van Swieten, J.C., Brice, A., Meco, G., van Duijn, C.M., Oostra, B.A., Heutink, P., 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299, 256–259.

Do, C.B., Tung, J.Y., Dorfman, E., Kiefer, A.K., Drabant, E.M., Francke, U., Mountain, J.L., Goldman, S.M., Tanner, C.M., Langston, J.W., Wojcicki, A., Eriksson, N., 2011. Web-based genome-wide association study identifies two novel loci and a substantial genetic component for Parkinson's disease. PLoS Genet. 7, e1002141.

Duran, R., Mencacci, N.E., Angeli, A.V., Shoai, M., Deas, E., Houlden, H., Mehta, A., Hughes, D., Cox, T.M., Deegan, P., Schapira, A.H., Lees, A.J., Limousin, P., Jarman, P.R., Bhatia, K.P., Wood, N.W., Hardy, J., Foltynie, T., 2013. The glucocerobrosidase E326K variant predisposes to Parkinson's disease, but does not cause Gaucher's disease. Mov. Disord. 28, 232–236.

Edwards, T.L., Scott, W.K., Almonte, C., Burt, A., Powell, E.H., Beecham, G.W., Wang, L., Züchner, S., Konidari, I., Wang, G., Singer, C., Nahab, F., Scott, B., Stajich, J.M., Pericak-Vance, M., Haines, J., Vance, J.M., Martin, E.R., 2010. Genome-wide association study confirms SNPs in SNCA and the MAPT region as common risk factors for Parkinson disease. Ann. Hum. Genet. 74, 97–109.

Escott-Price, V., International Parkinson's Disease Genomics Consortium, Nalls, M.A., Morris, H.R., Lubbe, S., Brice, A., Gasser, T., Heutink, P., Wood, N.W., Hardy, J., Singleton, A.B., Williams, N.M. IPDGC consortium members, 2015. Polygenic risk of Parkinson disease is correlated with disease age at onset. Ann. Neurol. 77, 582–591.

Fung, H.-C., Scholz, S., Matarin, M., Simón-Sánchez, J., Hernandez, D., Britton, A., Gibbs, J.R., Langefeld, C., Stiegert, M.L., Schymick, J., Okun, M.S., Mandel, R.J., Fernandez, H.H., Foote, K.D., Rodríguez, R.L., Peckham, E., De Vrieze, F.W., Gwinn-Hardy, K., Hardy, J.A., Singleton, A., 2006. Genome-wide genotyping in Parkinson's disease and neurologically normal controls: first stage analysis and public release of data. Lancet Neurol. 5, 911–916.

Gelb, D.J., Oliver, E., Gilman, S., 1999. Diagnostic criteria for Parkinson disease. Arch. Neurol. 56, 33–39.

Hamza, T.H., Zabetian, C.P., Tenesa, A., Laederach, A., Montimurro, J., Yearout, D., Kay, D.M., Doheny, K.F., Paschall, J., Pugh, E., Kusel, V.I., Collura, R., Roberts, J., Griffith, A., Samii, A., Scott, W.K., Nutt, J., Factor, S.A., Payami, H., 2010. Common genetic variation in the HLA region is associated with late-onset sporadic Parkinson's disease. Nat. Genet. 42, 781–785.

Hernandez, D.G., Nalls, M.A., Ylikotila, P., Keller, M., Hardy, J.A., Majamaa, K., Singleton, A.B., 2012. Genome wide assessment of young onset Parkinson's disease from Finland. PLoS One 7, e41859.

Huttenlocher, J., Stefansson, H., Steinberg, S., Helgadottir, H.T., Sveinbjörnsdóttir, S., Riess, O., Bauer, P., Stefansson, K., 2015. Heterozygote carriers for CNVs in PARK2 are at increased risk of Parkinson's disease. Hum. Mol. Genet. 24, 5637–5643.

International HapMap Consortium, 2003. The International HapMap project. Nature 426, 789–796.

International Parkinson Disease Genomics Consortium, Nalls, M.A., Plagnol, V., Hernandez, D.G., Sharma, M., Sheerin, U.-M., Saad, M., Simón-Sánchez, J., Schulte, C., Lesage, S., Sveinbjörnsdóttir, S., Stefánsson, K., Martinez, M., Hardy, J., Heutink, P., Brice, A., Gasser, T., Singleton, A.B., Wood, N.W., 2011. Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. Lancet 377, 641–649.

International Parkinson's Disease Genomics Consortium (IPDGC)Wellcome Trust Case Control Consortium 2 (WTCCC2), 2011. A two-stage meta-analysis identifies several new loci for Parkinson's disease. PLoS Genet. 7, e1002142.

Kara, E., Xiromerisiou, G., Spanaki, C., Bozi, M., Koutsis, G., Panas, M., Dardiotis, E., Ralli, S., Bras, J., Letson, C., Edsall, C., Pliner, H., Arepalli, S., Kalinderi, K., Fidani, L., Bostantjopoulou, S., Keller, M.F., Wood, N.W., Hardy, J., Houlden, H., Stefanis, L., Plaitakis, A., Hernandez, D., Hadjigeorgiou, G.M., Nalls, M.A., Singleton, A.B., 2014. Assessment of Parkinson's disease risk loci in Greece. Neurobiol. Aging 35, 442.e9–442.e16.

Kay, D.M., Stevens, C.F., Hamza, T.H., Montimurro, J.S., Zabetian, C.P., Factor, S.A., Samii, A., Griffith, A., Roberts, J.W., Molho, E.S., Higgins, D.S., Gancher, S., Moses, L., Zareparsi, S., Poorkaj, P., Bird, T., Nutt, J., Schellenberg, G.D., Payami, H., 2010. A comprehensive analysis of deletions, multiplications, and copy number variations in PARK2. Neurology 75, 1189–1194.

Keller, M.F., Saad, M., Bras, J., Bettella, F., Nicolaou, N., Simón-Sánchez, J., Mittag, F., Büchel, F., Sharma, M., Gibbs, J.R., Schulte, C., Moskvina, V., Durr, A., Holmans, P., Kilarski, L.L., Guerreiro, R., Hernandez, D.G., Brice, A., Ylikotila, P., Stefánsson, H., Majamaa, K., Morris, H.R., Williams, N., Gasser, T., Heutink, P., Wood, N.W., Hardy, J., Martinez, M., Singleton, A.B., Nalls, M.A. International Parkinson's Disease Genomics Consortium (IPDGC) Wellcome Trust Case Control Consortium 2 (WTCCC2), 2012. Using genome-wide complex trait analysis to quantify "missing heritability" in Parkinson's disease. Hum. Mol. Genet. 21, 4996–5009.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., Shimizu, N., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392, 605–608.

Krebs, C.E., Karkheiran, S., Powell, J.C., Cao, M., Makarov, V., Darvish, H., Di Paolo, G., Walker, R.H., Shahidi, G.A., Buxbaum, J.D., De Camilli, P., Yue, Z., Paisán-Ruiz, C., 2013. The Sac1 domain of SYNJ1 identified mutated in a family with early-onset progressive Parkinsonism with generalized seizures. Hum. Mutat. 34, 1200–1207.

Lautier, C., Goldwurm, S., Dürr, A., Giovannone, B., Tsiaras, W.G., Pezzoli, G., Brice, A., Smith, R.J., 2008. Mutations in the GIGYF2 (TNRC15) gene at the PARK11 locus in familial Parkinson disease. Am. J. Hum. Genet. 82, 822–833.

Lee, S., Emond, M.J., Bamshad, M.J., Barnes, K.C., Rieder, M.J., Nickerson, D.A., NHLBI GO Exome Sequencing Project—ESP Lung Project Team, Christiani, D.C., Wurfel, M.M., Lin, X., 2012. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. Am. J. Hum. Genet. 91, 224–237.

Lill, C.M., Hansen, J., Olsen, J.H., Binder, H., Ritz, B., Bertram, L., 2015. Impact of Parkinson's disease risk loci on age at onset. Mov. Disord. 30, 847–850.

Lill, C.M., Roehr, J.T., McQueen, M.B., Kavvoura, F.K., Bagade, S., Schjeide, B.-M.M., Schjeide, L.M., Meissner, E., Zauft, U., Allen, N.C., Liu, T., Schilling, M., Anderson, K.J., Beecham, G., Berg, D., Biernacka, J.M., Brice, A., DeStefano, A.L., Do, C.B., Eriksson, N., Factor, S.A., Farrer, M.J., Foroud, T., Gasser, T., Hamza, T., Hardy, J.A., Heutink, P., Hill-Burns, E.M., Klein, C., Latourelle, J.C., Maraganore, D.M., Martin, E.R., Martinez, M., Myers, R.H., Nalls, M.A., Pankratz, N., Payami, H., Satake, W., Scott, W.K., Sharma, M., Singleton, A.B., Stefansson, K., Toda, T., Tung, J.Y., Vance, J., Wood, N.W., Zabetian, C.P., 23andMe Genetic Epidemiology of Parkinson's Disease Consortium International Parkinson's Disease Genomics Consortium Parkinson's Disease GWAS Consortium Wellcome Trust Case Control Consortium 2), Young, P., Tanzi, R.E., Khoury, M.J., Zipp, F., Lehrach, H., Ioannidis, J.P.A., Bertram, L., 2012. Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: the PDGene database. PLoS Genet. 8, e1002548.

Matarin, M., Simon-Sanchez, J., Fung, H.-C., Scholz, S., Gibbs, J.R., Hernandez, D.G., Crews, C., Britton, A., De Vrieze, F.W., Brott, T.G., Brown, R.D., Worrall, B.B., Silliman, S., Case, L.D., Hardy, J.A., Rich, S.S., Meschia, J.F., Singleton, A.B., 2008. Structural genomic variation in ischemic stroke. Neurogenetics 9, 101–108.

Nalls, M.A., Bras, J., Hernandez, D.G., Keller, M.F., Majounie, E., Renton, A.E., Saad, M., Jansen, I., Guerreiro, R., Lubbe, S., Plagnol, V., Gibbs, J.R., Schulte, C., Pankratz, N., Sutherland, M., Bertram, L., Lill, C.M., DeStefano, A.L., Faroud, T., Eriksson, N., Tung, J.Y., Edsall, C., Nichols, N., Brooks, J., Arepalli, S., Pliner, H., Letson, C., Heutink, P., Martinez, M., Gasser, T., Traynor, B.J., Wood, N., Hardy, J., Singleton, A.B. International Parkinson's Disease Genomics Consortium (IPDGC) Parkinson's Disease meta-analysis consortium, 2015a. NeuroX, a fast and efficient genotyping platform for investigation of neurodegenerative diseases. Neurobiol. Aging 36, 1605.e7–1605.e12.

Nalls, M.A., Escott-Price, V., Williams, N.M., Lubbe, S., Keller, M.F., Morris, H.R., Singleton, A.B. International Parkinson's Disease Genomics Consortium (IPDGC), 2015b. Genetic risk and age in Parkinson's disease: continuum not stratum. Mov. Disord. 30, 850–854.

Nalls, M.A., McLean, C.Y., Rick, J., Eberly, S., Hutten, S.J., Gwinn, K., Sutherland, M., Martinez, M., Heutink, P., Williams, N.M., Hardy, J., Gasser, T., Brice, A., Price, T.R., Nicolas, A., Keller, M.F., Molony, C., Gibbs, J.R., Chen-Plotkin, A., Suh, E., Letson, C., Fiandaca, M.S., Mapstone, M., Federoff, H.J., Noyce, A.J., Morris, H., Van Deerlin, V.M., Weintraub, D., Zabetian, C., Hernandez, D.G., Lesage, S., Mullins, M., Conley, E.D., Northover, C.A.M., Frasier, M., Marek, K., Day-Williams, A.G., Stone, D.J., Ioannidis, J.P.A., Singleton, A.B. Parkinson's Disease Biomarkers Program and Parkinson's Progression Marker Initiative investigators, 2015c. Diagnosis of Parkinson's disease on the basis of clinical and genetic classification: a population-based modelling study. Lancet Neurol. 14, 1002–1009.

Nalls, M.A., Pankratz, N., Lill, C.M., Do, C.B., Hernandez, D.G., Saad, M., DeStefano, A.L., Kara, E., Bras, J., Sharma, M., Schulte, C., Keller, M.F., Arepalli, S., Letson, C., Edsall, C., Stefansson, H., Liu, X., Pliner, H., Lee, J.H., Cheng, R., International Parkinson's Disease Genomics Consortium (IPDGC) Parkinson's Study Group (PSG) Parkinson's Research: The Organized GENetics Initiative (PROGENI) 23andMe GenePD NeuroGenetics Research Consortium (NGRC) Hussman Institute of Human Genomics (HIHG) Ashkenazi Jewish Dataset Investigator Cohorts for Health and Aging Research in Genetic Epidemiology (CHARGE) North American Brain Expression Consortium (NABEC) United Kingdom Brain Expression Consortium (UKBEC) Greek Parkinson's Disease Consortium Alzheimer Genetic Analysis Group, Ikram, M.A., Ioannidis, J.P.A., Hadjigeorgiou, G.M., Bis, J.C., Martinez, M., Perlmutter, J.S., Goate, A., Marder, K., Fiske, B., Sutherland, M., Xiromerisiou, G., Myers, R.H., Clark, L.N., Stefansson, K., Hardy, J.A., Heutink, P., Chen, H., Wood, N.W., Houlden, H., Payami, H., Brice, A., Scott, W.K., Gasser, T., Bertram, L., Eriksson, N., Foroud, T., Singleton, A.B., 2014. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat. Genet. 46, 989–993.

Paisán-Ruíz, C., Jain, S., Evans, E.W., Gilks, W.P., Simón, J., van der Brug, M., López de Munain, A., Aparicio, S., Gil, A.M., Khan, N., Johnson, J., Martinez, J.R., Nicholl, D., Carrera, I.M., Pena, A.S., de Silva, R., Lees, A., Martí-Massó, J.F., Pérez-Tur, J., Wood, N.W., Singleton, A.B., 2004. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44, 595–600.

Pankratz, N., Beecham, G.W., DeStefano, A.L., Dawson, T.M., Doheny, K.F., Factor, S.A., Hamza, T.H., Hung, A.Y., Hyman, B.T., Ivinson, A.J., Krainc, D., Latourelle, J.C., Clark, L.N., Marder, K., Martin, E.R., Mayeux, R., Ross, O.A., Scherzer, C.R., Simon, D.K., Tanner, C., Vance, J.M., Wszolek, Z.K., Zabetian, C.P., Myers, R.H., Payami, H., Scott, W.K., Foroud, T. PD GWAS Consortium, 2012. Meta-analysis of Parkinson's disease: identification of a novel locus, RIT2. Ann. Neurol. 71, 370–384.

Pihlstrøm, L., Axelsson, G., Bjørnarå, K.A., Dizdar, N., Fardell, C., Forsgren, L., Holmberg, B., Larsen, J.P., Linder, J., Nissbrandt, H., Tysnes, O.-B., Ohman, E., Dietrichs, E., Toft, M., 2013. Supportive evidence for 11 loci from genome-wide association studies in Parkinson's disease. Neurobiol. Aging 34, 1708.e7–1708.e13.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., Nussbaum, R.L., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–2047.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J., Sham, P.C., 2007. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575.

Reich, D.E., Lander, E.S., 2001. On the allelic spectrum of human disease. Trends Genet. 17, 502–510.

Saad, M., Lesage, S., Saint-Pierre, A., Corvol, J.-C., Zelenika, D., Lambert, J.-C., Vidailhet, M., Mellick, G.D., Lohmann, E., Durif, F., Pollak, P., Damier, P., Tison, F., Silburn, P.A., Tzourio, C., Forlani, S., Loriot, M.-A., Giroud, M., Helmer, C., Portet, F., Amouyel, P., Lathrop, M., Elbaz, A., Durr, A., Martinez, M., Brice, A. French Parkinson's Disease Genetics Study Group, 2011. Genome-wide association study confirms BST1 and suggests a locus on 12q24 as the risk loci for Parkinson's disease in the European population. Hum. Mol. Genet. 20, 615–627.

Satake, W., Nakabayashi, Y., Mizuta, I., Hirota, Y., Ito, C., Kubo, M., Kawaguchi, T., Tsunoda, T., Watanabe, M., Takeda, A., Tomiyama, H., Nakashima, K., Hasegawa, K., Obata, F., Yoshikawa, T., Kawakami, H., Sakoda, S., Yamamoto, M., Hattori, N., Murata, M., Nakamura, Y., Toda, T., 2009. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat. Genet. 41, 1303–1307.

Shojaee, S., Sina, F., Banihosseini, S.S., Kazemi, M.H., Kalhor, R., Shahidi, G.-A., Fakhrai-Rad, H., Ronaghi, M., Elahi, E., 2008. Genome-wide linkage analysis of a Parkinsonian-pyramidal syndrome pedigree by 500 K SNP arrays. Am. J. Hum. Genet. 82, 1375–1384.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., Barbosa, E.R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., Chen, C.-M., Clark, L.N., Condroyer, C., De Marco, E.V., Dürr, A., Eblan, M.J., Fahn, S., Farrer, M.J., Fung, H.-C., Gan-Or, Z., Gasser, T., Gershoni-Baruch, R., Giladi, N., Griffith, A., Gurevich, T., Januario, C., Kropp, P., Lang, A.E., Lee-Chen, G.-J., Lesage, S., Marder, K., Mata, I.F., Mirelman, A., Mitsui, J., Mizuta, I., Nicoletti, G., Oliveira, C., Ottman, R., Orr-Urtreger, A., Pereira, L.V., Quattrone, A., Rogaeva, E., Rolfs, A., Rosenbaum, H., Rozenberg, R., Samii, A., Samaddar, T., Schulte, C., Sharma, M., Singleton, A., Spitz, M., Tan, E.-K., Tayebi, N., Toda, T., Troiano, A.R., Tsuji, S., Wittstock, M., Wolfsberg, T.G., Wu, Y.-R., Zabetian, C.P., Zhao, Y., Ziegler, S.G., 2009. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med. 361, 1651–1661.

Simón-Sánchez, J., Schulte, C., Bras, J.M., Sharma, M., Gibbs, J.R., Berg, D., Paisan-Ruiz, C., Lichtner, P., Scholz, S.W., Hernandez, D.G., Krüger, R., Federoff, M., Klein, C., Goate, A., Perlmutter, J., Bonin, M., Nalls, M.A., Illig, T., Gieger, C., Houlden, H., Steffens, M., Okun, M.S., Racette, B.A., Cookson, M.R., Foote, K.D., Fernandez, H.H., Traynor, B.J., Schreiber, S., Arepalli, S., Zonozi, R., Gwinn, K., van der Brug, M., Lopez, G., Chanock, S.J., Schatzkin, A., Park, Y., Hollenbeck, A., Gao, J., Huang, X., Wood, N.W., Lorenz, D., Deuschl, G., Chen, H., Riess, O., Hardy, J.A., Singleton, A.B., Gasser, T., 2009. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat. Genet. 41, 1308–1312.

Simón-Sánchez, J., van Hilten, J.J., van de Warrenburg, B., Post, B., Berendse, H.W., Arepalli, S., Hernandez, D.G., de Bie, R.M.A., Velseboer, D., Scheffer, H., Bloem, B., van Dijk, K.D., Rivadeneira, F., Hofman, A., Uitterlinden, A.G., Rizzu, P., Bochdanovits, Z., Singleton, A.B., Heutink, P., 2011. Genome-wide association study confirms extant PD risk loci among the Dutch. Eur. J. Hum. Genet. 19, 655–661.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., Wood, N.W., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304, 1158–1160.

Vilariño-Güell, C., Wider, C., Ross, O.A., Dachsel, J.C., Kachergus, J.M., Lincoln, S.J., Soto-Ortolaza, A.I., Cobb, S.A., Wilhoite, G.J., Bacon, J.A., Behrouz, B., Melrose, H.L., Hentati, E., Puschmann, A., Evans, D.M., Conibear, E., Wasserman, W.W., Aasly, J.O., Burkhard, P.R., Djaldetti, R., Ghika, J., Hentati, F., Krygowska-Wajs, A., Lynch, T., Melamed, E., Rajput, A., Rajput, A.H., Solida, A., Wu, R.-M., Uitti, R.J., Wszolek, Z.K., Vingerhoets, F., Farrer, M.J., 2011. VPS35 mutations in Parkinson disease. Am. J. Hum. Genet. 89, 162–167.

Yang, J., Lee, S.H., Goddard, M.E., Visscher, P.M., 2011. GCTA: a tool for genome-wide complex trait analysis. Am. J. Hum. Genet. 88, 76–82.

Zimprich, A., Benet-Pagès, A., Struhal, W., Graf, E., Eck, S.H., Offman, M.N., Haubenberger, D., Spielberger, S., Schulte, E.C., Lichtner, P., Rossle, S.C., Klopp, N., Wolf, E., Seppi, K., Pirker, W., Presslauer, S., Mollenhauer, B., Katzenschlager, R., Foki, T., Hotzy, C., Reinthaler, E., Harutyunyan, A., Kralovics, R., Peters, A., Zimprich, F., Brücke, T., Poewe, W., Auff, E., Trenkwalder, C., Rost, B., Ransmayr, G., Winkelmann, J., Meitinger, T., Strom, T.M., 2011. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. Am. J. Hum. Genet. 89, 168–175.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R.J., Calne, D.B., Stoessl, A.J., Pfeiffer, R.F., Patenge, N., Carbajal, I.C., Vieregge, P., Asmus, F., Müller-Myhsok, B., Dickson, D.W., Meitinger, T., Strom, T.M., Wszolek, Z.K., Gasser, T., 2004. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 44, 601–607.