Kristoffer Haugarvoll

Identifying Genetic Causes of Parkinson's Disease in Norway

Thesis for the degree of philosophiae doctor

Trondheim, May 2008

Norwegian University of
Science and Technology
Faculty of Medicine
Department of Neuroscience

Norwegian University of
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NTNU
Norwegian University of Science and Technology

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ISBN 978-82-471-1064-5 (printed ver.)
ISBN 978-82-471-1065-2 (electronic ver.)
ISSN 1503-8181

Theses at NTNU, 2008:191

Printed by Tapir Uttrykk

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Doctoral thesis

for the degree of Philosophiae Doctor

Norwegian University of Science and Technology

Faculty of Medicine

Department of Neuroscience

Eit ord

Eit ord
- ein stein
i ei kald elv.
Ein stein til –
Eg lyt ha fleire steinar
skal eg koma yver.

One word

One word
- one stone
in a cold stream.
One more stone -
I need more stones
if I am to cross.

Olav H. Hauge, Norwegian poet

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APPENDIX: PAPERS I - IV

Acknowledgements

It is a privilege to be able to serve patients with Parkinson's disease (PD). During my first appointment as a resident in neurology at Stavanger University Hospital I was fortunate enough to meet many PD patients, all of them unique and inspiring human beings. Movement disorder neurologists can now offer effective symptomatic treatment for the motor signs (resting tremor, bradykinesia, rigidity and postural instability) that define the clinical syndrome we refer to as PD. It is clear, however that PD comprises much more than these motor signs and that we currently have no treatment beyond the alleviation of symptoms. Genetics may not cure PD, but the past 11 years have demonstrated that genetics offers tremendously powerful methods to identify the causes of PD. Understanding the causes of this disease is pivotal for the development of future treatments aimed at halting and preventing disease progression. Hence, genetics may help provide the tools to improve future care for PD patients. This is the reason for my interest in PD genetics. I would like to thank the patients, families and control subjects participating in genetic research, and thus making this thesis possible.

The work presented herein was carried out at the Department of Neuroscience, Faculty of Medicine, Norwegian University of Science and Technology (NTNU) from 2005 to 2008. Genetic analyses were conducted at the Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, Florida, USA. My work has been funded by the Faculty of Medicine, NTNU. I also received support from the US-Norway Fulbright Foundation and from Forsbergs and Aulies Legacy.

This thesis has been made possible because of contributions from a number of brilliant and enthusiastic people. First of all I would like to thank Profs. Linda R. White and Jan O. Aasly for welcoming me as a part of the Trønderbrain group and letting me join their productive collaboration with Mayo Clinic Jacksonville. Linda R. White has helped me in a number of ways and I would like to express my gratitude for her continuous support. Jan O. Aasly has collected DNA from all his Norwegian patients and controls and without his tremendous effort this

thesis would not have been possible. He has always been available for discussion and has supported me in every possible way over these three years. Prof. Matthew J. Farrer generously invited me to be a part of his inspiring laboratory at the Mayo Clinic Jacksonville. I thank him profoundly for giving me the opportunity to learn genetics and practical research in an outstanding environment. I am grateful to Prof. Zbiginew K. Wszolek for adopting me as one of his fellows in the Department of Neurology, Mayo Clinic Jacksonville. I had the opportunity to take part in several interesting projects under his gentle supervision. Prof. Dennis Dickson is a renowned neuropathologist specializing in neurodegeneration, and I am grateful for his continued dedication to education and his helpful attitude towards any eager young mind. I thank Dr. Mathias Toft for his participation in the studies presented herein and for excellent friendship. I thank my friend Dr. Owen A. Ross for looking after a poor 'physician' and for many vivid discussions and good times. My wholehearted thanks to my dear friends Drs. Justus Dächsel, Carles Vilariño-Güell and Christian Wider. Indeed, I owe an inestimable debt of gratitude to all members of the Farrer lab, past and present, for letting me be a part of their enthusiastic group. I am grateful to all other friends and contributors at the Department of Neuroscience, Mayo Clinic Jacksonville. Thanks to Dr. Sigrid B. Sando and all international collaborators contributing samples to the studies. I thank Sylvia Nome Kvam for technical assistance. My previous supervisors in Stavanger Profs. Jan Petter Larsen and Dag Aarsland, I thank for introducing me to the interesting and challenging world of PD research. A special thanks to my dear friend and colleague in Stavanger Dr. Guido Alves. Finally, I proudly thank my parents and my brothers, Anders and Erlend, for being my family. There are no words to describe their importance to me.

Trondheim, March 2008.

Kristoffer Haugarvoll

List of papers

Paper I

Haugarvoll K, Toft M, Skipper L, Heckman MG, Soto A, Ross OA,Hulihan MM, Kachergus JM, Sando SB, White LR, Lynch T, Gibson JM, Uitti RJ, Wszolek ZK, Aasly JO, Farrer JM, Fine-Mapping and Candidate Gene Investigation within the *PARK10* Locus. Submitted manuscript.

Paper II

Haugarvoll K, Toft M, Ross OA, Stone JT, Heckman MG, White LR, Lynch T, Gibson JM, Wszolek ZK, Uitti RJ, Aasly JO, Farrer MJ.
ELAVL4, *PARK10*, and the Celts. Mov Disord. 2007 Mar 15;22(4):585-7.

Paper III

Farrer MJ, Haugarvoll K, Ross OA, Stone JT, Milkovic NM, Cobb SA, Whittle AJ, Lincoln SJ, Hulihan MM, Heckman MG, White LR, Aasly JO, Gibson JM, Gosal D, Lynch T, Wszolek ZK, Uitti RJ, Toft M. Genomewide association, Parkinson disease, and *PARK10* Am J Hum Genet. 2006 Jun;78(6):1084-8.

Paper IV

Haugarvoll K, Toft M, Ross OA, White LR, Aasly JO, Farrer MJ.
Variants in the *LRRK1* gene and susceptibility to Parkinson's disease in Norway. Neurosci Lett. 2007 Apr 18;416(3):299-301.

Summary in English

Background and objectives

The prevalence of Parkinson's disease (PD), today affecting about 2% of the population aged 65 years and older, is expected to double by the year 2030. The disorder causes significant patient and caregiver burden, particularly as the disease progresses and additional problems such as depression, fatigue, hallucinations and cognitive impairment occur. Until recently, there was little insight into the causes of PD. Over the last 11 years several genes causing familial parkinsonism have been linked and identified through positional cloning. The identification of the first of these genes, *α-synuclein* defined PD as a synucleinopathy. The identification of *Leucine-rich repeat kinase 2 (LRRK2)* gene mutations in clinically typical, late-onset PD highlighted the role of genetics in this disorder. The aim of this thesis was to identify further genetic causes of PD. We did this by fine-mapping a genetic locus that had been linked to late-onset PD in the Icelandic population (*PARK10*) (Paper I), by conducting replication studies of reported associations to validate the findings (Paper II-III) and by investigating *LRRK1* as a candidate gene for PD (Paper IV).

Methods

- DNA was collected from PD patients and healthy controls for inclusion in patient-control cohorts that we genotyped i) to assess the frequencies of genetic markers, and ii) to investigate whether marker alleles were associated with susceptibility to disease.

- A clinical and genetic study of PD in central Norway was initiated several years ago by a movement disorder neurologist (Jan O. Aasly) at the Department of Neurology, St. Olav's Hospital (Trondheim University Hospital). He has now colleted DNA samples from more than 600 PD patients and more than 500 controls (Papers I-IV). Additional Norwegian controls for Paper I were recruited from a study on dementia in central Norway (Trønderbrain) by another neurologist (Sigrid B.

Sando). In Papers I-III we also employed PD patient-control series from Ireland and the United States.

- In Paper I, we genotyped single nucleotide polymorphisms (SNPs) across the *PARK10* linkage peak in Norwegian and Irish samples. We then followed-up on SNPs showing evidence of association ($P \leq 0.05$) by genotyping them in additional samples. The follow-up stage also included SNPs that had been associated with PD risk in previous studies.

- In Paper II, we genotyped SNPs in the *ELAVL4* gene, based on a previous study that had nominated these SNPs to be associated with age at onset (AAO) of PD.

- In Paper III, we followed-up on the top 11 'hits' from the first genome wide association (GWA) study in PD. Additionally, we followed up on two *PARK10* SNPs that showed evidence of association with susceptibility to PD in the GWA study.

- In Paper IV, we investigated the role of genetic variability in the *LRRK1* gene in the Norwegian population.

Results

- We found that SNPs in the *USP24* gene are associated with susceptibility to PD (Paper I). No other SNPs showed consistent evidence of association with PD or AAO in that study.

- *ELAVL4* markers were not associated with AAO or susceptibility to PD in the Norwegian or US patient-control series, but there was evidence for association with susceptibility to PD in the Irish series (Paper II).

- We could not replicate any of the genetic loci nominated to influence PD by the first GWA-study in PD (Paper III).

- None of the LRRK1 variants investigated appear to be implicated in the etiology of PD in the Norwegian population (Paper IV).

Conclusions

- The evidence implicating *USP24* in PD (Paper I) is in agreement with a previous smaller study. However, association does not imply a causal link and spurious associations may occur when simultaneously testing many hypotheses. Consequently, we highlight the need for replication of our findings. Furthermore, it is clear that the modest risk in terms of odds ratios (ORs) does not explain the *PARK10* linkage peak. We observed ORs of 0.78-0.80 for the minor allele, corresponding to ORs of 1.20-1.22 for the major allele.

- The lack of consistency for the association between *ELAVL4* SNPs and PD in Paper II may have two reasons; i) the finding may represent a spurious association or ii) it may represent a population specific association. Independent replication for these markers is warranted to clarify the issue.

- The work of others and our group (Paper III) indicates that all 13 SNPs identified by the first GWA-study in PD may be false positives. Future GWA-studies in PD may be successful if insights derived from this pioneer study are implemented in future study design.

- The findings in Paper IV indicate that genetic variability in *LRRK1* does not play a major role in the etiology of PD in Norway. However, a role for this gene in PD patients from other populations cannot be excluded.

Summary in Norwegian

Bakgrunn og målsetningar

Prevalensen av Parkinson sjukdom, som i dag råkar omlag 2% av dei som er 65 år gamle eller eldre, er venta å doblast til år 2030. Denne sjukdomen belastar både pasientane og dei pårørande, særleg er dette tilfellet i framskridne stadier av sjukdomsutviklinga der pasientane ofte opplever problem med depresjon, auka trøyttleik, hallusinasjonar og reduserte kognitive evner. Inntil nyleg hadde ein liten kunnskap om årsakene til Parkinson sjukdom. Over dei siste 11 åra har fleire sjukdomsutløysande gener vorte identifisert ved bruk av genetiske koplingsstudiar og posisjonell kloning. Oppdaginga av det første av desse genene, *α-synuclein*, førde til at Parkinson sjukdom vart klassifiert som ein synucleinopati. Oppdaginga av at mutasjonar i *Leucine-rich repeat kinase 2 (LRRK2)* genet fører til klinisk typisk Parkinson sjukdom med sein sjukdomsdebut, stadfesta at genetikk speler ei rolle ved denne sjukdomen. Målet med denne avhandlinga var å identifisere fleire genetiske årsaker til Parkinson sjukdom. Framgangsmåten me nytta var å finkartleggje eit genetisk lokus som hadde vorte kopla til Parkinson sjukdom i ein populasjon frå Island *(PARK10)* i artikkel I. Vidare gjennomførde me replikasjonsstudiar for å validere funn frå tidlegare studiar (Artikklane II-III). I Artikkel IV undersøkte me *LRRK1* genet, avdi dette genet var ein klar kandidat som kunne vere involvert i prosessen som fører til Parkinson sjukdom.

Metodar

- DNA vart innhenta frå pasientar med Parkinson sjukdom og friske kontrollindivid. Desse prøvane vart nytta i pasient-kontroll studiar for å undersøkje om frekvensen av allelar var ulik mellom gruppene, dvs. om desse allelane var assosierte med sjukdomen.

- Ein kliniskgenetisk studie i Midt Noreg vart starta for fleire år sidan av ein nevrolog (Jan O. Aasly) ved St. Olav's Hospital. Han har no samla prøvar frå meir enn 600 pasientar og over 500 kontroll individ (Artikklane I-IV). I tillegg nytta me kontrollar samla gjennom ein studie

av demens i Trøndelag i artikkel I. Desse kontrollane var rekrutterte av ein annan nevrolog (Sigrid B. Sando). I artikklane I-III nytta me også pasient-kontroll seriar frå Irland og USA.

- I artikkel I gjennomførde me genetiske analysar av genetiske markørar (einskildnukleotidpolymorfismar: SNPs) spreidde over *PARK10* locuset i norske og irske prøvar. Deretter gjennomførde me oppfølgjingsstudiar for markørar som synte assosiasjon ($P \leq 0.05$) ved å analysera desse i eit utvida materiale. Dette oppfølgjingstrinnet inkluderte også markørar som hadde synt assosiasjon i andre studiar.

- I artikkel II analyserte me markørar i *ELAVL4* genet, avdi desse hadde vorte assosiert med alder for sjukdomsdebut for Parkinson sjukdom i ein tidlegare studie.

- I artikkel III gjorde me ein replikasjonsstudie av funna frå den første genomomfattande assosiasjonsstudien gjennomførd i Parkinson sjukdom. I tillegg analyserte me to *PARK10* markørar som synte assosiasjon med risiko for Parkinson sjukdom i den nemde studien.

- I artikkel IV undersøkte me om genetiske variantar i *LRRK1* genet var risikofaktorar for Parkinson sjukdom i den norske befolkinga.

Resultat

- Me fann at genetiske markørar i *USP24* genet er assosierte med predisposisjon for Parkinson sjukdom (artikkel I). Ingen andre gener/markørar synte teikn til ein slik assosiasjon.

- Markørar i *ELAVL4* genet er ikkje assosierte med sjukdomsdebutalder eller risiko for Parkinson sjukdom i den norske eller amerikanske serien, derimot var det ein assosiasjon med sjukdomsrisiko i den irske serien (artikkel II).

- Me kunne ikkje reprodusera noko av funna frå den første genomomfattande assosiasjonsstudien i Parkinson sjukdom (Artikkel III).

- Dei genetiske variantane som vart undersøkte i artikkel IV var ikkje assosiert med risiko for Parkinson sjukdom i den norske populasjonen.

Konklusjonar

- Assosiasjonen mellom genetiske markørar i *USP24* genet og predisposisjon for Parkinson sjukdom stemmer overeins med ein tidlegare, men mindre studie (artikkel I). Det er viktig å understreka at assosiasjon ikkje er det same som årsakssamanheng og tilfeldige assosiasjonar kan opptre når mange hypoteser vert undersøkte samstundes. Av denne grunn understrekar me at det er naudsynt å reprodusera resultata våre. Vidare er det klart at våre resultat ikkje forklarar *PARK10*-koplinga, avdi den observerte effektstorleiken er for liten (odds ratios [ORs]) 0.78-0.80 for den lågfrekvente allelen, noko som korresponderer til ORs 1.20-1.22 for den høgfrekvente allelen.

- Assosiasjonen mellom *ELAVL4* markørar og Parkinson sjukdom i den Irske serien stemmer ikkje overeins med funna i den norske og amerikanske serien. Denne observasjonen kan ha to ulike forklaringar:

i) Assosiasjonen kan vere eit tilfeldig funn eller ii) assosiasjonen kan representere populasjons spesifikke risikofaktorar. Uavhengige reproduksjonsstudiar trengst for å avklare dette.

- Vår forsking og studiar frå andre forskingsgrupper syner at dei 13 genetiske markørane som vart identifisert i den første genomomfattande assosiasjonsstudien i Parkinson sjukdom truleg var falsk positive funn. Denne typen assosiasjonsstudiar kan framleis ha stort potensiale til å finna årsaker til denne sjukdomen dersom ein byggjer vidare på erfaringane frå denne tidlege studien.

- Funna som er presanterte i artikkel IV tyder på at genetisk variabilitet i *LRRK1* genet ikkje spelar ei stor rolle i molekulærbiologien ved Parkinson sjukdom. Me kan ikkje utelukka at dette genet spelar ei rolle i andre populasjonar.

Abbreviations and definitions

AAO	Age at onset
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ATP13A2	*ATPase type 13A2*
CI	Confidence interval
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid (containing the genetic code)
EOPD	Early-onset Parkinson's disease
Genotype	The particular set of alleles that an individual has at a given region of the genome.
GIGYF2	*Grb 10-Interacting GYF Protein 2*
GWA-study	Genome wide association study
Haplotype	A particular combination of alleles that are closely linked on a chromosome.
KRS	Kufor Rakeb Syndrome
LBD	Lewy body disease
LD	Linkage disequilibrium
LOD-score	Logarithm of odds score
LOPD	Late-onset Parkinson's disease
LRRK2	*Leucine-rich repeat kinase 2* (gene)*;* Lrrk2 (protein)
MAF	Minor allele frequency
MAPT	*Microtubule-associated protein tau*
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
Mutation	An alteration in a genome compared to some reference state with frequency <1%, but not necessarily pathogenic.
OMI/HTRA	*OMI/HTRA serine peptidase 2*
OMIM	Online Mendelian Inheritance in Man (see; http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim)
ORs	Odds ratios
PD	Parkinson's disease
PDD	Parkinson's disease dementia

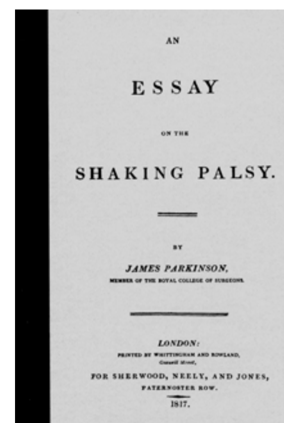
PET	Positron emission tomography
PGRN	*Progranulin*
Phenotype	The observable properties and characteristics of an individual or a locus
PINK1	*PTEN-induced kinase 1*
PRKN	*Parkin*
RLS	Restless legs syndrome
Polymorphism	A region on the genome that varies between individual members of a population with frequency >1%.
PSP	Progressive supranuclear palsy
RBD	REM sleep behavior disorder
SNCA	*α-synuclein*
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computer tomography

1. Introduction

1.1 Historical background

The clinical syndrome we refer to as Parkinson's disease (PD [OMIM #168600]) was first described in 1817 by James Parkinson (1755-1824) in his classical publication "An essay on the shaking palsy" (Paralysis Agitans) (1). In his work Parkinson described the motor signs that define this disorder:

Shaking Palsy (Paralysis Agitans):
"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured."


AN
ESSAY
ON THE
SHAKING PALSY.

BY
JAMES PARKINSON,
MEMBER OF THE ROYAL COLLEGE OF SURGEONS.

LONDON:
PRINTED BY WHITTINGHAM AND ROWLAND,
FOR SHERWOOD, NEELY, AND JONES,
PATERNOSTER ROW.
1817.

Figure 1. James Parkinson's original publication from 1817

Interestingly, Parkinson also described nonmotor features of this disorder, e.g. his mentioning of symptoms that would now be attributed to REM sleep behavior disorder (RBD); *"the sleep becomes much disturbed. The tremulous motion of the limbs occurs during sleep, and augment until they awaken the patient, and frequently with much agitation and alarm."* Finally, in the end stage of the disease Parkinson's description includes fatigue and hallucinations: *"..at the last, constant sleepiness, with slight delirium, and other marks of extreme exhaustion, announce the wished-for release."*

It was the work of the great French neurologist Jean-Martin Charcot (1825 – 1893) who brought general attention to Parkinson's description. Charcot further defined the syndrome by adding rigidity to the motor signs, and he coined the term *maladie de Parkinson*.

For a long time PD remained a devastating disorder with no effective treatment. In their 1957 publication, Carlsson et al. first demonstrated that levodopa, a

dopamine precursor that crosses the blood-brain barrier, reverses reserpine-induced parkinsonism in mice and rabbits (2) and later that dopamine is a neurotransmitter present in the brain (3). This was followed by the work of Ehringer and Hornykiewicz demonstrating striatal dopamine deficiency in brains of PD patients (4). Birkmayer and Hornykiewicz built on this work by demonstrating for the first time that levodopa improves parkinsonism in humans (5). After some initial controversy, Cotzias and colleagues established the efficacy of dopamine replacement therapy in PD when they reported dramatic improvement in PD patients with oral administration of levodopa in increasing amounts over long periods (6).

The levodopa revolution is to date the basis of motor symptom treatment in PD. Although a wide range of compounds have been developed since 1960, levodopa remains the single most effective symptomatic treatment for PD. Surgical treatment for PD has been available for decades, and was significantly improved by the introduction of continuous deep brain stimulation (DBS) in the 1990's. DBS of the subthalamic nucleus (STN) is now established as a well-documented treatment for carefully selected, particularly young patients, with fluctuating motor symptoms and dyskinesias (7-9).

Over the last decades it has become increasingly clear, however, that PD patients suffer from symptoms that go beyond the classical motor features that define the disease. These nonmotor symptoms result in disability, diminish quality of life for patients and contribute substantially to caregiver burden (10). Most nonmotor symptoms do not respond to levodopa treatment (11-13). This insight has increasingly highlighted PD as a multisystem brain disorder and that novel treatment that reaches beyond the nigrostriatal system is needed (14, 15).

Genetics has proven effective in identifying key proteins involved in neurodegeneration (16). The identification of these proteins opens new avenues of research and will advance our insight into the molecular mechanisms that underlie PD. Furthermore, unaffected mutation carriers may be ideal candidates to benefit from future neuroprotective treatments. The goal of the work presented in this thesis was to identify further genes implicated in PD, and thus

contribute to novel insight that may result in improved future therapies aimed at halting and preventing disease progression.

1.2 Definitions

Parkinsonism

Table 1. Six cardinal clinical features of parkinsonism (from ref. 14)

- **Tremor at rest**
- **Bradykinesia**
- Rigidity
- Loss of postural reflexes
- Flexed posture of neck, trunk, and limbs
- Freezing phenomenon

Fahn defined the term parkinsonism as any combination of six specific motor features: tremor at rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture and the freezing phenomenon (where the feet are transiently “glued to the ground”), see table 1 (17). Two of the six motor features should be present, and at least one of them should be tremor at rest or bradykinesia, before a diagnosis of parkinsonism is made (17). There are numerous causes of parkinsonism, see table 3.

Parkinson’s disease

PD is the most common cause of parkinsonism. The diagnosis of PD is based on the clinical identification of some combination of the four cardinal motor signs (tremor at rest, bradykinesia, rigidity and postural instability), with most criteria requiring at least the presence of tremor at rest or bradykinesia. Additional features include; asymmetry at disease onset and favorable response to levodopa treatment. There should be an absence of atypical symptoms e.g. early falls, severe autonomic dysfunction, early dementia or supranuclear gaze palsy. In addition, imaging and clinical examination should exclude other causes of parkinsonism (Table 3).

No biomarker is established for the routine diagnosis of PD, and PD remains a clinical diagnosis. Routine blood tests, structural imaging of the central nervous system and other paraclinical tests are mainly used to distinguish other forms of parkinsonism. Functional imaging assessing neurotransmitter activity in the nigrostriatal dopaminergic system, such as SPECT and PET, can be helpful to reveal dopaminergic deficiency and to differentiate PD from other causes of parkinsonism (18).

The clinical signs of parkinsonism may be subtle at disease onset and this can lead to misdiagnosis (19). Application of strict diagnostic criteria has been shown to increase the diagnostic accuracy (20). Furthermore, following the patients longitudinally is important to achieve high diagnostic accuracy (21). Several groups have therefore proposed diagnostic criteria for a diagnosis of PD, to reliably distinguish PD from other forms of parkinsonism. The criteria proposed by Gelb and colleagues were used for the diagnosis of PD in papers I-IV (22). Gelb et al. differentiated three levels of diagnostic confidence; definite, probable and possible. The diagnoses of possible and probable PD are based on clinical criteria alone, whereas neuropathological confirmation is required for the diagnosis of definite PD (Table 2) (22).

Table 2. Diagnostic criteria for PD (from Gelb et al., ref. 19)

Grouping of clinical features according to diagnostic utility
Group A features: characteristic of PD
Resting tremor
Bradykinesia
Rigidity
Asymmetric onset
Group B features: suggestive of alternative diagnoses
Features unusual early in the clinical course
- Prominent postural instability
- Freezing phenomena
- Hallucinations
- Dementia preceding motor symptoms or in the first year
- Supranuclear gaze palsy or slowing of vertical saccades
- Severe, symptomatic dysautonomia
- Documentation of a condition known to produce parkinsonism plausibly connected to the symptoms

Proposed diagnostic criteria for Parkinson's disease
Criteria for POSSIBLE diagnosis of PD
- At least 2 of 4 features in Group A, at least one of these is tremor or bradykinesia
- None of the features in Group B (or symptoms for less than 3 years)
- Response to dopaminergic treatment upon adequate trial
Criteria for PROBABLE diagnosis of PD
- At least 3 of 4 features in Group A
- None of the features in Group B
- Response to dopaminergic treatment
Criteria for DEFINITE diagnosis of PD
- All criteria for POSSIBLE PD are met
- Histopathological confirmation

Familial and sporadic PD

In this thesis, the term PD describes any patient fulfilling diagnostic criteria (22), and a family history of parkinsonism is not an exclusion criterion. In the literature, patients with a clinical syndrome indistinguishable from typical PD caused by known genetic mutations have been referred to using the terms PD and parkinsonism. It could be argued that patients affected by parkinsonism with a known etiology should not be referred to as having PD, and that this term

should be reserved for idiopathic cases. However, in many cases these patients fulfill all proposed diagnostic criteria (23, 24). Patients with an unknown cause of PD are referred to as having idiopathic PD.

Sporadic PD is in this thesis defined as PD in a patient without any known first or second degree relative affected with parkinsonism or PD. Familial PD is defined as PD in a patient with at least one first or second degree relative with parkinsonism or PD.

Autosomal dominant and recessive PD

Autosomal dominant inheritance refers to genetic conditions that occur when mutations are present in one allele of a given gene i.e. one allele is dominant to the wild-type (standard) allele. If two or more members from the same family are affected by PD in at least two consecutive generations we considered the genetic transmission to be consistent with an autosomal dominant pattern of inheritance. Autosomal recessive inheritance refers to genetic conditions that occur only when mutations are present in both alleles of a given gene, i.e the allele is recessive to the wild-type (standard) allele.

Early-onset Parkinson’s disease (EOPD)

In this thesis a patient affected by parkinsonism at 50 years of age or earlier is considered to have early-onset Parkinson’s disease (EOPD).

Table 3. Classification of the parkinsonian states

Synucleinopathies

Lewy body disorders

- Parkinson’s disease
- Dementia with Lewy Bodies

Glial inclusion body disorders

- Multiple system atrophy

Other synucleinopathies

- Pantothenate kinase associated neurodegeneration
- Pallidonigroluysian atrophy

Tauopathies

- Progressive supranuclear palsy
- Corticobasal degeneration
- FTDP-17(*MAPT*)
- Pick’s disease
- Alzheimer’s disease
- Postencephalitic parkinsonism
- Parkinsonism-dementia complex of Guam

TDP-43proteinopathies

- FTDP-17(*PGRN*)
- Parkinsonism with alveolar hypoventilation and mental depression (Perry disease)

Other neurodegenerative disorders (including trinucleotide repeat disorders)

- SCA2
- SCA3
- Dentorubral-pallidoluysian atrophy
- X-linked dystonia-parkinsonism (Lubag)
- Levodopa responsive dystonia with parkinsonism (DYT5)
- Fragile X tremor/ataxia syndrome (FXTAS)

Secondary parkinsonism

Drugs (antipsychotic medications, calcium channel blockers and others)

Toxins: manganese, carbon monoxide, pesticides, MPTP, cyanide

Vascular

Brain tumors

Head trauma

Normal-pressure hydrocephalus

Linkage disequilibrium (LD)

Linkage disequilibrium describes a situation in which some combinations of alleles or genetic markers occur together more frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. Non-random associations between polymorphisms at different loci are measured by the degree of linkage disequilibrium (LD). If a genetic marker is in LD with a disease susceptibility locus, it would be expected that genotype and allele frequencies at the marker loci will differ between patients and control individuals. Investigations of such differences are known as association studies.

Haplotype

The combination of alleles found at neighboring loci on a single chromosome.

1.3 Epidemiology

After Alzheimer's disease (AD), PD is the most common neurodegenerative disorder affecting about 2% of the population aged 65 years and older (25). The prevalence of PD increases with age, which is the strongest risk factor for disease development. The mean age at disease onset is aproximately 60 years. PD is somewhat more likely to affect men than women (26). The age-adjusted prevalence rate in the county of Rogaland in Western Norway was found to be 102.4 per 100,000 (26). Interestingly, using similar methodology Wermuth et al. found the age-adjusted prevalence in the nearby Faroe Islands to be twice as high (218.0 per 100,000) (27). This suggests that a genetic mutation or an environmental risk factor may be particularly common in specific populations. It is clear from the identification of the Lrrk2 p.G2019S substitution that the frequency of a specific genetic cause may vary dramatically between different populations (24). The importance of PD as a public health issue was further highlighted by a recent report projecting that the number of affected PD patients in the five most populous nations will double to between 8.7 and 9.3 million by 2030 (28).

1.4 Pathology of Parkinson's disease

Meynert for the first time implicated the basal ganglia in disorders of abnormal movements in 1871 (29). In 1895 Brissaud suggested the *substantia nigra* to be affected in PD (30). Brissaud based this on a report by Blocq and Marinesco of a tuberculoma in the *substantia nigra* associated with hemiparkinsonian tremor (31). The authors were careful to point out that the pyramidal tract and the *brachium conjunctivum* above and below the level of the lesion were not affected (17). Tretiakoff confirmed the importance of the *substantia nigra* in nine cases of PD, one case of hemiparkinsonism with a contralateral lesion in the *substantia nigra* and three cases of postencephalitic parkinsonism (32). The *substantia nigra* got its name because of the high content of neuromelanin. The pathological findings consisted of depigmentation, neural cell loss and gliosis. Tretiakoff also confirmed a finding originally described by Lewy in 1914 (33); cytoplasmatic inclusions in PD. These inclusions are now widely recognized as the pathological hallmark of PD and referred to as Lewy bodies (LBs). Since the

identification of *α-synuclein* gene mutations as a cause of familial parkinsonism (34), it has become clear that LBs contain substantial amounts of α-synuclein (35). The pathological diagnosis is Lewy body disease (LBD) which comprises the clinical diagnoses PD and PD with dementia (PDD), as well as dementia with Lewy bodies (DLB). Immunohistochemical staining for α-synuclein is now recommended for the pathological evaluation of LBD (36).

Macroscopically, the PD brain is unremarkable or may display cortical atrophy and enlargement of the ventricles. The midbrain sections often show pallor of the *substantia nigra* and *locus ceruleus* (37). Notably, the *striatum* and *globus pallidus* appear normal. The microscopical findings feature LBs and Lewy neurites (LNs) that are associated with variable neuron loss in the brainstem, midbrain and other subcortical nuclei, in particular the *substantia nigra* and the *locus ceruleus*. There is severe depletion of melanized neurons (>50%) in the whole *substantia nigra* (37). Subregionally, nigral neuronal cell loss has been observed to be greatest in the ventrolateral part (94%) followed by the ventromedial part (70%), the dorsolateral part (65%), the dorsomedial part (58%) and *pars lateralis* part (57%) from control values, respectively (38).

Incidental LBs are found in increasing amounts in the brains of unaffected individuals as they get older. It has also become clear that as many as 60% of AD cases may have some degree of LB pathology, mainly in the *amygdala*. Recent recommendations for diagnosing LBD comprise a semiquantitative grading of LB pathology rather than just the presence of LBs and LNs. The concurrent existence of AD pathology should also be taken into account (36). The current LBD scheme also includes an evaluation of the regional involvement of the LB and LN pathology; i.e. brainstem, limbic and diffuse cortical types.

The identification of clinically typical, late-onset levodopa responsive PD caused by mutations in the *Leucine-rich repeat kinase 2* (*LRRK2*) gene has made it clear, however, that the clinical syndrome we refer to as PD may also be present in the absence of LBD (24, 39).

1.5 Heritability of Parkinson's disease

Two students of Charcot at the *Hôpital de la Salpêtrière* in Paris were probably the first to highlight PD as a hereditary trait. In 1880 and 1883 Leroux and Lhirondel, respectively published their theses on PD (40, 41). They both reported that PD was hereditary and Leroux went so far as to state that "a true cause of paralysis agitans, and maybe the only true cause, is heredity". This was followed by several reports, mainly from Europe, describing familial cases of PD (42-44).

The first genetic-statistical analysis of PD was carried out in Sweden by Mjönes in the 1940's (43). His study included 194 probands and their families, personally examining many probands and families. In addition he selected probands without regard to family history, and carefully recorded the family pedigrees.

1.6 Environmental risk factors for Parkinson's disease

Until recently idiopathic PD was considered to be caused mainly by environmental factors (45). In 1983 Langston and colleagues identified parkinsonism in 'the frozen addicts' (abusers who injected themselves with self-synthesized drugs and subsequently developed parkinsonism) to be caused by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (46). This toxin was found to selectively damage cells in the *substantia nigra* by inhibition of mitochondrial complex I (47). Since then, rural living, well-water drinking, pesticides, certain metals and professional exposure to such toxins have been implicated in nigrostriatal degeneration and parkinsonism. However, there is no conclusive evidence proving that any environmental toxin causes PD (48). Smoking has been inversely associated with susceptibility to PD, but smoking does not appear to have any effect on disease progression (49).

1.7 Genetics of familial Parkinson's disease

Table 4. Genes and loci involved in PD

Gene	Locus	Mode	Mutations	AAO	Clinical features
SNCA	*PARK1/4* (4q21)	AD	p.A30P, p.E46K and p.A53T substitutions; genomic CNVs	Onset earlier in triplications (24–48 years) than in duplications (38–65 years)	Progressive, levodopa responsive parkinsonism with autonomic dysfunction and dementia.
Parkin	*PARK2* (6q25.2-27)	AR	Homozygous/ compound heterozygous missense (>57) and exonic CNVs	<45 years (range 16–72 years)	Parkinsonism with slow progression, responsive to low doses of levodopa, with early dyskinesias, diurnal fluctuation and sleep benefit
Unknown	PARK3 (2p13)	AD	N/A	N/A	Typical parkinsonism
UCH-L1?	*PARK5* (4p14)	AD	p.I93M substitution	55–58 years	Typical parkinsonism
PINK1	*PARK6* (1p35-36)	AR	Missense and exonic CNVs	20–40 years	Slow progression, responsive to low doses of levodopa, some with dyskinesias
DJ-1	*PARK7* (1p36)	AR	Homozygous missense and deletion (delEx1–5) mutations, compound heterozygotes	20–40 years	EOPD; Slow progression, levodopa responsive.
LRRK2	*PARK8* (12q12)	AD	Substitutions; p.R1441C/G, p.Y1699C, p.G2019S and p.I2020T	50–70 years (range 32–79)	Predominantly levodopa responsive LOPD.
ATP13A2 ?	*PARK9* (1p36)	AR	Homozygous/ compound heterozygous CNVs	12–18 years	Juvenile levodopa responsive parkinsonism; pyramidal degeneration, gaze palsy, spasticity and dementia
Unknown	*PARK10* (1p32)	N/A	N/A	N/A	Typical parkinsonism
GIGYF2 ?	*PARK11* (2q36-37)	AD?	Missense mutations?	N/A	Typical parkinsonism
Unknown	*PARK12* (Xq21-25)	X-linked	N/A	N/A	
OMI/ HTRA2 ?	*PARK13* (2p12)	AD	p.G399S	49-77 years	Typical parkinsonism

AAO; Age at onset. AD, autosomal dominant; AR, autosomal recessive; CNVs; copy number variants, N/A; not available, ?; role in PD is unresolved.

Over the last 11 years genetic linkage studies and subsequent positional cloning have been tremendously successful in identifying causes of familial parkinsonism (Table 4). The first gene identified *α-synuclein (SNCA)* defined PD as a synucleinopathy (34, 35). In 2004 another breakthrough was reached when mutations in *Leucine-rich repeat kinase 2* (*LRRK2*) were identified in families with clinically typical, late-onset Parkinson's disease (LOPD) (39, 50). Furthermore, the Lrrk2 p.G2019S substitution was identified as a common cause of LOPD (24).

***α-synuclein* (*PARK1/4* [OMIM *163890])**

In 1996 a genome wide linkage analysis in an Italian family (known as the Contursi kindred) with autosomal dominant parkinsonism mapped a locus on chromosome 4q21 (*PARK1*) (51). This lead to the identification of a missense mutation (c.209G>A) in the *SNCA* gene as the cause of disease in the Contursi kindred and three further families (34). This mutation leads to a p.A53T amino acid substitution in the α-synuclein protein. Subsequently, two further missense mutations, c.88C>G (p.A30P) and c.188G>A (p.E46K) were identified in German and Spanish families respectively (52, 53). It is clear, however that these missense mutations are rare.

The three missense mutations in the *SNCA* gene alter the function of the protein and are believed to promote aggregation of the protein (54, 55). This is of particular interest as the aggregated α-synuclein is the major component of Lewy bodies (LBs) and Lewy neurites (LNs) (35). These are the hallmark neuropathological findings in sporadic PD.

Further insight was gained from the identification of genomic multiplications of the *SNCA* gene locus as a cause of parkinsonism (56). *SNCA* triplictions have been found in an American and a Swedish-American kindred (14, 56). Interestingly, the Swedish-American family has now been shown to be the largest family (the Lister family) described by Henry Mjönes in his 1949 thesis on familial aggregation of PD in Sweden (43, 57). It is also clear that the phenotype that segregates with disease even within the same family is

dependent on the *SNCA* gene dosage, with an earlier AAO and more severe autonomic dysfunction in triplication carriers compared to duplication carriers (57, 58). SNCA duplications have been identified in two French families (59, 60), the Lister family (57) and two Japanese families (61). Recently, SNCA duplications were identified in three apparently sporadic PD patients from Korea (62). SNCA multiplications are associated with increased α-synuclein mRNA and protein in brain tissue (14, 63). Screening large series of PD patients has demonstrated that *SNCA* multiplications are a rare cause of PD, though more common than *SNCA* missense mutations. However, their identification provides important insights by demonstrating that there is a link between *α-synuclein* expression and disease severity (14, 64, 65).

Importantly, genetic variability within the *SNCA* gene locus has consistently been associated with susceptibility to sporadic PD with odds ratios (ORs) of about 1.5 (66-71). The variants are located from the promoter to the 3' end of the *SNCA* gene and are in LD. The functional variant(s) underlying this association have yet to be discovered. It is tempting to hypothesize that such variants may cause PD through a modest increase in *α-synuclein* expression. Thus far, however there are no data proving this (72).

***Parkin (PRKN)* (*PARK2* [OMIM *602544])**

AAO before age 45 years is a typical feature in autosomal recessive forms of familial EOPD; see table 4 (73, 74). A locus for EOPD was mapped to chromosome 6q25.2-27 in 1997 and the causative gene was identified as *parkin* (*PRKN*) (75, 76). The first *parkin* mutations were large homozygous deletions of one and five exons, respectively (76). Since then numerous mutations have been identified, including deletions, duplications and triplications of exons, frameshift mutations and point mutations (missense, nonsense and splice-site mutations) (77). Mutations have been found in each domain of *parkin*. *Parkin* is reported to act as an E3-ubiquitin ligase and play a functional role in proteosomal degradation and receptor trafficking (78). *Parkin* mutations usually occur either as homozygous or compound heterozygous mutations (with different mutations in both alleles). A role for heterozygous *parkin* mutations has been debated. In a large European study of patients with an age of onset <45

years (or with an affected sibling showing such an early onset), *parkin* mutations were identified in up to 50% of familial cases, and in 18% of sporadic disease (79-81). However, affected individuals with parkinsonism having AAO <45 years account for only 1% of idiopathic PD patients. The role of heterozygous *parkin* mutations and other *parkin* variants in idiopathic PD disease is not clear (82-84).

***Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1)* (*PARK5* [OMIM +191342])**

In 1998, Leroy et al. identified a missense mutation in the *UCH-L1* gene c.277C>G; p.I93M) in a German sib-pair with PD (85). It is clear that the findings of Leroy et al. have never been replicated and thus the association is uncertain (86). Lincoln et al. sequenced the entire *UCH-L1* coding region in 11 families with autosomal dominant inheritance and only identified one missense polymorphism p.S18Y (87). This polymorphism was found in approximately 20% of chromosomes in a Caucasian population, suggesting that it is unlikely to be pathogenic. Association between the p.S18Y variant and susceptibility to PD has been reported (88-91). Recently, however two large studies did not find evidence supporting the role of p.S18Y as a susceptibility factor for PD (86, 92).

***PTEN-induced kinase 1 (PINK1)* (*PARK6* [OMIM*608309])**

Valente et al. linked autosomal recessive EOPD in a large Sicilian family (known as the Marsala kindred) to *PARK6*, a 12.5-cM region of 1p36-p35 (93). Subsequently, they identified mutations in *PTEN-induced putative kinase 1 (PINK1)* as the cause of disease (94). Several mutations in *PINK1* have since been found in different populations. The first pathogenic substitutions identified were two G>A transitions in nucleotide 926 (p.G309D) and 1311 (p.W437X), in families from Spain and Italy (94). Mutations were also found in two early-onset sporadic patients suggesting this gene may play a role in the more frequent sporadic form of PD, but this only rarely seems to be the case (95). A heterozygous deletion of the entire *PINK1* gene and a splice-site mutation on the remaining copy were recently described (96). The role of heterozygous *PINK1* mutations in PD has yet to be fully resolved (97, 98). Common genetic variants within *PINK1* do not appear to influence the risk of sporadic PD (99).

Oncogene DJ-1 (*PARK7* [OMIM *602533])

PARK7 was first mapped to chromosome 1p36 in two families from a genetically isolated community in the Netherlands (100). This finding was later replicated in two independent families (101). In 2004, mutations in *DJ-1* were found to cause early-onset autosomal recessive PD in these families (102). Both homozygous deletions and missense mutations in *DJ-1* have been identified. However, these mutations only account for a small number of EOPD. Maraganore et al. investigated four *DJ-1* haplotype tagging SNPs in PD. None of the four SNPs was associated with PD overall, but two SNPs were associated with PD in women (*P*=0.03 and *P*=0.002) (103).

***Leucine-rich repeat kinase 2 (LRRK2)* (*PARK8* [OMIM *609007])**

The *PARK8* locus on chromosome 12q12 was linked to clinically typical, LOPD in a large Japanese family known as the Sagamihara kindred (104). The mode of transmission was autosomal dominant with reduced penetrance. The *PARK8* linkage was confirmed in several PD families of European descent (105). Positional cloning of *Leucine-rich repeat kinase 2 (LRRK2)* as the disease causing gene was reported in 2004 by two groups (39, 50). This represented a major breakthrough in PD genetics as the identification of *LRRK2* mutations in levodopa responsive LOPD made it clear that genetics definitively plays a role in this disorder. Shortly thereafter, a 6055G>A mutation (p.G2019S) in the kinase domain was identified as a relatively common cause of PD (106). Interestingly, most carriers of this mutation can be linked back to a common founder that lived several thousand years ago (24, 106, 107). The frequency of Lrrk2 p.G2019S parkinsonism is strikingly different across populations. It is particularly prevalent in the Middle East and in North Africa, where it accounts for up to 13% and 41% of sporadic PD cases, respectively (108, 109). The frequency generally decreases with distance from the Mediterranean. This pattern is consistent with a common founder, whose diaspora disseminated the mutation (24). To date, five *LRRK2* missense mutations that lead to Lrrk2 protein substitutions have been proven to be pathogenic (p.R1441C, p.R1441G,

p.Y1699C, p.G2019S and p.I2020T). A variety of other mutations have been described, but they have not been conclusively proven to be pathogenic (110).

The pathology in *LRRK2* mutation carriers is probably LBD in most cases, though pure nigral cell loss with gliosis and tau pathology have also been reported (24, 104, 111-113). This highlights the fact that the clinical syndrome we refer to as PD may also be present in the absence of LBD (23)

Given the apparent overlap between Lrrk2 p.G2019S parkinsonism and idiopathic PD, there has been much interest in *LRRK2* as a candidate gene for genetic association studies in sporadic PD. Two comprehensive studies did not find *LRRK2* variants to be associated with sporadic PD in Caucasian populations (114, 115). An early study in patients and controls of Chinese descent indicated that such 'risk' variants may exist in other populations (116). Indeed, it is now clear that the Lrrk2 p.G2385R substitution is consistently found in about 10% of PD patients versus 5% of controls in the Han Chinese population (117-122). A recent study pooling three patient-control series of Chinese descent have highlighted a second Lrrk2 substitution (p.R1628P) to be associated with susceptibility to PD in this population (123). Together with the insights from Lrrk2 p.G2019S parkinsonism these findings strongly emphasize the different effects of specific genetic variants in different populations. This may lead us to conclude that PD is a syndrome rather than a disease.

***ATPase type 13A2 (ATP13A2) (PARK9* [OMIM #606693])**

Kufor Rakeb syndrome (KRS) is a rare autosomal recessive, juvenile-onset parkinsonism. The disorder is responsive to levodopa, but patients suffer from atypical symptoms that include signs of pyramidal degeneration, upward gaze palsy, spasticity and dementia. KRS was first reported in a consanguineous Jordanian family (124). In 2001, the disease was mapped to chromosome 1p36 (125). The disease-causing gene was found to be *ATPase type 13A2 (ATP13A2)* when a compound heterozygous deletion (exon 26: delC3057; G1019fsX1021) and splice site (exon 13: 1306+5G>A) mutation was identified in affected members of a large Chilean family. This study also identified a homozygous 22 bp duplication (exon 16: 1632_1653dup22 Leu552fsX788) in

affected members of the original Jordanian family (126). These mutations lead to loss of function in the 1,180 amino acid ATP13A2 protein, and a link between aggregation of mutant protein in the endoplasmic reticulum and proteasomal or lysosomal dysfunction has been suggested (126). Di Fonzo et al. reported a homozygous missense mutation (exon 15: 1510G>C; Gly504Arg) in one juvenile parkinsonism patient with disease-onset at age 12 years and it is possible that this variant is the cause of disease (127). This study also identified two further heterozygous variants (exon 2: 35C>T; Thr12Met & exon 16: 1597G>A; Gly533Arg) in EOPD patients. However, the role of this gene in PD has yet to be resolved.

***PARK11* [OMIM %607688])**

Linkage to 2q in a sample of sib pairs with PD was reported in 2002 (128). The authors expanded on their work by showing that the evidence for a chromosome 2q36-q37 PD susceptibility gene was primarily due to families with a strong family history of PD. A strong family history of PD being defined as at least 4 affected family members or an affected sib pair with an affected parent (129). Very recently, heterozygous missense mutations in the *Grb10-Interacting GYF Protein-2 (GIGYF2)* gene within the *PARK11* locus were reported (130). The families included in the study did not show definitive co-segregation with disease. Further studies are needed to clarify the role of *GIGYF2* in PD.

***OMI/HTRA serine peptidase 2* (*PARK13* [OMIM *606441])**

Strauss et al. first reported the presence of mutations within the *OMI/HTRA2* gene in PD patients (131). The gene is located within the *PARK3* linkage region on chromosome 2p13, and it encodes a 458 amino acid protein that is reported to function as a serine protease (132).

A missense mutation 1195G>A resulting in a p.G399S amino acid substitution in the protein was observed in four sporadic PD patients and was not seen in 370 healthy controls. The p.G399S patients share a haplotype, suggesting a common ancestor (131). p.G399S is located in the PDZ binding domain of the protein and may result in decreased protease function. A second 421G>T variant that results in a p.A141S substitution was reported to be associated with

increased risk of disease. The p.A141S substitution was observed in 6.2% of PD patients (n=414) versus 3% of controls (n=331). A recent comprehensive study sequenced the entire coding region of *OMI/HTRA2* in a series of 644 North American PD cases with both young- and late-onset disease and in 828 North American neurologically normal controls. The results showed that neither of the variants previously related to PD were associated with PD in this large cohort and that the risk variants were present in neurologically normal controls (133).

1.8 Parkinson’s disease as a complex trait

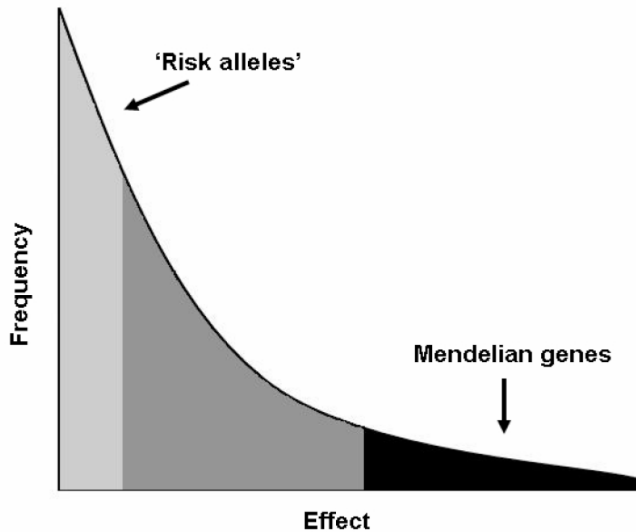


Figure 2. Model of the relationship between allele frequencies and effect size in complex traits. Mendelian genes may be rare, but they exert a significant biological effect and may be the sole cause of disease in some cases. ‘Risk alleles‘ are commonly found in the population and are associated with a modest increase in disease risk, e.g. odds ratio (OR) ≤ 2.0.

Despite the tremendous progress in PD genetics over the past 11 years, the cause of disease remains unaccounted for in most PD patients. Genetic association studies have been utilized to investigate differences in allele frequency in PD patient and control series. The goal of such studies is to identify marker alleles whose frequencies are significantly different between cases and controls, and thus may be associated with susceptibility to disease. Association studies may potentially have better power to detect subtle effect sizes (Figure 2) as traditional linkage studies cannot detect subtle effects, i.e. ORs ≤ 2.0 (134). The manifestation of disease may be a result of several such genetic and possibly environmental risk factors. Investigating ‘candidate genes’ is a widely used approach in genetic association studies, because these methods are widely available and relatively inexpensive; see www.PDGene.org for an updated collection of genetic association studies in PD. However, ‘candidate gene’ studies may not be the best approach as it is hard to select the best candidates among the >25,000 genes in the human genome. This may be

a particularly relevant problem in PD, where we have limited *a priori* insight into the key molecular players involved in neurodegeneration. Indeed, the main goal of genetic mapping studies is to identify these molecular players in the first place. This may explain the fact that the most convincing associations between markers and susceptibility to PD have been discovered in the familial parkinsonism genes *SNCA* and *LRRK2* (see previous section). Possible solutions to the problem of 'not knowing where to start' when investigating 'risk-alleles' may include fine-mapping within genetic loci identified through linkage studies, genome wide association (GWA) studies and candidate gene approaches based on insight from familial genes.

2. Aims of the studies

Paper I

- Describe the pattern of LD within the *PARK10* locus by genotyping SNPs across the locus.
- Increase the number of SNPs in the region until reasonable coverage of the genetic variability within *PARK10* was achieved.
- Identify markers that are associated with susceptibility to PD
- Follow-up on findings from other studies that have nominated candidate genes within *PARK10*.

Paper II

- Replicate a reported association between AAO in PD and SNPs in the *ELAVL4* gene (Nouriddine et al., 2005)
- *ELAVL4* is located within the *PARK10* locus. Consequently, we wanted to investigate whether genetic markers within the gene were associated with susceptibility to PD.

Paper III

- The first GWA-study in Parkinson's disease nominated two *PARK10* SNPs to be associated with susceptibility to PD, so we attempted to replicate this finding and fine-map the region surrounding these two SNPs (Maraganore et al., 2005)
- Replicate the other 11 SNPs nominated by the GWA-study.

Paper IV

- Assess whether genetic variability across the *Leucine-rich repeat kinase 1* (*LRRK1*) gene, the only human homologue of the important *LRRK2* gene, is associated with susceptibility to PD in the Norwegian population.

3. Materials

3.1 Human subjects

All four papers included in this thesis made use of DNA and clinical data obtained from PD patients and control subjects:

PD patients and controls from central Norway

A clinical based series now consisting of >600 PD patients from central Norway has been recruited since 1998. 530 unrelated PD patients were included in Paper I (Table 5). All patients have been examined and followed longitudinally by one movement disorder neurologist (Jan O. Aasly) at the outpatients clinics of three hospitals in central Norway; St. Olav's Hospital (Trondheim University Hospital), Ålesund Hospital (Ålesund) and Helgeland Hospital (Mosjøen). The patients were referred from general practitioners or other hospitals. Patients reporting a family history of PD were asked to inform family members of this ongoing study. All patients and family members willing to do so were invited to take part in the study.

A medical history, including a family history was obtained from each patient. Each patient underwent neurological examination, including the Unified PD Rating Scale (UPDRS) and Hoehn and Yahr staging (135, 136). The clinical interview and the Mini Mental State Examination (MMSE) were used to assess cognitive impairment (137). The clinical presentation was consistent with a diagnosis of possible or probable PD according to the criteria proposed by Gelb et al. (22). Patients with atypical symptoms such as severe autonomic dysfunction, early dementia, unfavorable response to levodopa therapy and supranuclear gaze palsy were excluded. The patient assessment included routine laboratory tests. Blood samples were also obtained for DNA extraction.

A total of 1143 controls were also collected as a part of the ongoing study. These were healthy individuals with no signs of neurodegenerative disease and no family history of PD. About 50% of the controls genotyped in Paper IV were recruited through an ongoing study on dementia in the Trondheim region by a second neurologist (Sigrid B. Sando).

Table 5. Demographics for patients and controls in Paper I (Stage 3)

Variable	*PD cases*	*Controls*
Norwegian samples	*n = 530*	*n = 1,142*
Age	72 ± 11 (29 – 98)	73 ± 11 (29 – 98)
Gender		
Male	324 (61%)	541 (47%)
Female	206 (39%)	601 (53%)
Age at onset (AAO)	59 ± 11	N/A
Irish samples	*n = 173*	*n = 173*
Age	61 ± 12 (33 – 90)	61 ± 12 (33 – 90)
Gender		
Male	70 (40%)	70 (40%)
Female	103 (60%)	103 (60%)
Age at onset (AAO)	50 ± 11 (18 – 77)	N/A
US samples	*n = 221*	*n = 221*
Age	70 ± 10 (36 – 89)	70 ± 10 (36 – 89)
Gender		
Male	106 (48%)	106 (48%)
Female	115 (52%)	115 (52%)
Age at onset (AAO)	62 ± 12 (23 – 85)	N/A

The sample mean ± SD years (range) is given for *age* and *age at PD onset*.

PD patients and controls from Ireland

Papers II-IV include DNA obtained from 186 PD patients and 186 controls from Ireland. The Irish patients were examined and followed longitudinally by two movement disorder neurologists (Timothy Lynch and J. Mark Gibson). PD was diagnosed according to the Gelb criteria (22). Controls were also ethnic Irish and individually matched to a patient by age (± 4 years) and gender.

PD patients and controls from Mayo Clinic Jacksonville

DNA from samples included in Papers II-IV was also collected at the Department of Neurology, Mayo Clinic Jacksonville. These were Caucasian individuals of European decent. Patients were examined and followed by to movement disorder neurologists (Ryan J. Uitti and Zbigniew K. Wszolek). PD was diagnosed according to published criteria (22). Controls recruited from Mayo Clinic Jacksonville had no evidence of neurodegenerative disease.

4. Methods

4.1 Molecular biology

DNA was extracted from whole blood using standard protocols.

For Paper I genotyping was conducted by PCR amplification using one primer pair for each SNP. PCR products were digested with restriction enzymes and resulting fragments separated by agarose gel electrophoresis. Additional genotyping was performed by running available and designed (Available-by-Design or Available-by-Demand) TaqMan chemistry on an ABI7900 (Applied Biosystems, Foster City, CA, USA) and analyzed with SDS 2.2.2 software. In cases where genotype data was only available for one sample of a matched pair, the other subject was retained in the analysis. TaqMan chemistry was also used for the genotyping in Papers II-IV, including positive controls for mutation screening in Paper IV. Mulitplexed genotyping of SNPs for the last part of Paper I (Stage 3) was performed on a Sequenom MassArray iPLEX platform (San Diego, CA).

4.2 Statistical methods

Association

Genetic association examines whether single-locus alleles or genotype frequencies differ between the patient and control groups. In Papers I-III we used logistic regression to estimate odds ratios (ORs) and the corresponding *P*-values for each marker. In Paper IV the association was tested using Pearson's chi-square test. In paper I-III we used permutations to correct for multiple testing (138).

Linkage disequilibrium (LD) mapping

Pairwise LD between markers was assessed by calculating r^2 as implemented in Haploview software (139). In addition we constructed a LD-unit (LDU) map across the locus of interest in Paper I and III using the LDMAP software (140).

4.3 Ethical considerations

All individuals who participated in the studies provided informed consent. Study protocols for the samples from central Norway were approved by the Regional Committee for Medical Research Ethics in Central Norway. Studies conducted at Mayo Clinic Jacksonville were approved by the Mayo Clinic Institutional Review Board (IRB). The Irish collaborators also have IRB approval. The biobanks in Trondheim have approvals in compliance with the Norwegian Biobank Law.

5. Results

5.1 Review of Paper I

Fine-Mapping and Candidate Gene Investigation within the *PARK10* Locus

Kristoffer Haugarvoll, Mathias Toft, Lisa Skipper, Michael G. Heckman, Julia E. Crook, Alexandra Soto, Owen A. Ross, Mary M. Hulihan, Jennifer M. Kachergus, Sigrid B. Sando, Linda R. White, Timothy Lynch, J. Mark Gibson, Ryan J. Uitti, Zbigniew K. Wszolek, Jan O. Aasly, and Matthew J. Farrer

Background: *PARK10* has been linked to LOPD in Iceland. We investigated associations between SNPs within the *PARK10* locus and susceptibility to PD or AAO of PD.

Methods and results: One-hundred and eighty-eight SNPs were genotyped across the *PARK10* locus in 180 PD patients and 180 controls from central Norway (Stage 1). The pattern of LD from stage 1 was used to select 75 SNPs for genotyping in 186 patients and 186 controls from Ireland (Stage 2). Finally, we selected 19 SNPs from this and previous studies for follow-up in an extended Norwegian series (530 patients and 1,142 controls), the Irish series and a US series (221 patients and 221 controls) (Stage 3). In the overall combined series, associations with PD are significant for two SNPs in the *USP24* gene (rs13312: OR 0.78; $P \leq 0.001$, rs487230: OR 0.80; $P=0.001$) after correction for multiple testing. The association trends are comparable across the series included in this study. No marker showed consistent association with AAO. Markers in other candidate genes were not associated with susceptibility to PD.

Conclusion: The *USP24* SNPs included herein were previously reported to be associated with PD. Our data indicate that *USP24* may indeed be implicated in PD. However, the observed effect size does not explain the *PARK10* linkage peak.

5.2 Review of Paper II

***ELAVL4*, *PARK10* and the Celts**

Kristoffer Haugarvoll, Mathias Toft, Owen A. Ross, Jeremy T. Stone, Michael G. Heckman, Linda R. White, Timothy Lynch, J. Mark Gibson, Zbigniew K. Wszolek, Ryan J. Uitti, Jan O. Aasly, and Matthew J. Farrer

Background: Genetic variation within the gene encoding *ELAVL4* (embryonic lethal, abnormal vision-like protein) was reported to be associated with AAO in a familial series of PD patients originating from the United States.

Methods and results: We examined 5 SNPs spanning the *ELAVL4* gene in Norwegian, American, and Irish PD patient–control samples. No association was found between the examined markers and AAO or PD in Norwegian or American samples. However, *ELAVL4* markers (rs967582; OR 1.53, CI 1.13-2.07, *P*=0.007 and rs3902720; OR 1.55, CI 1.14-2.13, *P*=0.006) were significantly associated with susceptibility to PD in our Irish series. This association is significant after correction for multiple testing.

Conclusion: Our data suggest that the association between *ELAVL4* and susceptibility to PD previously observed may be explained by a Celtic-founder effect. However, independent replication is needed to confirm *ELAVL4* as a population-specific risk factor and exclude the possibility of a spurious association.

5.3 Review of Paper III

Genome wide association, Parkinson's disease, and *PARK10*

Matthew J. Farrer, Kristoffer Haugarvoll, Owen A. Ross, Jeremy T. Stone, Nicole M. Milkovic, Stephanie A. Cobb, Andrew J. Whittle, Sarah J. Lincoln, Mary M. Hulihan, Michael G. Heckman, Linda R. White, Jan O. Aasly, J. Mark Gibson, David Gosal, Timothy Lynch, Zbigniew K. Wszolek, Ryan J. Uitti, and Mathias Toft

Background: The first genome wide association (GWA) study in PD nominated 13 SNPs to be associated with susceptibility to disease. Two of these SNPs were in LD in the *CUB domain containing protein 2 (CDCP2)* gene. *CDCP2* (previously known as *LOC200008*) is located within the *PARK10* locus.

Methods and results: We genotyped 28 SNPs (including rs682705 and rs7520966) within a 132 kb interval around *CDCP2* in patient-control samples from Norway and Ireland. rs682705 and rs7520966 were also genotyped in a US series. In addition, we attempted to replicate the 11 'top-hits' from the GWA-study. There was no evidence of association with susceptibility to PD for any the 28 *CDCP2* SNPs. None of the other 11 SNPs nominated by the GWA-study were associated with PD in our samples, independently or as a combined sample set. No marker was associated with age at onset (AAO).

Conclusion: The negative findings in this study indicate that the conclusions derived from the first GWA-study in PD may be based on spurious associations. These findings highlight the need for independent replication of association studies.

5.4 Review of Paper IV

Variants in the *LRRK1* gene and susceptibility to Parkinson's disease in Norway

Kristoffer Haugarvoll, Mathias Toft, Owen A. Ross, Linda R. White, Jan O. Aasly, and Matthew J. Farrer

Background: The identification of *LRR2* gene mutations as an important cause of LOPD has highlighted the genetic component of this disorder. The Lrrk2 p.G2019S substitution has been identified in nine Norwegian families. *LRRK1* is the single homologue of *LRRK2*, indicating that their gene products share functions and pathways. A sequencing study in probands from PD families identified four novel missense variants in the *LRRK1* gene, one of them in a Norwegian proband.

Methods and results: We investigated whether five SNPs or the four novel missense variants are associated with susceptibility to PD in the Norwegian population. The nine markers were genotyped in 338 PD patients and 338 matched controls from central Norway. In addition, we genotyped the same markers in 249 further unmatched controls. Two rare coding variants; ss65713826 (p.K203E) and ss65713830 (p.T967M), were more frequent in patients than controls, However, this difference was not significant after correction for multiple testing.

Conclusion: The identification of disease-causing mutations in *presenilin 1 and 2* in Alzheimer’s disease shows that homologous proteins can cause the same disease phenotype. Two rare *LRRK1* coding variants; ss65713826 (p.K203E) and ss65713830 (p.T967M) were more frequent in patients than controls, but their identification in healthy controls and lack of co-segregation with disease suggest they may represent benign variants. Thus, we found no evidence implicating *LRRK1* in the etiology of PD.

6. Discussion

6.1 Fine-mapping within the PARK10 locus

The *PARK10* locus was first linked to LOPD in Iceland (141). Li et al. linked an overlapping locus to AAO in the US population (142). Population history and genetic analysis of X- and Y-chromosomal markers have shown that the Icelandic population is mostly of Scandinavian descent, with a minor Celtic matrilineal contribution (143, 144). We hypothesized that if a *PARK10* mutation antedates the settlement of Iceland, this mutation may be identified in the Norwegian or Irish population. In Paper 1, we investigated this by fine-mapping 188 SNPs across the maximum LOD score minus one (LOD-1) interval in 180 PD patients and controls from Norway (Stage 1). Subsequently, 75 of those SNPs were selected based on the LD structure from Norwegian controls in Stage 1, to investigate the locus in 186 patients and 186 controls from Ireland (Stage 2). Markers that showed evidence of association with susceptibility to PD ($P \leq 0.05$) were selected for genotyping in Stage 3. Stage 3 included an extended Norwegian series (530 patients and 1,142 controls), the Irish series and a series from the US (221 patients and 221 controls). None of the SNPs from the fine-mapping studies (5 from Stage 1, Norwegian; and 2 from Stage 2, Irish) was associated with susceptibility when followed-up in the extended series.

These results indicate that initial associations from Stages 1 and 2 were spurious, and highlight the need for replication in association studies. Our fine-mapping series did not have power to detect subtle effects. However, Hicks et al. estimated that carriers of one *PARK10* ‘risk allele’ have 30 times increased risk of PD compared to non-carriers (141). We estimated that the fine-mapping series used in Paper I (Stages 1 and 2) had 80% power to detect ORs≥1.9 at the 5% significance level. This assumes an additive model with minor allele frequency (MAF) ≥10%. We genotyped more than one SNP every 50 kb. This is not sufficient to capture all genetic variability within the *PARK10* locus. However, LD in a United States population of north-European descent was shown to typically extend 60 kb from common alleles (145). Empirical data from the *LRRK2* gene tells us that most carriers of the common c.6055G>A mutation (p.G2019S) mutation share an ancestral haplotype spanning between 145 to

154 kb around the mutation (106). Likewise, carriers of the Chinese ‘risk allele’ c.7153G>A (p.G2385R) share a 239 to 294 kb haplotype around the disease-associated allele (119). These observations demonstrate that LD-mapping may indeed be possible for PD associated alleles that occur frequently in a population using the SNP density employed in Paper I. A caveat of the approach taken in Paper I is that a gene containing several mutations, each with low individual frequency, may not be detected.

6.2 Candidate gene investigation within PARK10

Papers I-III investigated SNPs in candidate genes within the *PARK10* locus. Several studies had investigated *PARK10* for SNPs that are associated with susceptibility to PD or AAO, nominating several genes during the course of our fine mapping study; *human immunodeficiency virus type I enhancer binding protein 3 (HIVEP3*) (146, 147), *eukaryotic translation initiation factor 2B, subunit 3 gamma (EIF2B3*) (147)*, embryonic lethal, abnormal vision-like 4 (ELAVL4)* (148) and *ubiquitin specific peptidase 24 (USP24)* (147, 149). A recent genome-wide association study in PD identified two *PARK10* SNPs in LD within the *CUB domain containing protein 2 (CDCP2)* gene (150). Furthermore, a gene expression study recently highlighted *ring finger protein 11 (RNF11)* as a *PARK10* candidate gene (151). It remains unresolved whether any of these candidate genes could account for the *PARK10* linkage or are involved in the etiology of PD.

In paper II we investigated 5 SNPs within the *ELAVL4* gene. These markers had previously been associated with AAO in PD by Noureddine and colleagues (148). We could not replicate the association between these markers and AAO. The use of AAO as a quantitative trait in genetic association studies may be questionable, e.g. the AAO may differ by decades even in *LRRK2* mutation carriers from the same family. Furthermore, most PD cases have an AAO around 60 years and one may need two large groups that have distinctly younger and older AAO, respectively, in order to make an AAO approach work. These problems may explain why the group that originally reported *ELAVL4* markers to be associated with AAO could not replicate their own findings in a follow-up study (147, 148). In contrast, we found two *ELAVL4* SNPs (rs967582

and rs3902720) to be significantly associated with susceptibility to PD in the Irish population, after correction for multiple testing. No *ELAVL4* SNP is associated with PD in the Norwegian or US series. This lack of consistency may be due to a population specific effect. The Lrrk2 p.G2385R 'risk' substitution in the Asian population is an example of such population specific effects (117-122). Alternatively, the observed association in the Irish series may be spurious. Independent replication is imperative to resolve this issue (152, 153).

In Paper I, we found no evidence of association between *HIVEP3* SNPs and susceptibility to PD. This is inconsistent with two previous reports including the same markers (146, 147). However, these two studies included essentially the same samples, and so do not represent independent replication of the initial finding (147). Maraganore et al. also examined two of the *HIVEP3* SNPs in their GWA-study, but the results were inconsistent for the two *HIVEP3* SNPs in the two tiers (150). Together with our results, this does not support a role for *HIVEP3* in the etiology of PD.

SNPs in the *RNF11, EIF2B3* and *CDCP2* genes were not associated with susceptibility to PD in our studies (Papers I and III, respectively). Furthermore, we found no association with markers in *EIF2B3* and USP24 and AAO (Paper I).

6.3 Association between USP24 SNPs and PD

SNPs within the *USP24* gene are associated with susceptibility to PD in the combined Norwegian, Irish and US series; see paper I. The association is significant for two markers. Joint analysis of the Norwegian, Irish and US series was significant after correction for multiple testing for rs13312 (OR: 0.78, 95% CI: 0.67 – 0.90, P=0.0007) and rs487230 (OR: 0.80, 95% CI: 0.70 – 0.91, P=0.0013), whereas rs287235 (OR: 0.81, 95% CI: 0.71 – 0.93, P=0.0035) and rs1165226 (OR: 0.86, 95% CI: 0.76 – 0.97, P=0.015) showed the same trend. In the Norwegian series all four SNPs in *USP24* were individually associated with susceptibility to PD. However, the association trends were not significant after correction for multiple testing. Interestingly, the Irish series showed identical

ORs for *USP24* SNPs and there was a similar, albeit weaker, effect in the US series. However the associations were not significant in these smaller samples. A previous study in the US population first nominated markers in *USP24* to be associated with susceptibility to PD (149). Our data supports a role for this gene in the etiology of PD. Nevertheless, the association between *USP24* SNPs and PD does not explain the *PARK10* linkage peak, as linkage studies do not have the power to detect subtle effect sizes, e.g. ORs≤2 (134). Thus, independent replication in a well-powered patient-control series is warranted to establish the role of *USP24* in PD.

6.4 Genome wide association (GWA) studies in PD

Maraganore et al. conducted the first GWA-study in a US Caucasian PD population. They employed a two-tiered study design: In tier 1, 443 sibling pairs (*n*= 886) discordant for PD were genotyped for 198,345 SNPs (172,420,019 genotype calls). In tier 2, an independent patient-control series was used to follow-up the strongest 1,892 associations (150). Eleven SNPs showing strongest evidence of association were nominated to be involved in the etiology of PD, in addtion to the 2 *PARK10* SNPs discussed above. In paper III we attempted to replicate these findings, but none of these SNPs was associated with susceptibility to PD in our study. Several other groups have also failed to support the original findings (154-159). One conclusion from this work is that there may be no major 'global' risk factor for PD (e.g. a risk allele with OR >2). In contrast, such 'risk' alleles were recently identified in another common movement disorder, restless legs syndrome (RLS) through GWA-studies (160-163).

The associations between common polymorphisms in the *SNCA* gene and PD across populations indicate that variants with smaller effects do exist (e.g. ORs≤1.5). However, GWA-studies need substantially better power to be able to detect these subtle effects (164, 165). Power is particularly important in GWA-studies, as correction for multiple testing is essential when genotyping >200,000 SNPs. There is no consensus on how best to perform correction for multiple testing in GWA-studies. The Bonferroni correction is inappropriate in GWA-studies as it assumes independent tests. In GWA-studies, however many SNPs

are dependent on each other due to some degree of LD between them. Permutations, as used in Papers I-IV, may be more appropriate (166). A second GWA-study in PD has now been published (167). As this study used a small US-series of 267 PD patient and 270 controls, little additional insight could be derived from it. GWA-studies may yet provide insight into the etiology of PD if we build on the experiences gained from the first studies. A powerful GWA-study in PD has not been performed to date. Alternatively, future studies may employ more homogenous populations, EOPD patients or endophenotypes (e.g. tremor dominant PD) (168-170).

6.5 Linkage and candidate gene studies

In Paper IV we investigated genetic variants in the *Leucine-rich repeat kinase 1 (LRRK1)* gene in Norwegian PD patients and controls. *LRRK1* is the single homolog of *LRRK2* (171-173), and the Lrrk2 p.G2019S substitution has been shown to be an important cause of PD in Norway (106, 174, 175). We know from *presenilin 1* and *2* in Alzheimer's disease (AD) that homologous proteins can cause the same neurodegenerative syndrome (176, 177). In paper IV, we found no evidence implicating *LRRK1* variants in the etiology of PD in Norwegian patients (173).

To date, familial linkage studies have been the most successful method to identify the molecular players involved in neurodegeneration. GWA-studies currently generate much enthusiasm, but if they only detect very subtle effects (e.g. ORs≤1.5) it is pertinent to ask to what degree they provide us with useful insight (178). In contrast, Mendelian forms of parkinsonism with large effect sizes (e.g. ORs >10) may only make up a small proportion of the entire PD population, though the biological insights derived from these discoveries can hardly be overstated, and genes with large effects are probably far more useful for functional studies and the generation of disease models (179, 180).

7. Conclusions

From the studies presented herein we have gained new and important knowledge about the genetics of PD and provided hints on how best to conduct genetic studies in the future. Furthermore, our work highlights the need for caution when interpreting the results from novel methods and that independent replication is pivotal when assessing the importance of a scientific finding:

- We identified no genetic variant exerting a major effect on PD risk within the *PARK10* locus (Papers I-III). This could be because the finding from the initial study nominating the locus may represent an artifact. More focus on familial studies to replicate linkage and identifying novel loci may be fruitful. Thus far, linkage has been the single most effective method to identify genetic causes of disease.

- However, we did identify markers in the *USP24* gene that are associated with susceptibility to PD. Our finding is in agreement with a previous study. The effect of these variants is modest, corresponding to ORs 1.20-1.22 for the major allele. Independent replication is needed to establish the role of *USP24* variants in the etiology of PD. Our results indicate that *USP24* may contain 'risk' alleles that are independent of *PARK10*. We also identified *ELAVL4* as a possible population specific 'risk' gene in PD, a finding that also warrants replication (Paper II).

- We and others, could not replicate the findings from the first GWA-study in PD despite comprehensive efforts (Paper III). Future GWA-studies may be fruitful if they build on insight derived from these early studies. Improvements may include substantially better power, studies in more homogenous populations and investigations of endophenotypes.

- *LRRK1* is the single homolog of *LRRK2*, and thus an excellent candidate gene for PD. Nevertheless, our results indicate that this gene does not seem to play a major role in the etiology of PD in Norway (Paper IV).

If independent studies replicate the findings from Papers I or II, PD may truly be classified as a complex trait, i.e. multiple modest risk factors (genetic and environmental) may be needed to jointly produce disease. This may have impact on future study design, as substantial power will be needed to identify such modest risk factors.

It is, however pertinent to ask what insight may be gained from such modest risk factors. They will not be meaningful for genetic counseling and may contribute little to functional research compared with Mendelian genes. Conversely, the identification of multiple 'risk' variants paired with increased insight into the normal function of proteins may highlight pathways involved in neurodegeneration.

'Translation' is a popular term in modern medicine. Basic research can generate knowledge about the process of neurodegeneration and may help create the platform necessary to achieve translational breakthroughs. Genetic research may be one way of achieving further insight. This will hopefully lead to substantially improved treatment in the future.

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Paper I

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Paper II

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Paper III

Jordi Clarimon,[1] Sonja Scholz,[1]
Hon-Chung Fung,[1,2] John Hardy,[1]
Johanna Eerola,[3,4] Olli Hellström,[5]
Chiung-Mei Chen,[2] Yih-Ru Wu,[2]
Pentti J. Tienari,[3,4] and Andrew Singleton[1]
[1]*Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda;* [2]*Department of Neurology, Chang Gung Memorial Hospital and College of Medicine, Chang Gung University, Taipei, Taiwan;* [3]*Department of Neurology, Helsinki University Central Hospital, and* [4]*Neuroscience Programme, University of Helsinki, Biomedicum-Helsinki, Helsinki; and* [5]*Department of Neurology, Seinäjoki Central Hospital, Seinäjoki, Finland*

Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp (for *SEMA5A* markers)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/entrez/Omim/ (for PD and *SEMA5A*)

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5. Dupont WD, Plummer WD Jr (1998) Power and sample size calculations for studies involving linear regression. Control Clin Trials 19:589–601

Address for correspondence and reprints: Dr. Jordi Clarimon, Molecular Genetics Section, Laboratory of Neurogenetics, National Institutes of Health, Building 35, Room 1A1000, MSC 3707, 35 Lincoln Drive, Bethesda, MD 20892. E-mail: jclarimon@santpau.es
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0002-9297/2006/7806-0020$15.00

Am. J. Hum. Genet. 78:1084–1088, 2006

Genomewide Association, Parkinson Disease, and *PARK10*

To the Editor:
Genomewide linkage analysis of rare familial forms of parkinsonism has identified mutations in seven genes, revealing a clinicopathologically and genetically heterogeneous syndrome.[1] Less progress has been made in the more typical late-onset form of Parkinson disease (PD [MIM 168600]), although the recently identified *LRRK2* (MIM 609007) G2019S substitution is estimated to account for ~1% of sporadic PD cases.[2] Common polymorphisms of familial genes may also influence susceptibility to idiopathic PD.[3,4] Of the 198,345 SNPs successfully genotyped in the recent genomewide association (GWA) study, 26 had notably different allele frequencies between patients and controls in both tiers ($P < .01$).[5] Fifteen of these SNPs had opposite directions of effect (disease risk or protection) in tiers 1 and 2. The remaining 11 SNPs were proposed as markers for new genes/chromosomal loci that influence susceptibility to PD. In addition, two SNPs in tier 2 (*rs682705* and *rs7520966*) were highlighted in the *PARK10* locus (MIM 606852), which nominated the gene *LOC200008* in disease susceptibility.

The *PARK10* locus on chromosome 1p32 was originally identified in a genomewide linkage analysis of 117 patients from 51 Icelandic families (maximum $Z_{lr} = 4.8$ at *D1S231,* with a LOD-1, 7.6-cM support interval from *D1S2874* to *D1S475*).[6] Iceland has a well-characterized genealogy that is powerful for family-based linkage studies. The ancestral founders of Iceland have Scandinavian patrilineal inheritance with a minor Celtic matrilineal component.[7] Assuming that the *PARK10* mutation predates the Icelandic settlement, we reasoned that the 1p32 susceptibility gene might be more readily found in patients with PD originating from Scandinavian or Celtic populations. In parallel to the study of Maraganore et al.,[5] we have been mapping the *PARK10* locus. Genotypes from 28 SNPs (including *rs682705* and *rs7520966)* within a 132-kb region of chromosome 1p32 located around the *LOC200008* gene have been analyzed in two well-characterized case-control series from Norway and Ireland. In addition, we attempted to replicate findings for the two *PARK10* SNPs in a U.S. series collected at the Mayo Clinic in Jacksonville, FL. We then employed all three case-control series to investigate the genotype/allele frequencies of the main 11 SNPs nominated to influence PD susceptibility.[5] Power was comparable to the original study (>80% at $\alpha = 0.05$ for odds ratios [ORs] >2.0 and for disease-allele frequencies >0.035), and genotyping call rates were >95% for all markers (table 1).

In total, Norwegian samples included 676 subjects (cases and controls) with a mean age ($\pm$SD) of 70 $\pm$ 11 years, Irish samples included 372 subjects with a mean age ($\pm$SD) of 61 $\pm$ 13 years, and the U.S. samples included 522 subjects with a mean age ($\pm$SD) of 71 $\pm$ 10 years. All patients were examined and were observed longitudinally by a movement-disorders neurologist (J.O.A., J.M.G., D.G., T.L., Z.K.W., and R.J.U.), and they were

Table 1

Genotype/Allele Frequencies of the Main 11 SNPs Nominated to Influence PD Susceptibility

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

given a diagnosis of PD in accordance with published criteria.[8] Each patient was individually matched, on the basis of age (± 4 years) and ethnicity, to an unrelated control without evidence of neurological disease. The ethical review boards at each institution involved approved the study, and all participants provided informed consent.

SNP genotyping was performed using TaqMan chemistry on an ABI7900 genetic analyzer; in cases where genotype data was available for only one subject of a matched pair, the other subject was retained in the analysis. For the controls in each population, χ^2 tests of Hardy-Weinberg equilibrium (HWE) were implemented using Haploview.[9] Optimal SNP coverage for association analysis of the *LOC200008* gene was determined empirically by the construction of linkage-disequilibrium (LD) maps in Norwegian and Irish samples, onto which haplotype blocks were assigned (fig. 1).[10,11] ORs for disease association, with corresponding 95% CIs, were subsequently calculated using logistic-regression models adjusted for age and sex. Overall ORs combining data from all three sites were additionally adjusted for site. Previous studies have nominated the *PARK10* locus as an age-at-onset modifier in PD[12]; thus, we also assessed the influence of 1p32 SNPs variability on this disease trait, using linear-regression models adjusted for sex.

There was no evidence of association with PD for any of the 28 genotyped 1p32 SNPs in our study (all SNP $P > .05$ after applying Bonferroni correction in both population samples). Haplotype frequencies between patients and controls were not significantly different for the haplotype blocks identified; nor was the age at onset in patients associated with any single marker or haplotype (all corrected $P > .05$). Of note, the ancestral recombination and haplotype blocks apparent within Norwegian and Irish samples were comparable for this interval at this marker resolution. The average number of SNPs per LD unit (LDU) was 6.8 (mean LDU between markers 0.15, range 0–0.63), indicating that the number of SNPs genotyped within and flanking *LOC200008* should be sufficient for examination of the region.[11] In addition, the two *PARK10* SNPs showed no significant association within the U.S. series ($P > .05$). None of the other 11 SNPs nominated by the GWA study had different allele frequencies or genotype distributions between affected subjects and matched controls (all SNP $P > .05$ in all populations independently or as a combined sample set) (table 2). There was no evidence of departure from HWE in controls ($P > .01$ in all population controls).

Our study indicates that genetic variability within the *LOC200008* gene is unlikely to explain the *PARK10* susceptibility locus for PD. Sadly, the lack of disease association and replication in an independent U.S. series of comparable power suggests that the original findings may be spurious. Failure to nominate *LOC200008* as the *PARK10* gene in our population samples provides empirical support for statistical caveats concerning GWA studies. Implicit in multiple testing is false discovery, even in well-designed studies, and there are several potential sources of bias.[13] Of note, neither *PARK10* SNP *rs682705* nor *rs7520966* fulfilled the main criterion for being genotyped in tier 2 ($P < .01$ in tier 1 overall analysis), but each was included with a less stringent association criterion ($P < .05$ in tier 1 overall analysis) because of its physical position within a *PARK* locus. Interestingly, the combined P value for *rs682705* ($P = 9.07 \times 10^{-6}$) is the second-lowest P value of the overall study, even though it did not fulfill the inclusion criteria. Individual-level data from the GWA study is not yet available, but, in our study, these two SNPs also appear to be in LD (pairwise $r^2 > 0.9$), as suggested by Maraganore et al.[5]; in addition, the minor-allele frequencies (MAFs) of the two SNPs are comparable across studies and populations. The former suggests less-than-optimal haplotype tagging in the initial study, whereas the latter argues against technical errors in genotyping, but neither provides sufficient explanation for the positive findings observed elsewhere.[5]

We found no evidence of direct association between the 11 SNPs nominated in the GWA study and disease in the three independent populations or in a combined sample group ($n = 1{,}570$) (table 2). However, for these loci, we did not employ a gene-based approach (nor did we fine-map each region as with *PARK10*), as advocated elsewhere[14]; we await the results of further replication studies. Of note, in the study by Maraganore et al.,[5] the *rs7702187* SNP within *SEMA5A* (MIM 609297) had the lowest combined P value ($P = 7.62 \times 10^{-6}$); however, a total of 53 SNPs were examined in this gene in tier 1. Only *rs7702187* was significant before correction ($P = .001$), which supports the possibly spurious nature of this and the other associations. The MAFs observed in our three populations and in that of the GWA study are comparable, which argues against population bias/heterogeneity (table 2).

The number of SNPs highlighted in each tier of the original study is consistent with chance—that is, 1% of SNPs use a significance level of $P < .01$. None of the P values obtained by Maraganore et al.[5] meets a Bonferroni correction for multiple testing, although this standard may be too conservative in GWA, since it fails to account for LD and incorrectly assumes that chromo-

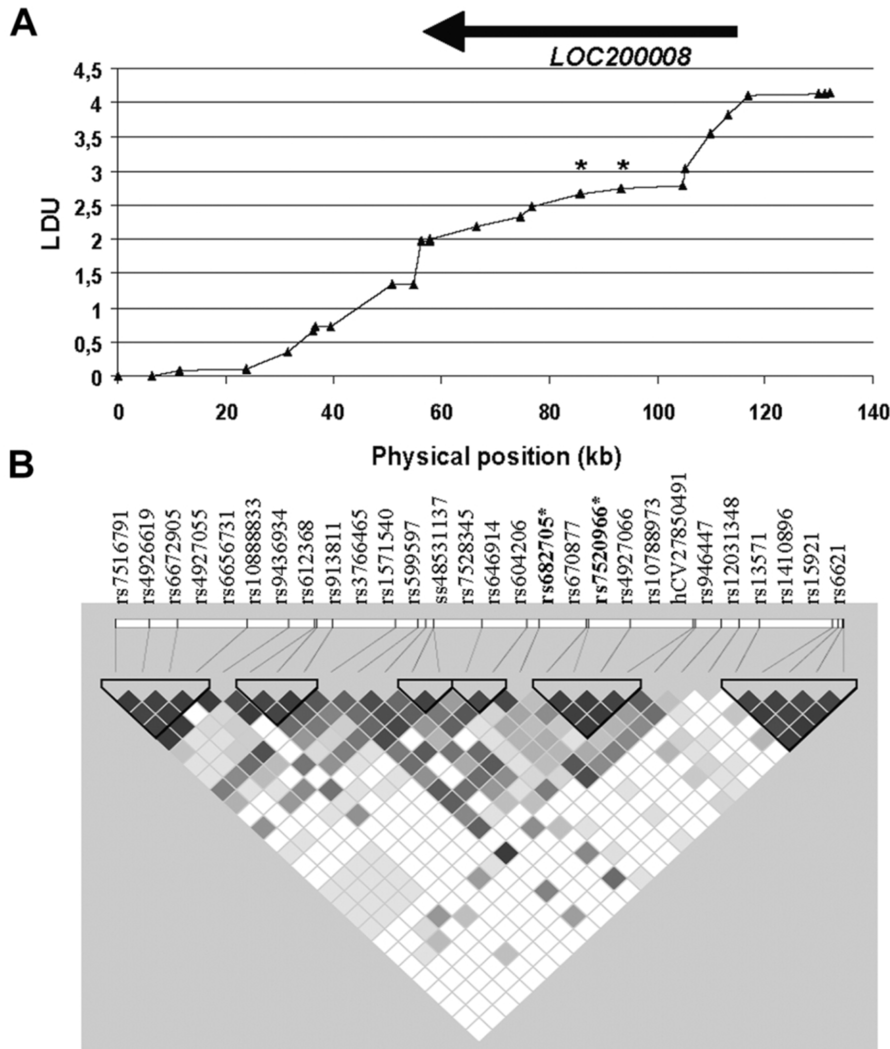

Figure 1 Metric LD map and haplotype block structure of the investigated region. *A,* LD map providing information about LD patterns in the investigated candidate region, through locations expressed in LDUs. LDUs have an inverse relationship with LD, with regions of extensive recombination having many LDUs. The physical position of the gene in the region *LOC200008* is marked with an arrow. All 28 SNPs genotyped are reported, although the symbols (▲) may be obscured for SNPs that lie in close physical proximity and high LD. SNPs *rs682705* and *rs7520966* are denoted by an asterisk (*). *B,* LD structure of the candidate region. *Black* and *dark gray cells,* strong LD; *gray cells,* intermediate; and *light gray* and *white cells,* evidence for historical recombination. The haplotype block structure of the region is defined according to Gabriel et al.[10] An asterisk denotes SNPs *rs682705* and *rs7520966*. The LD map and haplotype structure were constructed using genotypes from the Norwegian sample. Similar results were obtained for the Irish population.

somal markers are independent. A consensus on the most appropriate correction for multiple testing has yet to be reached. Now that genomewide data sets have been generated, there exists the possibility to use these to develop appropriate statistical methods to identify true positive results.[15]

In the interim, we recommend that enthusiasm for positive findings should be tempered by the strength of the evidence, the population-attributable risk, and the differences in SNP allele/genotype frequencies between cases and controls. If allele frequencies are significantly different, genomic controls might be used to assess population substructure. It is important that future studies employ multiple independent sample series, each with sufficient power to verify significant genetic associations, before publication.[16] However, lack of evidence for an association is not the same as evidence against one; thus, lack of replication should also be interpreted with caution.

Over the few next years, the number of GWA studies

Table 2

11 SNPs Nominated in GWA Study as Genetic Susceptibility Loci for PD

dbSNP Accession Number	Chromosome	Position (bp)	This Study: Control MAF, Ireland	This Study: Control MAF, Norway	This Study: Control MAF, United States	This Study: Estimated OR (95% CI)[a]	This Study: Combined P (n = 1,570)	Maraganore et al.[5]: Control MAF, Tier 1	Maraganore et al.[5]: Control MAF, Tier 2	Maraganore et al.[5]: P, Tier 1	Maraganore et al.[5]: P, Tier 2	Maraganore et al.[5]: Estimated OR (95% CI)[b]	Maraganore et al.[5]: Combined P (n = 1,550)
rs7702187	5p15.2	9385281	.17	.18	.18	.88 (.74–1.06)	.18	.18	.20	.001	.002	1.74 (1.36–2.24)	7.62×10^{-6}
rs10200894	2q36	228642637	.13	.11	.09	.96 (.77–1.21)	.74	.12	.13	.009	.001	1.84 (1.38–2.45)	1.70×10^{-5}
rs2313982	4q31.1	139145665	.05	.11	.09	.93 (.73–1.18)	.54	.07	.06	.006	.002	2.01 (1.44–2.79)	1.79×10^{-5}
rs17329669	7p14	36625169	.13	.12	.14	1.01 (.82–1.24)	.92	.13	.11	.008	.001	1.71 (1.33–2.21)	2.30×10^{-5}
rs7723605	5p15.3	5407615	.13	.14	.13	.91 (.75–1.12)	.38	.12	.09	.010	.002	1.78 (1.35–2.35)	3.30×10^{-5}
ss46548856	10q21	58986929	.09	.08	.11	.93 (.73–1.19)	.58	.09	.11	.003	.002	1.88 (1.38–2.57)	3.65×10^{-5}
rs16851009	2q24	166456214	.11	.11	.12	.95 (.76–1.18)	.63	.09	.08	.002	.009	1.84 (1.36–2.49)	4.17×10^{-5}
rs2245218	1p36.2	13885132	.19	.17	.15	.95 (.79–1.14)	.57	.11	.13	.002	.002	1.67 (1.29–2.14)	4.61×10^{-5}
rs7878232	Xq28	150516943	.25	.23	.25	1.10 (.97–1.25)	.15	.29	.26	.003	.010	1.38 (1.17–1.62)	6.87×10^{-5}
rs1509269	4q31.1	139111329	.08	.13	.13	.94 (.76–1.17)	.58	.10	.09	.005	.008	1.71 (1.30–2.26)	9.21×10^{-5}
rs11737074	4q27	125438978	.21	.20	.21	1.05 (.89–1.25)	.55	.19	.19	.007	.005	1.50 (1.21–1.86)	1.55×10^{-4}

NOTE.—In this study, MAFs are not significantly different between the populations. No P values are corrected for multiple testing. SNPs are ordered by combined P value, per Maraganore et al.[5]

[a] The direction of effect of the estimated OR observed in this study for each SNP is shown (i.e., >1 risk and <1 protective).

[b] Estimated ORs in the study by Maraganore et al.[5] do not indicate the direction of effect relative to the MAF.

will increase, and it is important to learn from the experiences gained by the few studies performed to date. Although our negative findings suggest that the conclusions drawn from the study by Maraganore et al.[5] might be based on spurious associations, further analysis of individual-level raw data is now necessary. The recent identification of a complement factor H polymorphism in age-related macular degeneration in a GWA study and the identical findings by two other groups using other study designs demonstrates that this approach can be used successfully.[17–19] It may be that, because of the heterogeneous nature of PD, associations with a gestalt phenotype are masked by background variation in SNP informativeness, population strata, and insufficient power. It is, therefore, crucial that future associations are validated and that analysis is performed to resolve the underlying cause of association in the sample population. GWA studies may still provide direction for the genetic analysis of heterogenous complex traits, but, in the short term, they may exacerbate the problem of replication failure in association studies.

Acknowledgments

The authors thank the patients and families for their participation in this study. Mayo Clinic Jacksonville is an M. K. Udall Parkinson's Disease Research Center of Excellence (National Institute of Neurological Disorders and Stroke grant P01 NS40256), and the authors thank all collaborators at the Udall Center. This study was also supported by the National Institutes of Health grant R01 NS36960, the Research Council of Norway grant 153487/V50, and Reberg's Legacy. The Ireland research consortium was supported by a Programme for Research in Third-Level Institutions neurosciences award and by the Research and Development Office of the Health and Personal Social Services. O.A.R. and M.T. are partly funded by National Parkinson Foundation and Parkinson's Disease Foundation fellowships, respectively. We thank Minnie Schreiber for technical assistance.

MATTHEW J. FARRER,[1] KRISTOFFER HAUGARVOLL,[1,2] OWEN A. ROSS,[1] JEREMY T. STONE,[1] NICOLE M. MILKOVIC,[1] STEPHANIE A. COBB,[1] ANDREW J. WHITTLE,[1] SARAH J. LINCOLN,[1] MARY M. HULIHAN,[1] MICHAEL G. HECKMAN,[1] LINDA R. WHITE,[2] JAN O. AASLY,[3] J. MARK GIBSON,[4] DAVID GOSAL,[5,6] TIMOTHY LYNCH,[5,6] ZBIGNIEW K. WSZOLEK,[1] RYAN J. UITTI,[1] AND MATHIAS TOFT[1,2]

[1]*Departments of Neuroscience and Neurology, Mayo Clinic College of Medicine, Jacksonville, FL;* [2]*Department of Neuroscience, Norwegian University of Science and Technology, and* [3]*Department of Neurology, St. Olav's Hospital, Trondheim, Norway;* [4]*Department of Neurology, Royal Victoria Hospital, Belfast, Ireland;* [5]*Department of Neurology, Mater Misericordiae Hospital, and* [6]*The Conway Institute, University College, Dublin, Ireland*

Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PD, *LRRK2, PARK10,* and *SEMA5A*)

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Address for correspondence and reprints: Dr. Owen A. Ross, Department of Neuroscience, Mayo Clinic College of Medicine, Birdsall Building, 4500 San Pablo Road, Jacksonville, FL 32224. E-mail: ross.owen@mayo.edu
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0002-9297/2006/7806-0021$15.00

Am. J. Hum. Genet. 78:1088–1090, 2006

No Evidence for Association with Parkinson Disease for 13 Single-Nucleotide Polymorphisms Identified by Whole-Genome Association Screening

To the Editor:

The 13 SNPs identified by Maraganore et al.[1] as being potentially associated with Parkinson disease (PD [MIM 168600]) represent some of the first fruit produced by the whole-genome association screening era and are clearly worthy of follow-up. To further explore these exciting candidates, we typed each SNP in 538 patients with idiopathic PD and in 516 control individuals from the United Kingdom. Cases included 160 patients involved in a community-based epidemiological study of incident PD and 378 consecutive patients with prevalent PD attending our research clinic. All cases met United Kingdom Parkinson's Disease Society Brain Bank criteria for the diagnosis of PD. The mean age at disease onset was 63 years (range 25–91 years); 2% of patients had early-onset disease (⩽40 years), and 14% of patients reported a family history of one or more first-degree relatives with parkinsonian symptoms or tremor. The control group consisted of 146 spouses of patients with PD and 370 blood donors. All individuals were white, except for four patients and one spouse. All gave written informed consent and a blood sample from which DNA was extracted using standard methods. Genotyping was performed using Taqman Assay-on-Demand (*rs2245218*) and Assays-by-Design products on a 7900HT Sequence Detection System (Applied Biosystems). Only samples that typed successfully for at least one-third of markers were included in the analysis (520 cases and 499 controls). Genotyping success rates were all ⩾97%, and no marker showed evidence of deviation from Hardy-Weinberg equilibrium. Two pairs of SNPs (*rs2313982* and *rs1509269*; *rs682705* and *rs7520966*) were found to be in strong linkage disequilibrium ($D' = 1.0$, $r^2 > 0.69$), which reduced the number of independent tests to 11. Allele frequencies in cases and controls were compared using the COCAPHASE program in the UNPHASED package.[2] Our study provides, on average, 85% power (range 68%–96%) to detect the case-control differences averaged over tier 1 and tier 2, as observed by Maraganore et al.[1]

Table 1

Genotype/Allele Frequencies of the Main 11 SNPs Nominated to Influence PD Susceptibility

dbSNP Accession Number	Genotype	Ireland Controls	Ireland Cases	Norway Controls	Norway Cases	United States Controls	United States Cases
rs2245218	AA:AG:GG	118:56:8	124:53:8	230:92:10	219:85:11	179:65:5	176:65:5
rs7878232	TT:TG:GG	116:39:20	105:46:27	223:38:49	212:48:52	168:46:34	157:39:46
rs1509269	CC:CT:TT	155:26:1	155:25:1	245:75:8	240:59:7	186:60:4	189:53:4
rs11737074	GG:GA:AA	105:56:10	115:63:5	210:100:13	199:109:9	168:69:14	144:92:8
rs7702187	TT:TA:AA	124:46:6	120:59:2	206:102:12	216:89:8	163:71:12	172:52:13
rs10200894	CC:CG:GG	135:42:3	142:36:3	257:73:1	253:66:3	207:41:1	198:41:1
rs2313982	CC:CT:TT	163:19:0	163:20:0	258:60:8	263:54:4	205:47:1	204:39:2
rs17329669	AA:AG:GG	143:32:6	136:47:2	232:59:6	244:65:6	182:67:1	174:67:2
rs7723605	TT:TC:CC	138:44:2	138:43:1	248:77:7	237:77:7	185:61:5	196:47:4
rs16851009	CC:CT:TT	146:30:2	143:38:2	244:63:7	241:55:2	198:49:3	191:48:5
ss46548856	GG:GC:CC	145:27:2	146:31:2	275:49:1	279:44:1	180:52:1	195:43:3
rs682705	GG:GA:AA	86:72:16	107:55:12	162:104:26	170:127:27	132:91:23	121:107:12
rs7520966	CC:CT:TT	84:77:15	108:57:11	168:116:36	170:122:30	135:94:22	123:106:13

Am J Hum Genet. 2006 June; 78(6): 1084–1088.
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Paper IV

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Neuroscience Letters 416 (2007) 299–301
Neuroscience Letters
www.elsevier.com/locate/neulet

Variants in the *LRRK1* gene and susceptibility to Parkinson's disease in Norway

Kristoffer Haugarvoll [a,b,*], Mathias Toft [a,b], Owen A. Ross [a], Linda R. White [b], Jan O. Aasly [b,c], Matthew J. Farrer [a]

[a] *Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, Florida, USA*
[b] *Department of Neuroscience, Norwegian University of Science and Technology, Trondheim, Norway*
[c] *Department of Neurology, St. Olav's Hospital, Trondheim, Norway*

Received 5 December 2006; received in revised form 5 February 2007; accepted 7 February 2007

Abstract

The discovery of *LRRK2* gene mutations in late-onset Parkinson's disease (PD) has irrevocably established the role of genetics in the etiology of PD. The *LRRK1* gene is the single homolog of *LRRK2*. A high degree of homology exists between *LRRK1* and *LRRK2*, indicative of shared functions and/or pathways. One study has examined *LRRK1* in familial parkinsonism by complete sequencing of the gene, reporting 4 novel non-synonymous coding variants within the *LRRK1* gene. One of these variants (ss65713826) was identified in a Norwegian proband. We investigated whether five common polymorphisms or these recently identified coding changes within *LRRK1* are associated with PD in the Norwegian population. Two rare coding variants ss65713826 and ss65713830 were more frequent in patients than controls. However, their identification in healthy controls and lack of co-segregation with disease suggests they may represent benign polymorphisms.
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Keywords: Parkinson's disease; Genetics; *LRRK2*; *LRRK1*; Norway

The etiology of Parkinson's disease (PD, OMIM #168600) remains unknown in the majority of patients. Although a number of genes have been identified in familial forms of parkinsonism, little progress has been made in elucidating the genetic component of late-onset PD [3]. The identification of pathogenic variants within the *leucine-rich repeat kinase 2* gene (*LRRK2*; OMIM*609007), causing both familial parkinsonism and sporadic PD has reignited research interest. *LRRK2* is located on chromosome 12q12, contains 51 exons and encodes the 2527 amino acid protein Lrrk2 [3]. The Lrrk2 G2019S substitution is an important cause of PD in Norway [1]. Recently, G2019S has been identified in 30–37% of familial and 13–41% of sporadic PD patients in Ashkenazi Jews and North African Arabs [6].

The identification of pathogenic mutations in *presenilin 1* and *2* in Alzheimer's disease is evidence that homologous proteins can cause the same disease phenotype [8,10]. *LRRK2* has a single homolog, *LRRK1* (Chr 15q26.3, 156.5 kb, 33 Exons) [9]. There is a high level of DNA/protein sequence and protein structure homology suggesting that they may have similar functionality [7]. Interestingly, *Caenorhabditis elegans* and *Drosophila melanogaster* harbor only a single ortholog (*Lrk-1*) indicating that *LRRK1* and *LRRK2* in vertebrates have diverged from this common ancestral gene [7]. Furthermore, phylogenetic analyses using the functional domains of the *LRRK2* gene show that *LRRK2* emerged from a gene duplication [9].

Our group recently identified four novel non-synonymous coding variants within the *LRRK1* gene through complete sequencing of all exons in 95 probands with autosomal dominant parkinsonism (Table 1). These variants could not conclusively be shown to segregate with disease in these families [11]. Herein, we investigate five common single nucleotide polymorphisms (SNPs) and the four novel non-synonymous coding SNPs within the *LRRK1* gene to see if they are associated with susceptibility to PD in the Norwegian population.

All patients were examined and observed longitudinally by a movement disorder specialist (JOA) and diagnosed with PD according to published criteria [5]. Patients with atypical symptoms or a known genetic cause of PD were excluded. Norwegian

* Corresponding author at: Mayo Clinic Jacksonville, Department of Neuroscience, 4500 San Pablo Rd., Jacksonville, FL 32224, USA.
Tel.: +1 904 953 2483; fax: +1 904 953 7370.
E-mail address: haugarvoll.kristoffer@mayo.edu (K. Haugarvoll).

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doi:10.1016/j.neulet.2007.02.020

Table 1
Allelic association between *LRRK1* SNPs and PD

Marker	Position	Amino acid	Alleles	MAF in cases/controls	χ^2 (1 df)	*P*-value*	Permuted *P*-value
ss65713826	Exon 4	K203E	A/G	0.011/0.002	4.548	0.03	0.17
rs7169323	Intron 6	–	C/G	0.448/0.496	3.064	0.08	0.40
rs4965778	Exon 10	–	A/G	0.413/0.393	0.576	0.45	0.97
rs11853661	Exon 15	T844T	C/T	0.461/0.488	0.995	0.32	0.90
rs2925203	Intron16	–	A/C	0.441/0.415	0.926	0.34	0.91
ss65713830	Exon 19	T967M	C/T	0.005/0	3.173	0.07	0.36
ss65713838	Exon 31	P1796H	C/A	0.006/0.006	0.006	0.94	1
ss65713839	Exon 31	R1850C	C/T	0.012/0.008	0.718	0.40	0.95
rs2278953	3′	–	C/T	0.334/0.31	0.904	0.34	0.92

Results from the matched case-control series. (*) Two tailed *P*-value for single markers. MAF = Minor allele frequency. df = degrees of freedom. Correction for multiple testing was performed by permutations ($n = 10{,}000$).

samples consisted of 676 subjects (338 patients and 338 controls) with mean age 70 ± 11 SD years (range 40–96 years), all originating from Central Norway. The male-to-female ratio was 1.4:1, and the mean age at onset for patients was 58 ± 11 years (range 25–88 years). About 15% of the PD patients in our series had a first degree relative also affected by PD. Each patient was matched based on age (±4 years), gender and ethnicity to an unrelated control without evidence of neurological disease. These and 249 further unmatched controls originated from the same geographical region in Central Norway. Participants provided informed consent and the ethical review boards at each institution involved approved the study.

Genomic DNA was extracted from whole blood using a standard protocol. We selected five SNPs displaying moderate inter-marker linkage disequilibrium (LD; $r^2 = 0.5$–0.6) located across the *LRRK1* locus (Available-by-Demand), and designed four TaqMan Available-by-Design probes for the novel coding variants; Applied Biosystems, Inc. (Sequences available on demand). Genotyping was performed on an ABI 7900 genetic analyzer and analyzed using SDS 2.2.2 software.

All SNPs were tested for Hardy–Weinberg equilibrium (HWE) in controls ($P > 0.05$). Individual allelic associations between PD and each SNP were investigated by χ^2-square test as implemented in Haploview software [2]. To correct for multiple testing, we calculated empirical *P*-values by permutation analysis ($n = 10000$).

One novel non-synonymous *LRRK1* variant ss65713826 (607A > G, K203E) was more frequent in PD patients than controls in our Norwegian series (Table 1). However, this difference was not significant after correction for multiple testing ($P > 0.05$). Interestingly, this variant was first identified in a Norwegian proband from a family displaying autosomal dominantly inherited PD (Fig. 1). The variant did not co-segregate with disease in this family [11]. A further novel variant, ss65713830 (2900C > T, T967M), first identified in a family of Eastern European descent without evidence of co-segregation with disease was found in 3 patients, but no controls in our case-control study. No association with PD was found for the other SNPs investigated, including ss65713838 and ss65713839 identified in the previous study (Table 1).

In order to obtain a better estimate of the frequency of the two novel variants (ss65713826 and ss65713830), we screened a further 249 Norwegian controls. ss65713826 (K203E) was identified in two controls aged 87 and 98 years, respectively. ss65713830 (T967M) was seen in one sixty years old control. These results, together with the lack of co-segregation with disease previously demonstrated for these two variants indicate that they most likely represent rare polymorphisms [11].

However, the reduced penetrance that has characterized Lrrk2-associated disease demonstrates that caution should be used when classifying polymorphisms even if they are observed in controls. Although *LRRK1* is a candidate gene for PD, our data suggest that *LRRK1* variants are not associated with PD in the Norwegian population. Considering that the Lrrk2 G2385R substitution has been shown to be a population specific risk factor for PD, we cannot rule out a role for *LRRK1* variants in other populations [4].

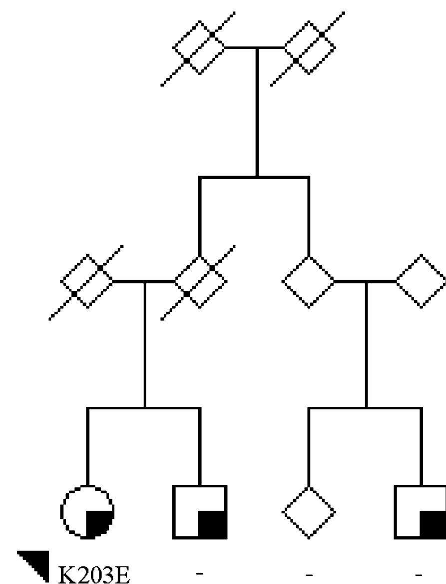

Fig. 1. The Norwegian pedigree F07 (Lrrk1 K203E). Squares represent males and circles represent females, whereas a diamond is used to disguise the gender/number of unaffected subjects. Deceased individuals are indicated with a diagonal line. The novel non-synonymous *LRRK1* variant ss65713826 (607A > G, K203E) was identified in the proband, indicated by (▶), but in no further family members, indicated by (–).

LRRK2 has one single homolog, *LRRK1* [7]. The functions and pathways of their gene products remain unknown, however there is evidence supporting a kinase activity for both [7,12]. Furthermore, there might be important functional interactions between Lrrk1 and Lrrk2, possibly implicating both proteins in the pathology of PD. Further studies will hopefully advance our understanding of the mechanisms through which these proteins act.

Acknowledgements

We thank the patients and controls who made this study possible. Mayo Clinic Jacksonville is a M. K. Udall Parkinson's Disease Research Center of Excellence (National Institute of Neurological Disorders and Stroke grant P01 NS40256) This work was supported by a Robert H. and Clarice Smith Fellowship (OAR) and grants from Forsberg and Aulies Legacy and the Fulbright Foundation (KH).

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