A genetic-epidemiologic study of Parkinson's disease

Marieke C.J. Dekker

Cover: quaking grass (*Briza media*), vegetation endemic to the GRIP area (in local dialect: 'schuddekopkes'). Photography/artwork by WJ Renger
Printed by Print Partners Ipskamp, Enschede

A genetic-epidemiologic study of Parkinson's disease

Een genetisch-epidemiologisch onderzoek naar de ziekte van Parkinson

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 11 juni 2003 om 9:45 uur

door

Marieke Cornelia Johanna Dekker

geboren te Sluis

PROMOTIECOMMISSIE

Promotoren   Prof.dr. C.M. van Duijn
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To David Holmes Morton- on Frost's errand

CHAPTER 1

INTRODUCTION

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CHAPTER 1.1

Parkinson's disease: piecing together a genetic jigsaw

Abstract

The role of genetics in the pathogenesis of Parkinson's disease (PD) has been subject to debate for decades. In recent years, the discovery of five genes and several more loci has provided important insight into its molecular aetiology. Some PD genes possibly cause PD by protein aggregation. The presence of Lewy bodies in carriers of mutations in one gene and their absence in carriers of another, however, still point towards a complex pathogenic network with PD as a common clinical end point. The recent identification of the fourth and fifth PD genes suggests multiple pathways: an impaired oxidative-stress defense for mutations in DJ-1, and a defect in another signaling pathway for mutations in NR4A2. Despite knowledge of genetics in familial PD, our knowledge of the common, late-onset form of PD remains limited. In non-familial PD, genes and environment probably interact to give rise to the disease. We review advances in the genetics of PD, focusing on the monogenic forms and their clinical and population-genetic consequences.

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Introduction

Parkinson's disease (PD) is a movement disorder and an important cause of morbidity and mortality in later life (prevalence 1.6 percent in the elderly) [1]. PD is clinically characterised by the 'cardinal signs': resting tremor, rigidity and bradykinesia. In some studies, postural instability is considered a fourth cardinal sign.[2] Asymmetric onset of symptoms and a good, prolonged response to levodopa endorse the clinical diagnosis. Neuropathological examination shows degeneration of the substantia nigra and various other regions of basal ganglia, brain stem, autonomic nervous system and cerebral cortex. The dopaminergic tract is predominantly affected in PD, but the cholinergic, noradrenergic and serotinergic systems are also involved, albeit to a lesser degree.[3] Lewy bodies, intracytoplasmatic protein aggregates considered to be the neuropathological hallmark of PD, are found throughout the brain. Although in a specialist setting the clinical diagnosis of PD could be neuropathologically confirmed in up to 90 percent, this level of agreement elsewhere is usually less than 80 percent.[4,5] Yet Lewy bodies are not sufficient to establish the neuropathological diagnosis of PD.[6] Furthermore, recent developments in genetics indicate that some monogenic forms of parkinsonism are not always associated with Lewy bodies.[7,8] The term parkinsonism embodies all clinical syndromes in which clinical features are similar to PD, regardless of pathology or concomitant symptoms. Parkinsonism occurs in other primary neurodegenerative diseases such as multiple system atrophy, progressive supranuclear palsy, and in vascular disease, major depression and dementia. Exogenous factors (such as neuroleptic medication and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or MTPT)[9] leading to secondary parkinsonism have long supported the view that PD was likely to be a non-genetic disorder. From the recent developments in genetics, however, the role for genetic components in at least a proportion of PD becomes evident.

Pathogenesis of PD

A consistent risk factor for PD is age.[1] The list of putative environmental risk factors is long, but few studies have shown a consistent association. Exposure to pesticides is one of the most established environmental risk factors for PD.[10] The debate about the role of genes in typical, idiopathic PD is ongoing. The estimated proportion of patients in the general population who have at least one relative with PD varies from 6.4 to 10.3 percent, whereas studies using hospital-based patient series report frequencies of up to 33 percent.[11-14] The increased frequencies in clinic-based populations could truly be due to a more frequent family history amongst clinic-based patients, yet such patients may also have a recollection bias for PD in relatives. A large study in twins suggested strong inheritance in PD with onset before the age of 50 years. Beyond this onset age, however, only weak evidence for a genetic aetiology was found.[15] Another twin study using PET neuroimaging

revealed, however, (preclinical) disease concordance in twins regardless of onset age.[16]

From a genetic perspective, several modes of inheritance for PD have been postulated, ranging from autosomal dominant inheritance with variable penetrance to mitochondrial inheritance.[17,18] Most studies of recent date indicate a complex, multifactorial mode of inheritance, with several genes interacting with environmental risk factors. An incident possibly illustrating a combination of environmental and genetic factors in PD is the small epidemic of parkinsonism in MPTP users.[9] MPTP was a component of a party drug, giving rise to parkinsonism clinically indistinguishable from idiopathic PD in relatively young individuals. Not all those exposed to MPTP, however, developed parkinsonism. Regardless of the dose, a certain genetic make-up may therefore have made certain people more vulnerable to the effects of MPTP, or have protected others.

Yet there are a number of families in which PD segregates in an unambiguously mendelian fashion. Since 1997, linkage to eleven genes and loci has been reported.[8,19-28] Six of these are implicated in PD with an autosomal dominant pattern of inheritance,[8,19,21-23,28] four in that with autosomal recessive inheritance,[20,24-26] and one in late-onset, seemingly sporadic PD.[27]

Table 1: Genes for familial Parkinson's disease

Gene/locus	Location	Inheritance	Onset	Distinctive clinical features	LB
parkin	6q25	AR	early-juvenile	frequent dyskinesia/dystonia; slow progression	no[a]
DJ-1	1p36	AR	early	focal dystonia; slow progression; psychiatric symptoms	n.a
PARK6	1p35-36	AR	early	slow progression	n.a
PARK9	1p36	AR	juvenile	spasticity; dementia; supranuclear ophthalmoparesis	n.a.
alpha-synuclein	4q21	AD	late	lower prevalence tremor; more rapid progression	yes
UCH-L1	4p14	AD	late	none	n.a.
NR4A2	2q22-23	AD	late	none	n.a.
PARK3	2p13	AD	late	dementia in some patients	yes
PARK4	4p14-16.3	AD	late	some relatives postural tremor; dementia; autonomic dysfunction; weight loss early in disease	yes
PARK8	12p11.2-q13	AD	late	none	no
PARK10	1p32		late	none	n.a.

[a] *Lewy bodies have been reported in one patient with parkin mutations[35]; AR = autosomal recessive; AD = autosomal dominant; LB = Lewy Bodies; n.a. = information not available*

PD genes and loci, clinical and neuropathological phenotypes

Several families with mendelian patterns of inheritance had been described in detail without hard evidence for a genetic basis of PD, until the discovery of the first familial PD gene in 1997.[19] At present, five genes (alpha-synuclein; parkin; UCH-L1; DJ-1; NR4A2) have been identified in familial PD. [19,20,23,25,28] A further six loci across the genome (*PARK3; PARK4; PARK6; PARK8; PARK9; PARK10*) harbour yet unknown genes (Table 1).[21,22,24,8,26,27] Despite the unknown causative genes contained in the latter loci, a characterisation of their clinical and pathological phenotype could shed light on possible overlap and differences with the phenotypes associated with the known PD genes. Recessive genes and loci are listed first, followed by dominant genes and loci.

Parkin (PARK2)

In 1998, mutations in a newly identified gene, parkin (*PARK2*, chromosome 6q25.2-q27), were described in Japanese families segregating early-onset parkinsonism as an autosomal recessive trait.[20] Several clinical features distinguish parkin-linked parkinsonism from sporadic PD, such as the wide range of ages at onset varying from early childhood to late adulthood, frequent dystonia and slow progression. Symptoms range from classical parkinsonism to dystonia in different degrees of severity, with frequently occurring dystonia and dyskinesia upon dopaminergic treatment.[29,30] On functional neuroimaging in parkin-linked parkinsonism, the uptake of dopamine tracer was reduced in both hemispheres in the putamen as well as in the caudate nucleus.[31,32] On the contrary, in sporadic, non-familial PD, the reduction in dopa uptake is initially unilateral and primarily involves the putamen.[33,34] Neuropathological studies in a small number of patients with mutations in the parkin gene showed selective degeneration of the nigrostriatal tract. Remarkably, Lewy bodies were absent.[7] One exception to this has been reported in a patient with parkin mutations who was found to have typical Lewy bodies at autopsy.[35] This could imply an occasional role for Lewy bodies in parkin-linked disease, and alternatively occasional Lewy-body neurodegeneration superimposed on parkin-linked parkinsonism.

Parkin is a large gene spanning 1.5 megabases and containing twelve exons, in which to date over 70 mutations have been identified (Bonifati, personal communication). New developments in the detection of mutations in the gene are expected to increase this number further. The protein product of parkin is an E3 ubiquitin ligase.[36] Ubiquitin is, as its name suggests, one of the most abundant proteins in the brain and, like alpha-synuclein, contributes to the formation of Lewy bodies.[37] A particular form of alpha-synuclein has been shown to be the substrate for parkin,[38] thus linking these two PD genes by the ubiquitin system.

DJ-1 (PARK7)

In 2001, shortly after the localisation of the *PARK6* locus,[24] a second locus on chromosome 1p36, *PARK7*, was reported. *PARK7* is a good 25 centiMorgans (cM) removed from the *PARK6* locus. Linkage to the *PARK7* locus was first identified in a kindred from a genetically isolated population in the South-West of The Netherlands segregating autosomal recessive early-onset parkinsonism,[39] and subsequently was confirmed in an Italian family.[40] DJ-1 parkinsonism is clinically characterised by variable severity of disease and slow progression of symptoms with sustained response to levodopa treatment. In addition to parkinsonism, in the original kindred and in one of the patients in the Italian DJ-1 family with DJ-1 mutations, psychiatric co-morbidity was reported.[39,41] Pathology of carriers of DJ-1 mutations is not yet available, but functional neuroimaging showed a symmetrical decrease in dopa uptake in putamen as well as in caudate nucleus, a picture resembling that in *parkin-* and *PARK6*-linked parkinsonism.[41]

Recently, mutations in the DJ-1 gene were reported to be associated with parkinsonism in these two kindreds.[25] The patients in the Dutch kindred carried a homozygous deletion of DJ-1 rendering the gene product absent, and affected individuals in the Italian family were homozygous for the L166P mutation. The function of DJ-1 is unknown, but there is evidence for a role in the cellular response to oxidative stress. The mutant DJ-1 protein may therefore have an impaired ability to limit oxidative damage. Transfection studies showed loss of the diffuse cytosolic patterns associated with the wild-type, and co-localisation of the mutant DJ-1 protein in mitochondria, although the mutant protein is also present in the nucleus. This points towards a pathogenic loss of cytoplasmic activity for mutant DJ-1.

PARK6

In 2001, linkage to chromosome 1p35-36 was reported in a large Italian family, the Marsala kindred.[24] This locus, named *PARK6*, is associated with early-onset parkinsonism with an autosomal recessive pattern. The clinical presentation of *PARK6*-linked parkinsonism resembled typical PD, apart from an early age at onset (ranging from 32 to 48 years) and a slow progression of symptoms. Furthermore, tremor was a predominant sign and dystonia was not reported. Response to levodopa treatment was good and lasting.[42] A functional neuroimaging study showed symmetrically decreased dopa uptake in putamen as well as in caudate, much like in *parkin*-parkinsonism.[43] No post-mortem data are yet available on *PARK6*-linked parkinsonism. Since its identification, linkage to *PARK6* has been confirmed in a number of small European families, reducing the candidate interval to 9 cM.[42]

PARK9

Kufor-Rakeb syndrome is a juvenile-onset neurodegenerative disorder with an autosomal recessive pattern of inheritance. The first clinical report on an Arab

consanguineous kindred dated from 1994.[44] The name of the syndrome denotes the area of origin of the kindred. Kufor-Rakeb syndrome clinically resembled typical PD with a good response to levodopa treatment with respect to the extrapyramidal dysfunction. Although listed as a mendelian parkinsonism, there were many additional features of the *PARK9* phenotype (spasticity, dementia and supranuclear-gaze paralysis), which did not resemble typical PD. On neuroimaging, there was significant atrophy of the globus pallidus, which in a later stage became generalised. No neuropathological data are available. Linkage to a region of 9 cM on chromosome 1p36 was described in 2001.[26] The *PARK9* status subsequently was assigned, although to date, no official report is available (The Genome Database, URL: http://www.gdb.org).

Alpha-synuclein (PARK1)

Two different mutations have been identified in the alpha-synuclein gene (*PARK1*) on chromosome 4q21. In 1997, the A53T mutation was found in a large Italian/American family (the Contursi kindred)[45] as well as in three unrelated families of Greek descent, in which PD was inherited with an autosomal dominant pattern.[19] The common haplotype in the families suggested a common founder due to the age-old historical ties between the two regions of origin. Since, this mutation has been found in a small number of other families. The second mutation, the A30P mutation, has been reported in one German family.[46] Clinical characteristics of the patients with the A53T mutation differed from sporadic PD with respect to a slightly younger age at onset, a considerably lower prevalence of tremor, a more rapid clinical deterioration. Furthermore, concomitant dementia, myoclonus and central hypoventilation have been reported in parkinsonism associated with the A53T mutation.[45,47,48] Conversely, the clinical phenotype of A30P alpha-synuclein parkinsonism closely resembled sporadic PD.[46] Neuroimaging features in alpha-synuclein parkinsonism were concordant with those observed in idiopathic PD.[49,50] Post-mortem examination in patients with a mutation in the alpha-synuclein gene showed the pattern of neuronal degeneration and the Lewy bodies so characteristic of PD.[19] In the original Contursi kindred,[45] a recent neuropathological study also revealed tau inclusions, suggesting that the alpha-synuclein neurodegenerative process is not entirely identical to that seen in typical idiopathic PD brains.[51]

The identification of alpha-synuclein's involvement in familial PD has been a breakthrough in the hitherto limited knowledge about the pathogenesis of the disease. Alpha-synuclein was first described as a presynaptic protein in *Torpedo californica*.[52] The protein is involved in synaptic plasticity, as was shown in an ortholog-protein study on song learning in the zebra finch.[53] Furthermore, alpha-synuclein transgenic *Drosophila* and mouse models exhibited progressive locomotor dysfunction and loss of dopaminergic neurons, mimicking the phenotype of PD.[54,55] Alpha-synuclein is abundantly present in brain, and, upon the identification of the mutations in its encoding gene, was identified to be a principal

component of Lewy bodies.[56] *In vitro* experiments suggested that the mutant protein facilitates fibril formation, giving rise to Lewy bodies. The A53T-mutated alpha-synuclein formed fibrils more easily than the A30P-mutant.[57] The lesser complexity of the A30P-linked clinical phenotype (resembling typical PD) may therefore reflect its lower degree of alpha-synuclein fibrillogenesis *in vitro*.

UCH-L1 (PARK5)

In 1998, the I93M mutation in the ubiquitin carboxy-terminal hydroxylase L1 (UCH-L1) gene on chromosome 4p14 was identified in a family of German descent in which PD was inherited in an autosomal dominant fashion.[23] By the time the described family came to attention, all reportedly affected individuals, except for two affected siblings, were deceased. The clinical phenotype in these siblings consisted of dopa-responsive parkinsonism, resembling idiopathic PD. Onset of symptoms in the two siblings occurred at the ages of 49 and 51 years. To date, neither radiological nor neuropathological data on this family are available. Mutations in the UCH-L1 enzyme reduce its catalytic activity *in vitro*, therefore possibly leading to a tendency of various protein metabolites to aggregate.[58] In immunofluorescence studies, Lewy bodies stained positive for UCH-L1, suggesting it also contributes to the ubiquitin-proteasome pathway implicated in alpha-synuclein and parkin-linked parkinsonism.[23]

NR4A2

A recent report described two mutations in the NR4A2, or NURR1 gene.[28] The clinical phenotype in the patients with mutations in NR4A2 was concordant with late-onset PD without atypical features. Radiological or neuropathological data are not available. NR4A2 (chromosome 2q22-23) is a gene involved in the differentiation and maintenance of dopaminergic neurons. Due to its function, previous studies had already suggested NR4A2 to be a candidate gene for PD.[59,60] NR4A2 was studied in a series of 107 individuals with familial PD (70 of whom had a history of PD over at least two generations). Two heterozygous mutations (–291Tdel and –245T→G) were revealed in 10 individuals, who were all of European descent. Both mutations affect a non-coding exon (exon 1) of the gene and lead to marked decrease in NR4A2 mRNA levels. The mechanism by which this mRNA transcription is targeted is not yet clear.

PARK3

In 1998, linkage was reported to chromosome 2p13 in six families in which PD was inherited in an autosomal-dominant fashion.[21] Clinically, there was typical dopa-responsive parkinsonism. However, dementia occurred in two of the *PARK3*-linked families. Ages at onset of disease ranged from 37 to 89 years. Autopsy findings showed degeneration of dopaminergic neurons in the substantia nigra and Lewy bodies, but also Alzheimer-like neurofibrillary tangles and neuritic plaques in some

patients.[61] The *PARK3* phenotype may therefore encompass a wide pathological spectrum, ranging from parkinsonism to dementia. The *PARK3*-critical region spans a distance of 2.5 Mb. Analysis of the genes contained within this region has not yet revealed any causal mutation.[62] The disease-associated haplotype was also observed in clinically unaffected relatives, suggesting a penetrance of less than 40 percent. Two families of Northern German and Southern Danish descent showed strongest evidence for linkage and a common haplotype at *PARK3*, raising the possibility of a common ancestor due to the vicinity of the regions of origin of these two families.

PARK4

In 1999, a haplotype on chromosome 4p (*PARK4*) was reported to be segregating with PD as well as with postural tremor in an autosomal dominant pattern.[22] Parkinsonism in this American kindred typically presented with asymmetrical limb 'heaviness' and rigidity, rather than with tremor. The postural tremor did not seem to be an early manifestation of parkinsonism as it remained a separate clinical entity over time. Many atypical features in the kindred were observed, i.e. autonomic dysfunction, dementia, early-stage weight loss, myoclonus and seizures, which are not concordant with typical PD. The onset age was considerably lower than in sporadic PD (mean 33.6 years), and progression to death was rapid.[63,65] On neuropathological examination in individuals with parkinsonism, Lewy bodies were found, the distribution of which was consistent with the neuropathological diagnosis of typical PD.[22] No neuropathological data are available on the family members with isolated postural tremor. Variable expression of the unknown gene is suggested by the occurrence of the *PARK4* haplotype not only in individuals with parkinsonism, but also in individuals with isolated postural tremor. The *PARK4* family was not large enough to achieve significant linkage, and linkage to the *PARK4* region has not yet been confirmed in other studies. In the 8.5-cM spanning locus, no causal mutations have been identified so far. UCH-L1 (*PARK5*) on chromosome 4p14[23] is just outside the candidate region, and could be excluded.

PARK8

In 2002, linkage to chromosome 12p11.2-q13.1 was described in a Japanese family with autosomal dominant parkinsonism, the Samigahara family.[8] The features of parkinsonism in this family resembled typical PD, with a good response to levodopa treatment. The mean age at onset of disease was 51 years. Neuropathological examination in four cases of *PARK8* parkinsonism revealed 'pure nigral degeneration' lacking the Lewy bodies so typical of PD. By parametric linkage analysis, the maximum LOD score was 4.32; non-parametric linkage analysis increased the LOD score to 24.9. The *PARK8*-linked haplotype was also observed in some unaffected family members, suggesting incomplete penetrance. So far, none of the analysed genes in the 13.6-cM wide candidate-gene interval have mutations.

PARK10

Unlike previously reported genes and loci, which all exhibit mendelian inheritance patterns, *PARK10* is a locus for late-onset non-mendelian PD.[27] Clinical features in the *PARK10*-linked families were concordant with typical, sporadic PD. Eighty-four percent of patients had onset of disease later than 50 years, with a mean age at onset of 65.8 years. No neuropathological or neuroimaging features have been reported. Based on a nationwide database of clinical and genealogical information, significant clustering for PD previously had been shown amongst these patients compared to control individuals.[66] Fifty-one families could be linked to one another in a large pedigree, suggesting a genetic aetiology. Significant linkage (LOD score 4.9) was reported for a haplotype of 7.6 cM on chromosome 1p32 in those families, each of which contained more than one PD patient.

Table 2. Population attribution of PD genes and loci

Gene/locus	*Replication elsewhere*	*Estimated attributable risk (percentage of PD explained)*
Parkin	yes	PD overall: 0.4-0.7 percent; within late-onset PD: small; within early-onset sporadic PD: 9-18 %; within early-onset recessive PD: 49 %
DJ-1	yes	locally in Dutch isolate: 33 % of early-onset cases; general population: unknown
PARK6	yes	within early-onset recessive PD: up to 15 %[a]
PARK9	no	(single report)
alpha- synuclein	yes	small
UCH-L1	no	(single report)
NR4A2	no	unknown
PARK3	no	small [b]
PARK4	no	(single report)
PARK8	no	(single report)
PARK10	Icelandic data set	unknown [c]

[a] *Based on linkage data;* [b] *PARK3 was implicated in onset age of PD in a genomic screen[101];*
[c] *Linkage to onset age of PD was reported to the region containing PARK10 in a genomic screen[9]*

PD genes and loci: population attribution

From a clinical perspective, the increasing knowledge of genes in PD raises the question to what extent mutations in these genes account for PD in the general population. Studies on polymorphisms (variants of a gene commonly occurring in the general population) in the familial-PD genes alpha-synuclein, parkin and UCH-L1 do not provide conclusive evidence for association with typical PD.[67-75] Amongst the 11 genes and loci in PD, mutations in some have not been replicated in families other than the originally reported kindred, whereas others are associated with PD in a substantial proportion of cases around the world (Table 2).

Population attribution by gene/locus

The parkin gene (*PARK2*) is one of the largest genes known (second-largest after dystrophin, the Duchenne muscular dystrophy gene[76]). A considerable proportion of cases of early-onset autosomal recessive parkinsonism and isolated juvenile-onset parkinsonism in several ethnic groups have mutations in the parkin gene.[30,77,78] In a multi-ethnic series of families with autosomal recessive early-onset PD, 49 percent were found to have parkin mutations (Figure 1a). In sporadic, non-familial PD patients with disease onset below age 45 years, parkin mutations were detected in 18 percent. The prevalence of parkin mutations in this study decreased rapidly with later onset: 77 percent of patients with onset of disease at or below 20 years, 26 percent of patients with onset between 21 and 30 years, and only three percent of those with onset over 31 years carried mutations in parkin.[30] Another study of community-derived PD patients with onset of disease below 50 years reported the proportion of parkin mutations to be nine percent.[78] Detailed data on the proportion of patients with early-onset, autosomal recessive forms amongst PD in the general population are not available, which hampers calculation of the attribution of early-onset families with recessive inheritance and parkin mutations. In the Rotterdam Study, a large, population-based cohort study in The Netherlands, the prevalence of PD patients with onset below 45-50 years at baseline was four percent.[1] The Rotterdam Study, being population-based, possibly generates less bias than hospital-derived patient series, in which a higher prevalence of early-onset cases by patient selection is expected. If parkin mutations account for 9-18 percent of sporadic early-onset PD,[30,78] the parkin gene explains approximately 0.36-0.72 percent (0.09*4 - 0.18*4) of all sporadic PD in the general population (Figure 1b). This is, however, expected to be an underestimation of the true proportion. Novel parkin mutations such as intronic and exonic rearrangements are likely to explain more disease cases in the future.

The contribution of the DJ-1 gene (*PARK7*) to the general population is still unknown. The DJ-1 deletion was not observed in a sample of 200 Dutch controls from elsewhere in The Netherlands, and the point mutation was not either in 160 controls from elsewhere in Italy.[25] In contrast, DJ-1 was shown to account for a considerable proportion of early-onset parkinsonism locally. In the genetically

isolated population of the original kindred in the Southwest of The Netherlands, four of 220 randomly drawn individuals from the local population were heterozygous for the DJ-1 deletion, yielding an estimated mutant allele frequency of almost one percent.[25] No other homozygotes were observed. In a survey on parkinsonism conducted in the isolated village from the original kindred,[39] six individuals with early-onset parkinsonism were observed, four of whom were homozygous for the DJ-1 deletion (Dekker et al., unpublished work). Therefore, two-thirds (67 percent) of early-onset parkinsonism in this population can be explained by DJ-1. Furthermore, regarding the observation of two mutations in the DJ-1 gene in families from different countries, mutations in the DJ-1 gene are expected in more ethnic groups.

It is difficult to estimate the contribution of mendelian-PD loci to PD overall. Until the causative gene is identified, such estimates only have limited value. Linkage to the PARK6 locus was replicated in families of Italian, British, German and Dutch descent.[42] The absence of a common haplotype amongst these families suggests that the PARK6-linked form of familial parkinsonism occurs in several European countries, possibly as a result of independent mutational events in the unknown gene. The eight PARK6-linked families from the study by Valente et al. were derived from a series of 28 families with early-onset autosomal recessive parkinsonism without evidence for parkin mutations.[42] No detailed information is available on the criteria for selection of these 28 families. Furthermore, caution should be taken when calculations are based on a small number of families, and on linkage to a locus rather than on the causal gene. Yet, based on the aforementioned numbers, PARK6 may account for approximately 29 percent (8/28) of parkin-negative early-onset cases with an autosomal pattern of inheritance, and thus for 15 percent of early-onset autosomal recessive PD overall (i.e., 29 percent of 51 percent families with early-onset recessive inheritance of PD without parkin mutations) (Figure 1a).

Figure 1a)

b)
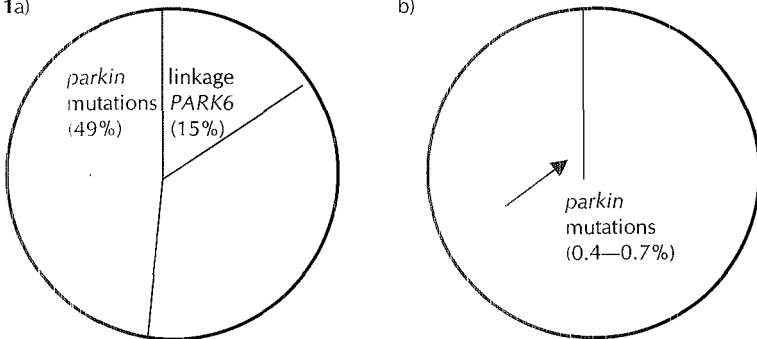
a) Attribution of mendelian genes to early-onset PD with autosomal recessive inheritance
b) Attribution of mendelian genes to PD in the general population

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The A53T mutation in the alpha-synuclein gene (PARK1) was reported in a dozen families, possibly with a common founder.[19] The Eastern-Mediterranean origin of the families reported to carry the A53T mutation further supports this notion.[47,79,80] The other mutation in the alpha-synuclein gene, A30P, has been reported in one German family only.[46] Studies in various populations have pointed out that mutations in the alpha-synuclein gene are very rare, explaining a small proportion of sporadic and familial PD overall.[81-85] Yet this gene was the first gene to be identified in familial PD, and unraveling the role of alpha-synuclein in PD has been a first step towards understanding the pathogenesis of the common form, or forms, of the disease.

Since the identification of the I93M mutation in the UCH-L1 gene (PARK5[23]) this mutation or other mutations have not been found in several other studies, indicating that mutations in the UCH-L1 gene are a very rare cause of PD.[23,86-88] This could mean that, similar to the paucity of reported mutations in the APP gene in presenile dementia,[89] the I93M mutation is one of the only viable mutations in the UCH-L1 gene, other mutations being incompatible with late-onset PD or even with life. Alternatively, the I93M mutation could be a rare polymorphism, coincidentally found in a sib pair with PD, rather than a causal mutation.[90]

Due to the recent identification of NR4A2,[28] few replication reports in other patient series are available at present.[91,92] A haplotype analysis performed in the originally reported families with the –291Tdel mutation showed a haplotype shared by six individuals from three families with German ancestry, raising the possibility of a common founder. Mutations in NR4A2 were observed neither in 94 individuals with sporadic PD and in 221 unaffected controls,[28] nor other series of familial-PD patients.[91,92] More information is to become available to assess the frequency of this gene at population level.

The PARK3 locus was reported to segregate with PD in six families, and strongest evidence for linkage was observed in two families, possibly with a common ancestor.[21] Neither in PD patients who originate from the same region, nor in patients from elsewhere in Germany was linkage to the PARK3 region confirmed.[93] Recently, however, suggestive linkage was reported to PARK3 in a PD sib-pair genome scan designed to detect modifiers of age at onset.[94]

The extent of involvement of the PARK10 locus[27] in PD is intriguing. Linkage results pointing towards the PARK10 locus are based on 117 individuals in 51 families, all of Icelandic descent and genealogically linked to one another. Data about what proportion of these 51 families is explained by PARK10 are, however, not available. A previous genealogic study by the same research group[66] described a group of 772 PD patients ascertained throughout Iceland, from which the 117 individuals who were used for the linkage study were derived. This would mean that the gene contained within the PARK10 locus may account for up to 15 percent (117/772) of PD in the Icelandic population. The role of the PARK10 in the remaining 655 Icelandic PD patients[66] is not yet known. Similarly, involvement of

PARK10 in populations outside Iceland remains to be confirmed. Shortly before the *PARK10*-report was published, another group also reported linkage to chromosome 1p32, yet this linkage concerned the age at onset of PD.[95]

The linkage results to the *PARK4*, *PARK8* and *PARK9* loci,[8,22,26] finally, have not been replicated in independent families.

Discussion

Eleven PD genes and loci have been identified since 1997. They provide valuable opportunities to study the genetic and phenotypical heterogeneity of PD, and thus the variety of pathogenic routes and their outcome. Nevertheless, even more questions have emerged. Some genes and loci are associated with Lewy bodies, others are not, but neuropathological data on many PD genes and loci is still unavailable. Two genes are involved in protein metabolism (parkin and UCH-L1), but others probably encode an antioxidant protein (DJ-1), or play a role in the mesencephalic genesis of dopaminergic neurons (NR4A2). Yet all these genes and loci lead to PD. The genes and loci reviewed in this paper at present explain only a minor fraction of PD in the general population. Despite this, they have had immediate implications for genetic counseling in particular families and individuals, as well as for the development of novel therapeutic strategies.

Genetic counseling

With the identification of the five PD genes, interest in genetic counseling and risk prediction in PD is growing. Genetic counseling particularly applies to patients with (familial or sporadic) juvenile to early onset of PD (parkin, DJ-1), and families with mendelian segregation of PD (alpha-synuclein; parkin; UCH-L1; DJ-1; NR4A2). The value of genetic testing in PD is not yet clear, since in most patients it is a clearly disabling, yet non-lethal condition. Furthermore, there is no detailed knowledge about the penetrance of the respective mutations. Outside the scope of genetic counseling are families testing negative for the established PD genes. If, however, linkage to other familial-PD loci (*PARK3;-4;-6;-8;-9;-10*) is present, these families could serve research purposes by reducing candidate-gene intervals and facilitate identification of the responsible gene.

Therapeutical implications

In terms of pharmacological applications, the PD genes mark the transition to a new era. Unlike currently used, mainly palliative, antiparkinson treatment, new neuroprotective and curative strategies may make use of protein targets from the newly uncovered neurodegenerative pathways. In this way, abnormal protein aggregation and excessive oxidative damage may be arrested, and even be reversed or prevented, in an early stage. Equally important in this respect is the identification of possible exogenous, environmental factors, which could initiate or accelerate nigral degeneration. In the future, treatment according to a patient's genetic make-

up could thus be tailored to fit individual genetic susceptibility, environmental-risk profile and drug-metabolism characteristics.

Further research strategies

The large body of genetic evidence in PD is overtaking the environmental hypothesis[9,96] Yet the role for certain environmental factors in the risk of PD, such as pesticides, is still to be clarified.[10] In spite of this revolution in PD genetics, however, the origin of PD in the vast majority of patients is unresolved. A major susceptibility gene for common, sporadic PD, such as apolipoprotein E (APOE) is in Alzheimer's disease,[97] could not be identified. Polymorphisms in various other (groups of) genes were candidate for a role in the pathogenesis of PD, a concise overview of which can be found elsewhere.[98] Inadequate numbers of cases and controls, inconsistencies in diagnostic criteria for PD, ethnic origin of the study population and composition of the control group all contribute to conflicting results across studies. As appeared from a large meta-analysis, only six polymorphisms showed evidence of association with PD overall, and further study is warranted to validate these results.[99] The *PARK10* locus[27,95] could prove to be a susceptibility factor in PD and needs to be validated in other populations to assess the extent of involvement in the common form of PD and its onset age. Similar to this approach, but using unrelated families, are genomic screens on large numbers of affected sib-pairs or on nuclear families segregating PD.[100,101] These studies reported association to, amongst others, loci containing the tau gene, the *PARK3* locus and the *PARK10* locus, respectively.[102,94,95] Some of these concern loci that may control age at onset of PD by an unknown mechanism.[94,95] The association with the tau gene, however, was refuted in another study.[103]

As more genetic pieces of the aetiological jigsaw emerge, the classical definition from 1817 by James Parkinson,[104] typical PD being of unknown aetiology, is gradually losing ground. Should mendelian forms be separated into nosological entities or be regarded as rare genetic, causes of PD ? A reclassification compatible with the 21st century on clinical-genetic grounds is required, as clinicopathological features alone no longer justify all PD to be clustered as one entity.

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CHAPTER 1.2

Scope of this thesis

Parkinson's disease (PD) encompasses several genetic sub-types, but a very small proportion of PD overall can be explained by presently known genes: the vast majority is of unknown cause. This thesis aims to genetically dissect PD using an isolated population. Such communities with small founding populations and minimal inward and outward migration are likely to harbour a more genetically homogeneous variant of PD, and have proven a powerful setting for genetic research. This applies to common, late-onset PD as well as to PD variants with early onset.

Within a decade, great progress has been made in the field of clinical genetics by the identification of five genes and several loci in familial PD, as outlined in **Chapter 1.1**. PD has been known to cluster in families for over a century. Although several PD genes have now been identified in families with mendelian inheritance patterns, the prevalence of PD with complex inheritance is far higher. In the latter, usually late-onset, form of PD, any inheritance pattern is difficult to define and is likely to be determined by multiple low-penetrance genes and environmental factors.

In a genetically isolated population in the Southwest of The Netherlands, we ascertained a kindred with early-onset PD with autosomal recessive inheritance (**Chapter 2**). By means of homozygosity mapping, a new PD locus (*PARK7*) was localised as described in **Chapter 2.1**. We identified the responsible gene, *DJ-1*, which is likely to play a role in the cellular defense against oxidative stress and is the fourth familial-PD gene identified worldwide (**Chapter 2.2**). Apart from typical early-onset parkinsonism, the phenotype in the Dutch patients with *DJ-1* mutations was characterised by additional psychiatric and neurological traits, which are addressed in **Chapter 2.3**. The phenotype observed in this family also encompassed other remarkable physical traits, upon which a clinical-genetic and radiographic analysis was performed (**Chapter 2.4**). Finally, carriers of a DJ-1 mutation, although clinically unaffected, may have preclinical disease visualised by functional neuroimaging. In order to assess cerebral dopamine and glucose metabolism properties, **Chapter 2.5** contains a PET neuroimaging study performed in heterozygotes and homozygotes for the DJ-1 deletion, as well as a non-carrier relative.

Chapter 3 describes genetic studies of sporadic PD, without an apparent familial pattern of inheritance. On a population level, the genetic basis of PD is assessed by genetic-epidemiologic methods. Firstly, **Chapter 3.1** reviews advances and prospects of the genetic epidemiology of neurological disease. The same genetic isolate as in Chapter 2 was the setting for a study on PD patients who were at first sight unrelated. Clinical characteristics of these PD patients, the extent of familial aggregation and results of a candidate-gene study are discussed in **Chapter 3.2**. Results of a genomic screen in the patients described in Chapter 3.2 are outlined in **Chapter 3.3**. The DJ-1 gene (Chapter 2.2) is likely to play a role in defense mechanisms against oxidative stress, which may therefore be a causal factor

in PD. Excessive iron deposition in the body is a condition with increased oxidative stress. Therefore, in **Chapter 3.4**, the role of mutations in the HFE gene, the major gene for the iron-storage disorder haemochromatosis, was studied in two series of patients with common, sporadic PD and, since the phenotype might include atypical clinical features, parkinsonism overall.

Finally, **Chapter 4** concludes this work by a discussion of methodological strategies and findings described in this thesis, and possible implications of our findings for patients and further research.

CHAPTER 2

FAMILIAL PARKINSONISM
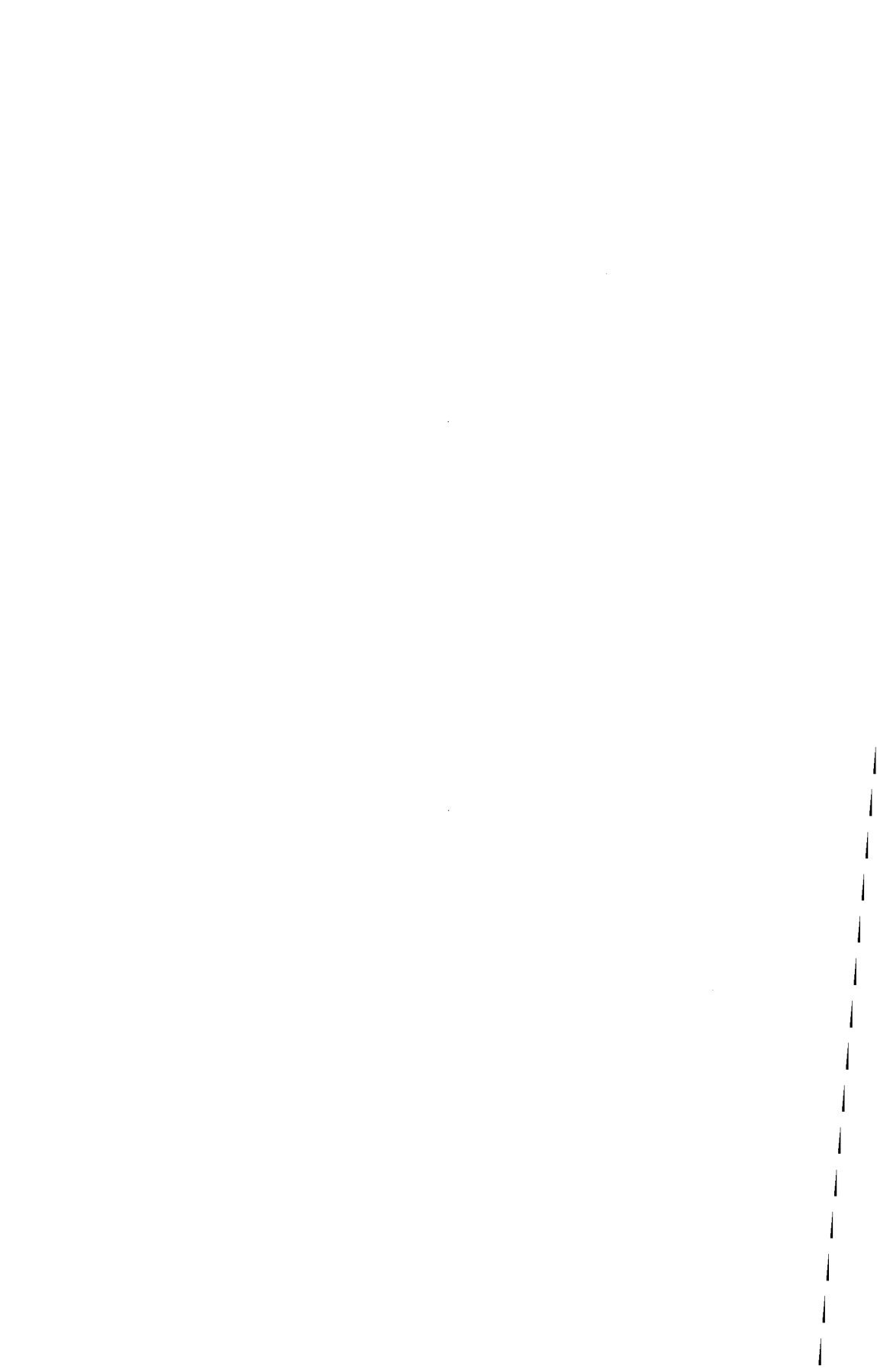
CHAPTER 2.1

PARK7, a novel locus for autosomal recessive
early-onset parkinsonism, on chromosome 1p36

Abstract

Although the role of genetic factors in the origin of Parkinson's disease has long been disputed, several genes involved in autosomal dominant and recessive forms of the disease have been localised. Mutations associated with early-onset autosomal recessive parkinsonism have been identified in the parkin gene, and recently a second gene, *PARK6*, involved in early-onset recessive parkinsonism was localised on chromosome 1p35-36. We identified a family segregating early-onset parkinsonism with multiple consanguinity loops in a genetically isolated population. Homozygosity mapping resulted in significant evidence for linkage on chromosome 1p36. Multipoint linkage analysis using MAPMAKER-HOMOZ generated a maximum LOD-score of 4.3, with nine markers spanning a disease haplotype of 16 cM. On the basis of several recombination events, the region defining the disease haplotype can be clearly separated, by 25 cM, from the more centromeric *PARK6* locus on chromosome 1p35-36. Therefore, we conclude that we have identified on chromosome 1 a second locus, *PARK7*, involved in autosomal recessive, early-onset parkinsonism.

Parkinson's disease (PD [MIM 168600]) is a neurodegenerative disorder characterised by bradykinesia, resting tremor, muscular rigidity, and postural instability. The cerebral pathology includes loss of dopaminergic neurons, in particular at the substantia nigra and cytoplasmatic eosinophilic inclusions (i.e., Lewy bodies). The role of genetic factors in the origin of PD has long been disputed. However, several families segregating the disease as a dominant or recessive trait have been identified. At present, four genes implicated in autosomal dominant forms of parkinsonism have been identified or localised, including the alpha-synuclein gene[1], the ubiquitin carboxy-terminal hydrolase-L1 gene,[2] and two yet-unidentified genes- PARK3 on chromosome 2p13[3] and PARK4 on chromosome 4p14-16.3.[4] Until now, most mutations have been found in the parkin gene[5] and lead to a recessive form of juvenile parkinsonism (ARJP [MIM 600116]). The onset age may vary from 20 to 65 years, but in most patients the onset is <40 years of age.[6] Parkin mutations are estimated to explain up to 50% of familial patients with ARJP.[6] Recently, a new locus, PARK6 (MIM 605909), involved in autosomal recessive early-onset parkinsonism was localised on chromosome 1p35-36 in a single Italian family.[7] In the present report, we describe a family segregating early-onset parkinsonism with multiple consanguinity loops that we identified in a genetically isolated population. We report the findings of a genome search using homozygosity mapping in an effort to localise the gene involved in the disease.

Patients were from a genetically isolated community in the southwestern region of The Netherlands. The study is part of a larger research program named Genetic Research In Isolated Populations (GRIP). The isolated population was founded 1750, by 150 individuals. The scientific protocol of GRIP has been approved by the Medical Ethical Committee of the Erasmus MC Rotterdam. From the population, initially three patients (VII-2, VII-3 and VII-7) diagnosed with early-onset parkinsonism were ascertained, including two affected siblings and one apparently sporadic patient (Figure 1).

The patients and their first-degree relatives were personally examined by the research physician, a neurologist, and a clinical geneticist. Clinical diagnosis of parkinsonism required the presence of at least two of the three cardinal signs (i.e., resting tremor, bradykinesia, and muscular rigidity) and absence of atypical features and of signs of involvement of other neurological systems (pyramidal, cerebellar, or autonomic). The extrapyramidal signs were evaluated by Hoehn-Yahr staging (HY)[8] and the Unified Parkinson's Disease Rating Scale (UPDRS; maximum of motor subscale = 108).[9] Furthermore, structural and functional brain-imaging studies were performed. The clinical examinations of available relatives revealed a fourth affected individual (VII-6), who was not known to have the diagnosis of parkinsonism.

The clinical details of the four affected subjects are reported in Table 1. Onset of symptoms was before age 40 years in three subjects (VII-2, VII-3, and VII-7). The fourth affected individual (VII-6), who was age 40 years at the time of the

Figure 1. Pedigree of family with autosomal recessive early-onset parkinsonism, and haplotypes of marker loci spanning the linked region on chromosome 1p36. *To protect patient confidentiality, a diamond symbol has been used to mask the sex of all family members. Individuals VI-2, VI-3, and VII-1 to VII-7 were examined clinically. Black symbols indicate definitely affected individuals; black bars denote the disease-associated haplotype.*
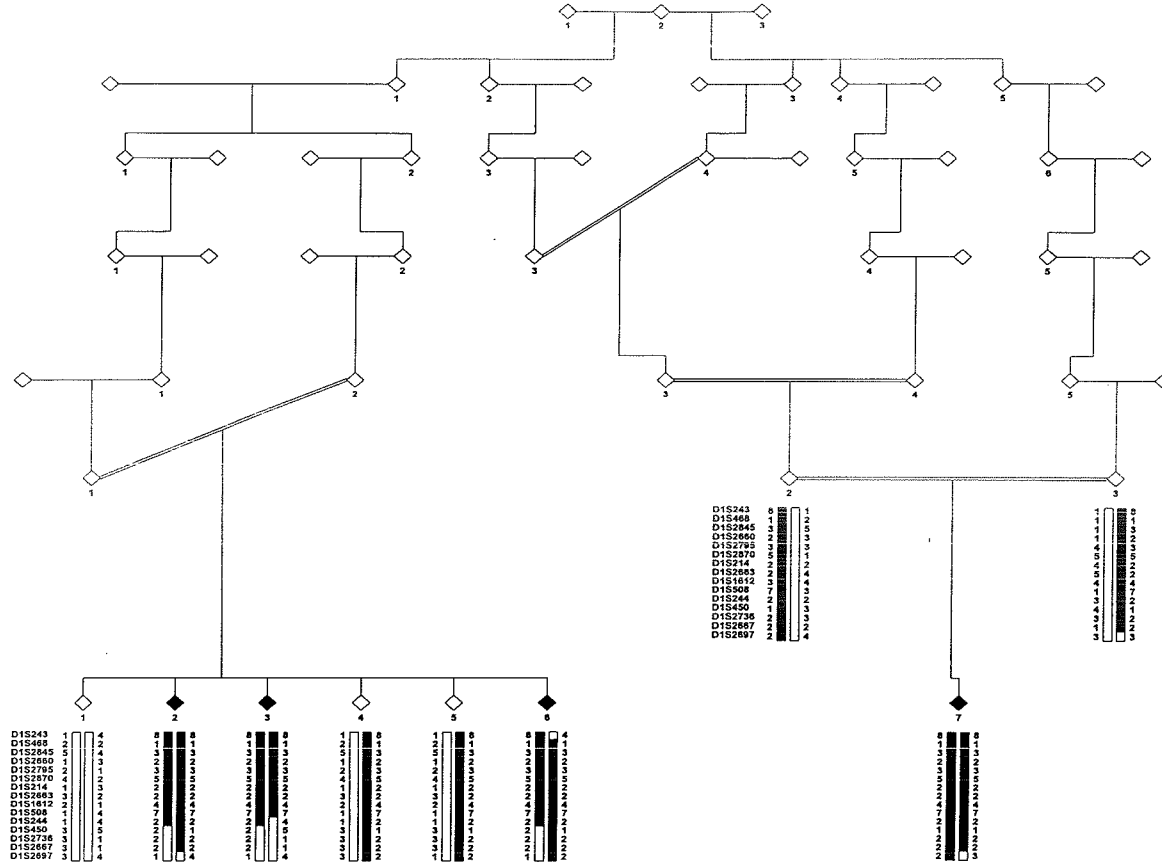
clinical examination, was newly diagnosed with parkinsonism and could not indicate the age at onset of the disease. None of the patients exhibited atypical features or signs of involvement of additional neurological systems. The parkinsonian signs responded well to therapy with L-dopa or dopamine agonists in two patients (VII-3 and VII-7); at the time of clinical examination, the two other patients still had not been treated with antiparkinsonian drugs. One patient (VII-7) showed motor fluctuations of the "wearing-off" type, levodopa-induced dyskinesias in the "on" phase, and "off"-phase dystonia (i.e., laterocollis). Mild blepharospasm was present in patient VII-3, who receives dopamine-agonist therapy. Tendon reflexes in lower limbs were brisk in three of the patients (VII-3, VII-6, and VII-7). Babinski sign was absent. Two of the patients also showed neurotic signs, and one had suffered from psychotic episodes. In all patients, the progression of disease was slow. Structural brain images were unremarkable, in agreement with the diagnosis of idiopathic parkinsonism. Brain single-positron emission computed tomography with dopamine-transporter tracer, performed in patients VII-2 and VII-3, showed severe abnormalities consistent with presynaptic dysfunction of nigrostriatal dopaminergic systems. Autopsy data are not available. At the time of clinical examination, the ages of unaffected individuals VII-1, VII-4 and VII-5 were 54, 50, and 46 years, respectively.

Table 1. Clinical phenotype of four patients with parkinsonism

					Symptom							
Patient	AaE	AaO	RT	PT	B	R	P	AS	Therapy	HY	UPDRS	Other characteristics
VII-2	50	40			X	X	X	X	untreated	II	29	psychotic episodes
VII-3	48	31	X	X	X	X	X	X	pergolide	III	57	neurotic signs; blepharospasm
VII-6	40	n.a.		X	X	X	X		untreated	III	22	neurotic signs
VII-7	38	27		X	X	X	X	X	L-DOPA/ IDD;enta- capone	III	42	wearing-off; dyskinesias; off- dystonia

AaE = age at exam; AaO = age at onset; n.a. = not available; RT = resting tremor; PT = postural tremor; B = bradykinesia; R = muscular rigidity; P = loss of postural reflexes; AS = asymmetric onset of symptoms; IDD = dopa-decarboxylase inhibitor; HY = Hoehn and Yahr score; UPDRS = Unified Parkinson's Disease Rating Scale

The genealogical history of the isolate has been computerised as far back as 1750. Although the patients were not aware of consanguinity in their family, the pedigree of each of the two sibships showed multiple consanguinity loops (Figure 1). The

pedigree is drawn on the basis of the two shortest consanguinity loops for both parent pairs (VI-1/V-2 and VI-2/VI-3), which links the two sibships to a common ancestor six generations ago. However, other connections between the sibships exist, including a second loop linking VI-1 and V-2 to a common ancestor, who is also related to VI-2.

The pedigree structure is consistent with an autosomal recessive mode of inheritance, and the kindred therefore was considered to be suitable for homozygosity mapping.[10] For all patients and available first-degree relatives, genomic DNA was isolated from peripheral blood, according to the method described by Miller et al.[11] For the systematic genome scan, short tandem-repeat polymorphisms (STRPs) from the ABI PRISM Linkage Mapping Set MD-10 (Applied Biosystems) were used. Additional markers for fine-mapping and the PARK6 region were obtained from the Généthon and Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) genetic-marker sets. Marker order and distances were obtained from the Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) integrated linkage map. Markers were amplified from genomic DNA, according to methods specified by the manufacturers. PCR products were pooled and loaded onto an ABI377 automated sequencer (filter set D; 5% denaturing FMC LongRanger acrylamide gel), and data were analysed by ABI GENESCAN3.1 and ABI GENOTYPER2.1 software.

In the initial screens, only three definitely affected individuals (VII-2, VII-3, and VII-7) were genotyped. We first tested for linkage of our family to known PD loci,[1-5] using STRPs from the ABI PRISM Linkage Mapping Set MD-10. At chromosome 4q22, the markers D4S414 and D4S1572 were assessed; at chromosome 4p14, markers D4S419 and D4S405; at chromosome 4p14-16.3, markers D4S419 and D4S1592; at chromosome 6q25.2-q27, markers D6S1581 and D6S264; and, at chromosome 2p13, markers D2S337 and D2S2368. The analysis of the markers flanking the known PD genes did not show evidence for homozygosity, indicating that the disease in the family cannot be explained by one of these known genes (data not shown). We then performed a systematic genome screen. LOD scores were generated by the program MAPMAKER-HOMOZ[12] under the assumptions of equal recombination for males and females, autosomal recessive inheritance, and gene frequency 0.004. In the initial genome screen, the family structure used in the analysis is depicted in Figure 1, and allele frequencies were estimated on the basis of the DNA of 51 spouses of patients participating in the various studies in GRIP. The three individuals included in the initial screen (VII-2, VII-3, and VII-7) were homozygous for six markers, located on chromosomes 1, 5, 11, 17, and 21. Only for the chromosome 1 region, two adjacent markers (D1S468 and D1S214) were found to be homozygous in all patients, leading to a maximum LOD score of 2.8.

The newly diagnosed patient (VII-6) was identified after this initial screen and was typed, together with unaffected first-degree relatives (VI-2, VI-3, VII-1, VII-4,

and VII-5), only for the five regions at which the first three patients were homozygous.

Homozygosity remained only for markers on chromosomes 1, 5, and 17. On chromosomes 5 and 17, homozygosity was found with very-frequent (34% and 42%, respectively) alleles of the markers D5S1981 and D17S944. For closely linked (i.e., <10 cM distant) markers, all four patients were heterozygous, indicating that the observed alleles for these markers could be identical by state (IBS). In contrast, the population frequency of the homozygous allele at D1S468 was 9%. The chances that the homozygous allele in our distantly related patients is IBS are very small. In addition, all four patients were homozygous for the flanking marker D1S214, indicating that the alleles were most likely identical by descent. We therefore saturated the region surrounding D1S468 and D1S214 with 13 additional markers and found that all patients were homozygous for 9 of the markers studied, whereas the unaffected parents and two unaffected siblings were all either heterozygous or non-carriers of the disease haplotype (Figure 1). When LOD scores for the disease haplotype were calculated, equal allele frequencies for each marker were assumed, because population allele frequencies were not available. Penetrance was assumed to be 100% by age 40 years. The most conservative analysis that is, under the assumption that there is a second-degree relationship between the parents of the sibships, resulted in a maximum multipoint LOD score of 4.3. Subsequent haplotype analysis showed homozygosity of all four patients, for a region of 16 cM on the sex-averaged linkage map. The first recombination events were observed for marker D1S243 on the telomeric side in individual VII-6 and for marker D1S244 on the centromeric side in individual VII-3.

Because of Valente et al.'s[7] recent report of linkage of an early-onset autosomal recessive form of parkinsonism to chromosome 1p35-36, we investigated whether we could exclude linkage to the *PARK6* region in the family that we studied; we tested nine markers from the *PARK6* region (Table 2). The patients in this family showed no homozygosity at any of these markers. The *PARK6* critical region, between markers D1S483 and D1S247, is localized 25 cM centromeric of our critical region. Considering the evidence that the critical region in the family that we studied does not overlap with the region encompassing the *PARK6* gene identified by Valente et al.[7] and taking into account the recommendations that Lander and Kruglyak[13] have made with regard to the reporting of linkage findings, we report a significant linkage finding for the presence, on chromosome 1p, of a second locus for autosomal recessive early-onset parkinsonism; and we propose to name it "*PARK7*."

Both *PARK6*[7] and *PARK7* were identified by homozygosity mapping. Although this is a very powerful approach in consanguineous pedigrees,[10] some possible methodological problems recently have been raised in articles published in the American Journal of Human Genetics.[14] Miano et al.[14] have shown that patients may be homozygous for large regions by chance in particular if there are multiple

loops in the pedigree, such as occur in the pedigree that we studied. However, our most conservative analysis, based on an analysis assuming a second-degree relationship, still yielded a LOD score >4. In addition, the fact that the affected individuals from the two sibships share an identical homozygous haplotype, whereas none of the unaffected first-degree relatives was homozygous for this haplotype, makes a false-positive finding less likely.

Table 2. Genotypes of affected individuals, for markers of the PARK6 region

Genotype of affected individual

Marker[a]	VII-2		VII-3		VII-6		VII-7	
D1S199	2	5	3	5	2	5	3	4
D1S483	1	1	1	2	1	1	2	2
D1S478	3	5	1	3	3	5	2	3
D1S2828	4	1	4	5	4	1	4	3
D1S2732	2	4	2	3	2	4	1	4
D1S2702	2	4	2	3	2	4	4	1
D1S2734	5	4	5	5	5	4	4	1
D1S2885	4	1	4	2	4	1	1	3
D1S247	4	2	1	6	4	2	3	5

Source: Valente et al.[7]; [a]Order is according to the Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) integrated linkage map.

Using homozygosity mapping, we have identified a third locus for autosomal recessive early-onset parkinsonism: PARK7. Similar to what has been reported in other studies of early-onset parkinsonism and parkin mutations,[6] the disease in the patients of the family that we studied is characterised by a slow progression. Dystonic features, including blepharospasm and laterocollis, and brisk tendon reflexes have also been found in patients with parkin mutations.[6,15,16] PARK7 is the second locus, in the chromosome 1p36 region, involved in autosomal recessive, early-onset parkinsonism. The region defining the disease haplotype can be clearly separated from that of the more centromeric PARK6 locus. Although the genomic sequence of the critical region is far from complete and many gaps remain, a first analysis of the Project Ensembl and Human Genome Project Working Draft at UCSC databases showed that inside the critical region 25 genes are located, including 7 genes with an unknown function. Possible candidate genes are the gene encoding the vesicle-associated membrane protein 3 (VAMP3) and the gene encoding protein kinase C, zeta (PRKCZ). Telomeric of D1S243, a ubiquitin-conjugating enzyme has been localised. Although this would be an obvious candidate, its location is outside the candidate region. The observation, on chromosome 1p, of two closely linked loci for early-onset parkinsonism raises the

question whether there might have been a duplication of this chromosomal region during evolution; however, comparison of the gene contents from the *PARK6* and *PARK7* critical regions and the syntenic region in the mouse does not support this idea.

Recessive mutations in the parkin gene have been shown to be a common cause of early-onset parkinsonism.[6] Our findings and those of Valente et al.[7] strongly suggest there may be other recessive mutations, in at least two unidentified genes involved in the disease. Analysis of additional families with early-onset parkinsonism lacking parkin mutations will reveal the importance of these two new loci for early-onset parkinsonism.

The frequency of recessive mutations in early-onset parkinsonism may even have masked the genetic origin of PD for a long time, since familial aggregation and the identification of consanguinity require extensive genealogical research. Therefore, it remains to be determined whether these common recessive mutations are involved in late-onset forms of the disease as well. Studies of inbred and/or isolated populations such as GRIP may therefore help to further dissect the pathogenesis of PD.

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Electronic database information

Accession numbers and URLs for data in this chapter are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://research.marshfieldclinic.org/genetics/ (for information on polymorphic markers and localisation of polymorphic markers)

Généthon, http://www.genethon.fr/ (for information on polymorphic markers and localisation of polymorphic markers)

Human Genome Project Working Draft at UCSC, http://genome.cse.ucsc.edu/ (for identification of candidate genes VAMP3 and PRKCZ)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PD [MIM 168600],ARJP [MIM 600116], and PARK6 [MIM 605909])

Project Ensembl, http://www.ensembl.org/ (for identification of candidate genes VAMP3 and PRKCZ)

CHAPTER 2.2

Mutations in the DJ-1 gene associated with
autosomal recessive early-onset parkinsonism

Abstract

The DJ-1 gene encodes a ubiquitous, highly conserved protein. Here, we show that DJ-1 mutations are associated with *PARK7*, a monogenic form of human parkinsonism. The function of the DJ-1 protein remains unknown, but evidence suggests its involvement in the oxidative stress response. Our findings indicate that loss of DJ-1 function leads to neurodegeneration. Elucidating the physiological role of DJ-1 protein may promote understanding of the mecghanisms of brain neuronal maintenance and pathogenesis of Parkinson's disease.

The causes of Parkinson's disease (PD), a common neurodegenerative disorder, remain largely unknown but genes identified for rare mendelian forms have increased our understanding of disease pathogenesis.[1,2] By homozygosity mapping in two consanguineous families from genetically isolated communities in The Netherlands and Italy, we localised a gene for autosomal recessive early-onset parkinsonism, *PARK7*, to a 20-cM interval on chromosome 1p36 (*Appendix*). [3,4]

Fine mapping by typing available and newly developed short tandem repeat (STR) or single nucleotide polymorphism (SNP) markers, reduced the critical region in the Dutch family to a 5.6-Mb region of homozygous sequence, spanning six contigs and five gaps in NCBI build 29 of the human genome[5] and containing as many as 90 genes (Figure 1).[6] We sequenced obvious candidate genes from genomic DNA, but no mutations were detected in the two families (*Appendix*).

We then performed a systematic screening of the transcripts in the region, using reverse-transcriptase-polymerase chain reaction (RT-PCR) material obtained from lymphoblastoid cell lines of one patient in each family and a normal control. One of the cDNAs analysed, corresponding to the whole DJ-1 gene open reading frame (ORF), could not be amplified in the Dutch patient (Figure A1 *Appendix*). Amplification of DJ-1 exons 1[A/B] to 5 from genomic DNA yielded no products in the same patient, whereas the two more centromeric exons 6 and 7 were normally amplified. This confirmed the presence of a homozygous genomic deletion and placed the centromeric border of the deletion in intron 5 of the DJ-1 gene. From the telomeric side, we could amplify exons of the neighboring gene TNFRSF9, as well as sequences from the intergenic region, delineating DJ-1 as the only gene involved in the deletion.

Using a PCR-based strategy, we cloned the deletion breakpoint (*Appendix*). A novel fragment of about 2000 base pairs (bp) was amplified only from the individuals carrying the disease allele. Sequencing this fragment confirmed the joining of the telomeric and centromeric borders, delineating a deletion of 14,082 bp, which includes 4 kb of sequence upstream of the DJ-1 gene ORF start. Interestingly, ALU elements flank the deleted sequence on both sides, and the breakpoint occurs within 16 bp of sequence identical in the two ALUs, suggesting that unequal crossing-over was likely at the origin of this genomic rearrangement (Figure A1 *Appendix*).

The deletion showed complete cosegregation with the disease allele in the Dutch family (Figure A1 *Appendix*), whereas it was absent in 380 chromosomes from the general Dutch population, which supports the position that it is a pathogenic mutation. We also investigated the frequency of the DJ-1 deletion within the genetically isolated population where the Dutch *PARK7* family was identified.[3] Among 440 independent chromosomes from the genetic isolate we found four heterozygous and no homozygous carriers of the deletion. Moreover, the deletion was absent in 400 chromosomes from the regions closely surrounding the genetic isolate. Together with our results from the general Dutch population, these findings

indicate that, although the mutant allele is present at 0.9% within the isolated population, it is likely confined to this population. None of 42 late-onset PD patients from the isolated community carried the deletion, indicating that they have a different aetiology.

Figure 1. Genetic and physical maps of the PARK7 region
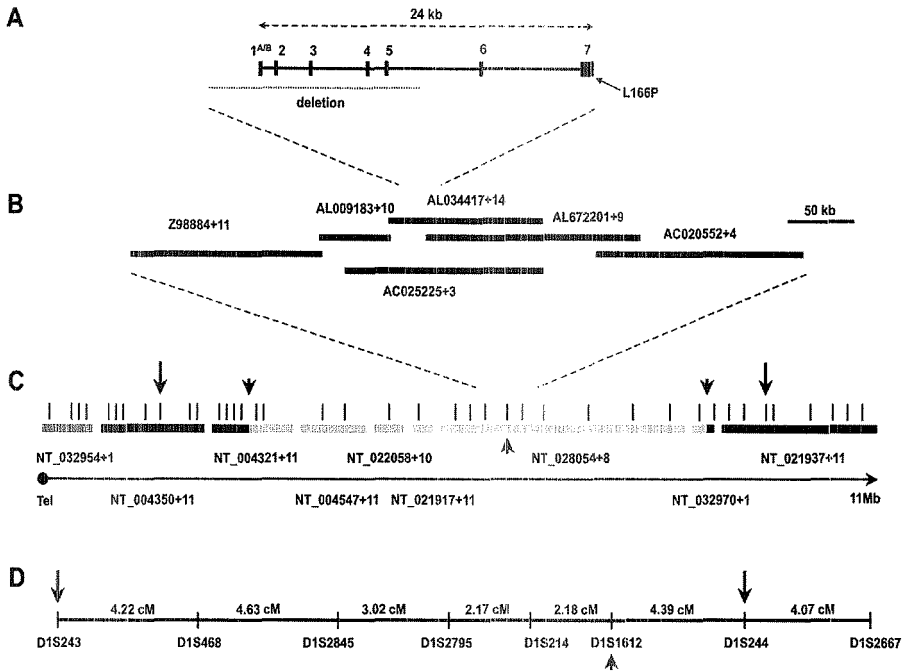
a) Genomic organisation of the DJ-1 gene. The location of the eight exons and the mutations found in the Dutch and Italian families are shown. b) BAC/PAC clone coverage of a 500-kb region spanning the DJ-1 gene. Grey and black bars indicate finished and unfinished clones, respectively. c) Physical map of the PARK7 region. Vertical bars above contigs indicate the location of the polymorphic markers typed in this study. The central contigs depicted in bright grey contain fully informative, homozygous markers in the Dutch family patients. The flanking regions depicted in dark grey contain heterozygous markers in at least one patient. The most telomeric contig, depicted in middle grey contains homozygous, non-informative markers. The contig NT 022058 contains informative homozygous markers and is therefore placed within the PARK7 critical region. Its position in the NBCI assembly (build 29) is more telomeric. The remaining contigs are ordered as in the NCBI assembly. Heterozygous markers placed in contig NT 004321 telomerically and NT 032970 centromerically, flank 5.6 Mb of homozygous sequence and define the new PARK7 critical interval (outer arrowheads). d) Genetic map; the intermarker genetic distance is given in centiMorgans, according to the Marshfield integrated sex average linkage map. Markers flanking the PARK7 locus in previous mapping studies are

indicated with the arrows. The marker D1S1612, located 80 kb centromeric to the DJ-1 gene, is indicated by the centrally placed arrowhead (c and d).

We then sequenced the DJ-1 cDNA from the Italian patient and identified a homozygous point mutation (T→C transition at position 497 from the ORF start in cDNA), resulting in the substitution of a highly conserved leucine at position 166 (Leu[166]) of the DJ-1 protein by a proline (Figure 2b). This change showed complete cosegregation with the disease allele in the Italian family (Figure A1 *Appendix*) and was absent from 320 chromosomes from the general Italian population. No further changes segregating with the disease in the Italian family and absent in the general population were detected.

Although *PARK6* and *PARK7* seem to be separate, close loci (*Appendix*), it will be interesting to search the published *PARK6* family for mutations in DJ-1.[7] We have tested a limited number of early-onset familial and sporadic PD patients that did not have mutations in the *parkin* gene; we did not find a mutation in DJ-1 (*Appendix*).

The DJ-1 gene contains 8 exons distributed over 24 kb (Figure 1a). The first two exons (1[A] and 1[B]) are noncoding and alternatively spliced in the DJ-1 mRNA.[5] One major transcript of about 1 kb contains a 570-bp ORF, encoding a protein of 189 amino acids, that is ubiquitously expressed in body tissues and brain areas, including those more affected in PD (Figure 2a). DJ-1 belongs to the ThiJ/PfpI family (pfam01965), which includes ThiJ, a protein involved in thiamine biosynthesis in prokaryotes; PfpI and other bacterial proteases; araC and other bacterial transcription factors; and the glutamine amidotransferases family (including bacterial catalases) (Figure 2d).

To investigate the structural consequences of the Leu[166]Pro mutation, a molecular model of DJ-1 was built using the programs WHAT IF[8] and YASARA.[9] With 23% sequence identity to the known structure of protease PH1704 from *Pyrococcus horikoshii*[10] and an almost gapless alignment spanning 170 residues, DJ-1 can safely be assumed[11] to adopt the same α/β sandwich structure as PH1704 (Figure 2c). According to the model, Leu[166] is placed right in the middle of the carboxyl-terminal helix. Proline is a strong helix breaker[12] and its presence in the DJ-1 mutant is therefore likely to destabilise the terminal helix. The PH1704 protease was found to form a hexameric ring structure made of a trimer of dimers.[10] The general pattern of salt bridges and hydrophobic packing in the trimerization region of PH1704 is conserved in the DJ-1 model, which makes it likely that DJ-1 also forms higher aggregates.

To explore the functional consequences of the DJ-1 mutation, we transfected wild-type and mutant DJ-1 in cell cultures (Figure 2, e to j). In COS and PC12 cells transfected with wild-type DJ-1 we observed diffuse cytoplasmic and nuclear DJ-1 immunoreactivity similar to findings from previous studies.[13,14] Transfection of the

DJ-1 carrying the Leu[166]Pro mutation showed a similar uniform nuclear staining, whereas the cytoplasmic staining appeared mostly colocalised with mitochondria.

Taken as a whole, our findings indicate that the DJ-1 protein is lacking in the Dutch family and is functionally inactive in the Italian family because of the Leu[166]Pro change. The mutant DJ-1 (Leu[166]Pro) is still present in the nucleus, which suggests that the loss of cytoplasmic activities is pathogenic, or that the nuclear activity is affected by the mutation even if the protein retains its translocation capability.

Figure 2. DJ-1 protein analysis, and transfection experiments
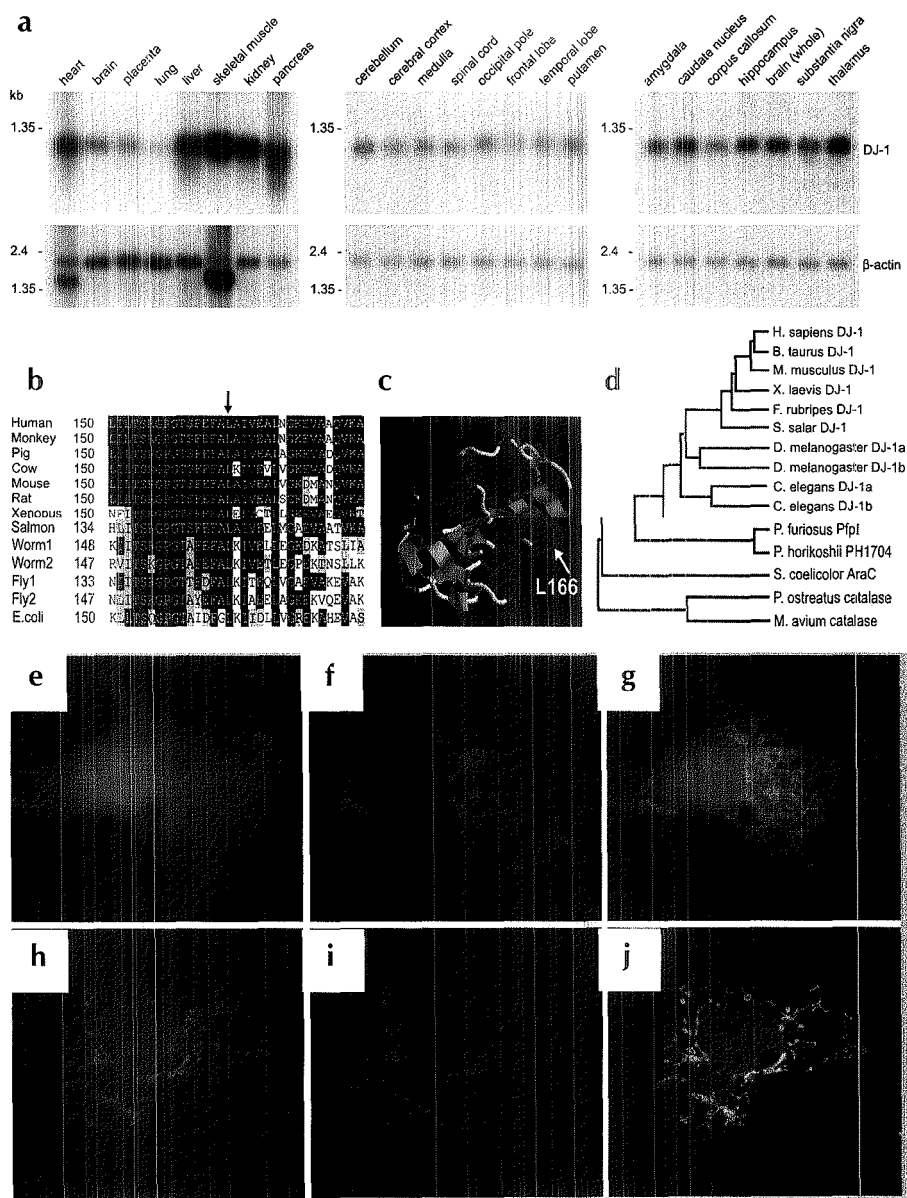
a) Northern blot – different human tissues and brain regions. Upper panels: DJ-1 probe. Lower panels: control probe (β-actin). DJ-1 expression is higher in subcortical regions (caudate nucleus, thalamus, s.nigra) than in cortical regions. b) Alignment of DJ-1 homologs showing the conservation of the amino acid mutated in the Italian family (Leu¹⁶⁶). c) Molecular model of DJ-1. The ribbons correspond to α-helix and β-sheet structures. Indicated is the position of the residue (Leu¹⁶⁶) mutated in the Italian family. d) Phylogenetic tree of DJ-1 and other ThiJ/PfpI family proteins. The length of the connecting lines reflects evolutionary distance between family members. e to j) COS cells transfected with constructs expressing wild -type (e to g) or Leu¹⁶⁶Pro mutant (h to j) v5-His-tagged DJ-1 protein. Immunostaining: v5-His tag [bright signal (e and h)]; HSP60, a mitochondrial marker [bright signal (f and g)]; v5-His tag and HSP60 merged (g and j).

The function of DJ-1 is unknown. Human DJ-1 was first identified as an oncogene[13] and later as a regulatory subunit of an RNA-binding protein (RBP).[14] Moreover, DJ-1 binds to PIAS proteins,[15] a family of SUMO-1 ligases that modulate the activity of various transcription factors.[16] DJ-1 itself is sumoylated at lysine 130.[15] Interestingly, in human and murine cell lines, DJ-1 has been identified as a hydroperoxide-responsive protein that is converted into a variant with a more acidic pI in response to oxidative stimuli like H2O2 or the herbicide Paraquat, which suggests a function as an antioxidant protein.[17,18] The transcription of YDR533C, a yeast DJ-1 homolog, is induced together with genes involved in the oxidative stress response.[19]

Oxidative damage has been implicated in the mechanisms of neuronal death and the pathogenesis of PD.[20] Normal dopamine metabolism produces reactive oxygen species, making nigral neurons particularly sensitive to oxidative stress, and signs of oxidative stress are found in postmortem studies of PD brains.[2,20] Emerging evidence also links oxidative stress to mutations in *alpha-synuclein* and *parkin*, two PD-related genes.[21,22]

It is possible that DJ-1 participates in the oxidative stress response by directly buffering cytosolic redox changes, and/or by modulating gene expression at transcriptional and post-transcriptional levels (Figure A2 *Appendix*), interacting with RBP complexes and transcriptional cofactors such as PIAS or other, unknown factors.

Our discovery of DJ-1 mutations in *PARK7* opens new avenues for understanding the neuronal function of DJ-1, that, when lost, causes neurodegeneration. Furthermore, the observation that DJ-1 may be involved in the oxidative stress response links a genetic defect in this pathway to the development of parkinsonism, with possible implications for understanding the pathogenesis of the common forms of PD.

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CHAPTER 2.2

APPENDIX

Materials and methods

Marker development and genotyping

Sequence data from selected BAC or PAC clones were downloaded from GenBank.[1] Short Tandem Repeat (STR) sequences were selected by repeat length and typed using the program FileProcessor.[2] Single Nucleotide Polymorphisms (SNPs) were downloaded from NCBI[3] or they were generated through use of the Celera Discovery System and Celera's associated databases. STR markers were typed by radioactive polymerase chain reaction, essentially according to Weber and May.[4] SNP markers were typed using a touch-down PCR protocol and digestion with restriction endonucleases. Heterozygosity of markers was determined using a panel of independent chromosomes from the general population. The complete lists of typed STRs and SNPs are available on request.

Genetic analyses

Genomic DNA was isolated from peripheral blood using standard protocols.

For mutation analysis of the *DJ-1* gene, PCR reactions were performed in 25 or 50 µl containing 1x GibcoBRL PCR buffer, 1.5mM $MgCl_2$, 0.01% W-1, 250µM of each dNTP, 0.4µM forward primer, 0.4µM reverse primer, 2.5 units of Taq DNA polymerase (GibcoBRL) and 50 ng genomic DNA/25 µl of PCR volume. For exon 2, the concentration of each primer was 2µM. For exon 1, DMSO 7% was used. Cycle conditions were: 5 min at 94°C; 35 cycles of 30 s denaturation at 94°C, 30 s annealing at temperatures reported below, and 90 s extension at 72°C; final extension 5 min at 72°C. Primers, annealing temperatures, and size of amplified fragments were as follows:

Exon	Fwd Primer 5'→3'	Rev Primer 5'→3'	annealing T	size (bp)
1[A/B]	GAGGTAGACTCG GCCGGAC	TTCTGGACGCTTC AGCGTTG	70°→60°(1°/cycle), then 60°	405
2	TAGGAAGTACTTA CTCTGCTTG	TATTTATTCTTATG TCATCTCTG	66°→58°(1°/cycle), then 58°	359
3	CAGCTGTGTAAAC GTTACTC	ATTCTGTATCAAG CAATTGCC	65°→56°(1°/cycle), then 56°	546
4	CTATCTCCTGTACT TCCCAC	ACAGAACATAAGC AGATGCTG	66°→58°(1°/cycle), then 58°	254
5	TGAGAAATGCCTT GCTTGGG	GCTATTTGGAATC AAACCATCG	70°→60°(1°/cycle), then 60°	354
6	TTTGCCAGATGTG CTCAGCAAATCG	ACTGCACTCCAGC CTGGGCGATGG	68°,total:35 cycles	495
7	CACATAGCCCATT AGGATGTC	AGCTGCAAATGAA GGTGATAC	66°→58°(1°/cycle), then 58°	504

Direct sequencing of both strands was performed using Big Dye Terminator ver. 3.0 chemistry (Applied Biosystems). Fragments were loaded on an ABI3100 automated

sequencer and analysed with DNA Sequencing Analysis (ver 3.7) and SeqScape (ver 1.1) packages (Applied Biosystems).

Figure A1. Mutation analyses
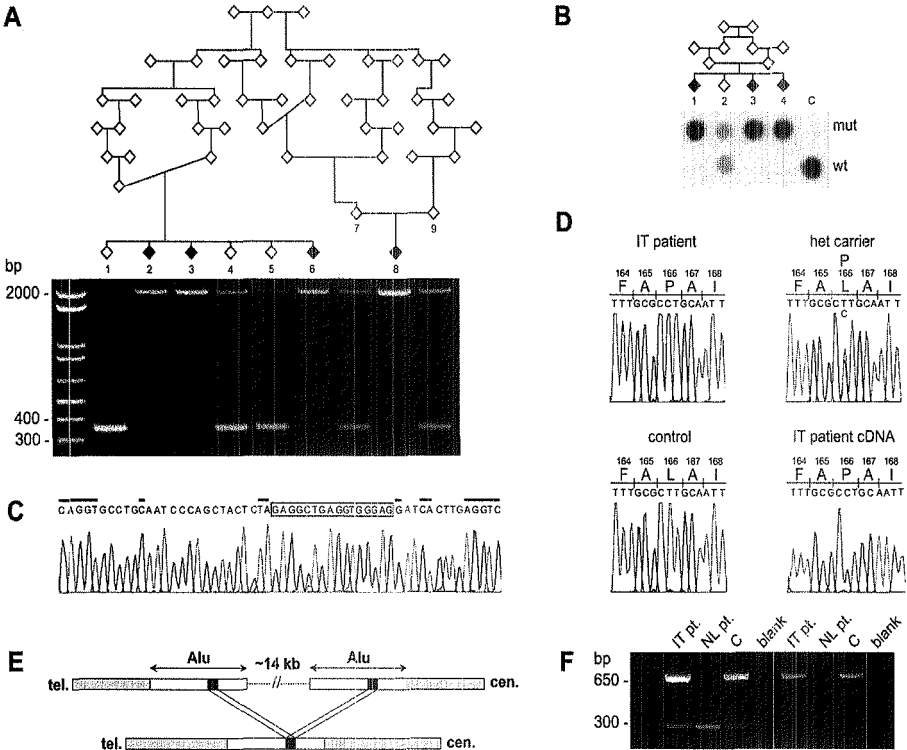
a) Cosegregation of the genomic deletion in the Dutch family. Duplex PCR from genomic DNA using primers designed to amplify the deletion breakpoint (2011 bp band) and one of the deleted DJ-1 exons (exon 5 - 354 bp band). Exon 5 is absent from all patients (homozygous deletion, lanes 2,3,6,8); the deletion breakpoint band is present in homozygous (lanes 2,3,6,8) and heterozygous (lanes 4,5,7,9) carriers of the mutant allele (note the dosage effect). Individual 1 is not carrying the disease haplotype, in agreement with data[11].b). Cosegregation of the L166P mutation in the Italian family. Allele specific oligo hybridisation: mut - mutant; wt - wild-type; C - unrelated normal control. c). Sequence of the deletion breakpoint in the Dutch family showing the joining of the telomeric and centromeric borders. The breakpoint lies within a 16 bp of sequence identical in the Alus and is depicted in a frame. The specific nucleotides in telomeric and centromeric sequences are indicated by upper lines. d). Electropherograms showing the L166P mutation in the Italian family. Sequencing from genomic DNA and cDNA. e). Scheme of the genomic rearrangement in the Dutch family. f). RT-PCR amplification of a 741 bp fragment

corresponding to the DJ-1 cDNA indicates absence of expression in the Dutch patient, and presence of expression in the Italian patient. To exclude the possibility of a polymorphism in one of the primer binding sites this was replicated using six different primer combinations (data not shown). A 285 bp fragment of the KIAA0833 cDNA is amplified as internal PCR control in the left lanes.

Analysis of the genomic deletion found in the Dutch family.
For sequencing purposes, a 1761 bp DNA fragment containing the deletion breakpoint was amplified using 2µM forward 5'-GCAAATAGCCAAAAGTGAAGTC-3' and 2µM reverse 5'-CGTACAAGACCTGCCAGGG-3' primers. PCR conditions were as above, with annealing temperatures of 70°→60° (-1°/cycle), then 60°, total 35 cycles.

For *testing the cosegregation* in the Dutch family and the occurrence in population controls, a 2011 bp fragment containing the deletion breakpoint was amplified using 1µM forward 5'-GCAAATAGCCAAAAGTGAAGTC-3' and 1µM reverse 5'-TACTTTCAGCAGAATCATAAGG-3' primers; a 354 bp fragment containing exon 5 of *DJ-1* gene was also amplified in the same PCR reaction using 0.2µM forward 5'-TGAGAAATGCCTTGCTTGGG-3' and 0.2µM reverse 5'-GCTATTTGGAATCAAACCATCG-3' primers. PCR conditions were as above, with annealing temperatures of 70°→60° (-1°/cycle), then 60°, total 35 cycles. Fragments were resolved using agarose 1.5% gel electrophoresis.

For *RT-PCR analyses*, lymphoblastoid cell lines were established from one patient of each PARK7-linked family and one unrelated normal control. Total mRNA was isolated, and cDNA was prepared according to standard protocols. The following primers (0.4µM each) were used to amplify the *DJ-1* cDNA: forward-I 5'-GGGTGCAGGCTTGTAAACAT-3', reverse-I 5'-TGACTTCCATACTTCCGCAAA-3', forward-II 5'-CGCGTCCGCAGGAAGAGG-3', reverse-II 5'-TAGACACAATTTGTAGGCTGAG-3', forward-III 5'-GCGTGCTGGCGTGCGTTC-3'. The primers were used in the following combinations: forward-I/reverse-I (product size 741 bp); forward-II/reverse-I (763 bp); forward-III/reverse-I (770 bp); forward-I/reverse-II (780 bp); forward-II/reverse-II (802 bp); forward-III/reverse-II (809 bp). In some experiments, a 285 bp fragment of the *KIAA833* cDNA was amplified as internal PCR control, using 0.4µM forward 5'-AGCTGCCGTCGTGCTGTAC-3' and 0.4µM reverse 5'-GGGTTGGTGCTTGCAATAAC-3' primers. PCR conditions were as above, with annealing temperatures of 66°→58° (-1°/cycle), then 58°, total 35 cycles.

For *allele specific oligo (ASO) hybridization*, PCR products containing *DJ-1* exon 7 were blotted into Hybond-N+ membranes (Amersham Biosciences). The blots were hybridised for 1 hour at 37°C in 5X SSPE, 1% SDS and 0.05 mg/ml single strand salmon sperm DNA with either the normal or mutated sequence oligo. Filters were washed until a final stringency of 0.3 x SSC/0.1% SDS at 37°C. The

following oligos were used for hybridisation: wild-type allele TTTGCGC̲TTGCAATT, mutated allele (L166P) TTTGCGCC̲TGCAATT.

Northern blot analysis was performed according to manufacturers protocols (Clontech).

GenBank accession numbers
DJ-1 genomic sequence: AL034417.
DJ-1 transcripts: AB073864, AF021819, BC008188, D61380.
DJ-1 protein: CAB52550, BAB71782, AAC12806, AAH08188, BAA09603.

Bioinformatics of DJ-1 protein
Multiple sequence alignments were produced with ClustalW[5] at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw). The phylogenetic tree of DJ-1 protein family was generated with TreeTop at GeneBee.[6]

Molecular modeling of DJ-1 protein
Initial alignments for DJ-1 were obtained from the 3D-PSSM fold recognition server[7] which identified PDB entry 1G2I as the closest homolog of known structure. The alignment and also the model does not cover the N-terminal three and C-terminal 16 residues of DJ-1 protein. Side chains were built using WHAT IF.[8] The model was energy minimized with the YASARA NOVA force field which was shown to improve models.[9] Validation of the model with WHAT_CHECK[10] showed a Ramachandran Z-score of -2.46 and all other quality Z-scores about one standard deviation below the average high resolution X-ray structure, which is good for a model based on only 23% sequence identity.

DJ-1 expression vectors
Wild-type and mutant (L166P) *DJ-1* cDNAs were cloned in pcDNA3.1/V5-His TOPO TA vector (Invitrogen) and checked by sequencing using the Big Dye Terminator ver. 3.0 chemistry (Applied Biosystems).

Cell culture and transfection
COS cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100□g/ml streptomycin and were kept at 37°C in 5% CO_2 (all from GIBCO BRL). PC12 cells were grown in DMEM supplemented with 10% horse serum (Sigma) and 5% fetal calf serum, 100 units/ml penicillin and 100μg/ml streptomycin and kept at 37°C in 10% CO_2. COS and PC12 cells were transiently transfected with the constructs mentioned above using the Lipofectamine + Reagent (Gibco) according to the manufacturer's recommendations. Transfected cells were fixed for immunofluorescence staining 24 and 48 h after transfection.

Immunofluorescence staining
Cells were rinsed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde (10 min), permeabilized with methanol (15 min), blocked with PBS/5% bovine serum albumin /0.15% glycine (15 min) and incubated for 1h with monoclonal mouse anti-V5 antibody (Invitrogen), dilution 1:500. FITC-conjugated goat anti-mouse immunoglobulin G (1:500 1h) was used as secondary antibody (Sigma). Cells were mounted in Vectashield media (Vector) containing DAPI and examined with a Zeiss Axioplan 2 microscope equipped with digital camera. Mitochondrial staining was performed by using rhodamine-conjugated MitoTrack (1:2000) or by double-labeling immunofluorescence with rabbit anti-HSP60 antibody (1:200, 1h Stressgen) and TRITC-conjugated goat anti-rabbit immunoglobulin (1:500, 1h).

Figure A2. Model of putative DJ-1 function
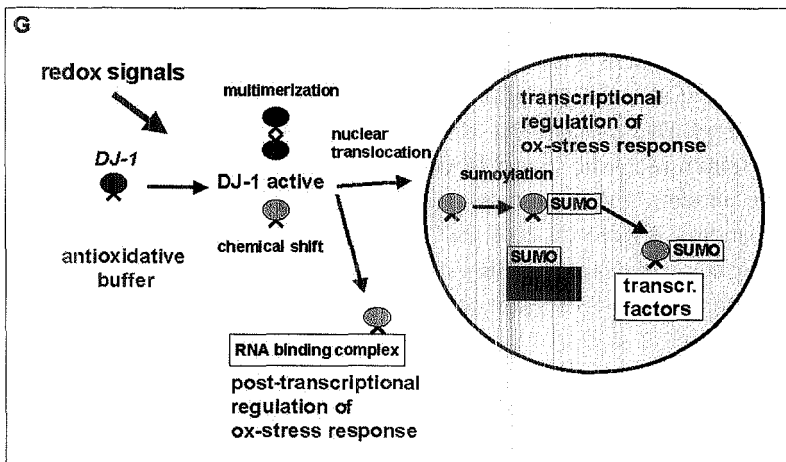
Modification of the DJ-1 protein (by chemical or multimerization changes) may directly buffer cytosolic redox fluctuations; activated DJ-1 may influence expression of genes for oxidative stress response post-transcriptionally (binding to RBP complexes); DJ-1 may also translocate to the nucleus, be sumoylated, and influence gene transcription.

Clinical studies

The Dutch and Italian *PARK7*-linked families have been described previously.[11,12] The diagnosis of parkinsonism required the presence of two of three cardinal signs (resting tremor, bradykinesia, muscular rigidity), absence of atypical features (dementia early in disease course, ophtalmoplegia, pyramidal or cerebellar involvement), and no other identifiable causes. Brain imaging with computed tomography and/or magnetic resonance was unremarkable. Response to levodopa

or dopamine agonist therapy was present in all patients, except two who were untreated at the time of study. Autopsy data are not available. Functional brain imaging studies performed in patients of the Dutch family using dopamine-transporter tracers and SPECT or PET showed severe abnormalities, indicating presynaptic dysfunction of the nigrostriatal dopamine system, the pattern typically observed in Parkinson's disease (PD).[13]

In comparison with classical, late-onset PD, the clinical phenotype in the Dutch and Italian *PARK7*-linked families is characterized by an early onset (in the thirties) and a slow disease progression. Furthermore, behavioral and psychic disturbances, and dystonic features (including blepharospasm) have been reported in both families.[11,12] However, more detailed genotype-phenotype correlation studies are needed to accurately define the clinical spectrum associated to mutations in *DJ-1* gene.

Fine-mapping studies

The *PARK7* locus as delineated by initial linkage studies[11,12] spans more than 8 Mb in the current physical map of the Human Genome (NCBI, build 29), including 9 contigs separated by 8 gaps, containing a large number of genes.

In the last four NCBI builds of the human genome, released during the course of this study, the order of several contigs inside and in the proximity of the *PARK7* region has fluctuated considerably, and for some contigs the internal assembly remains provisional. In order to overcome these difficulties, we have used extensive homozygosity mapping by typing available STR or SNP markers in the two definitely linked families to characterize each of the contigs located inside and flanking the *PARK7* genetic borders. For contigs where STRs or SNPs were not available or not informative we developed new polymorphic STR markers by scanning clones of interest for repetitive sequences.

Analysis of candidate genes

The refinement of *PARK7* critical interval allowed us to exclude many genes located in contigs showing heterozygous markers, including obvious candidates: *UBE4B* and *Ubc6p*, encoding enzymes involved in ubiquitination; *ISG15*, encoding an ubiquitin-like protein; *FLJ10782 (PANK4)*, encoding a homolog of the gene mutated in pantothenate kinase-associated neurodegeneration, an autosomal recessive neurodegenerative disease, and *FRAP1*, encoding an immunophilin ligand. Obvious candidate genes located in contigs showing homozygous markers were analysed by direct sequencing from genomic DNA (primers and PCR conditions are available on request).

We detected no mutations in the coding region of the following genes: *WDR8*, encoding a protein containing WD40 repeats, a domain also found in ubiquitin ligase protein complexes; *VAMP3*, encoding a vesicle-associated protein, possibly involved in docking or fusion of synaptic vesicles; *FLJ10737*, encoding a

protein with a DnaJ domain, also present in molecular chaperones; *KCNAB2*, encoding a subunit of the potassium voltage-gated channel, shaker-related subfamily.

Strategy for cloning the deletion breakpoint

In order to identify the deletion breakpoint, we designed primer sets to amplify fragments of DNA evenly distributed between the minimal deleted area and the two flanking areas of non-deleted sequence. Iteration of this technique reduced the interval between the deleted region and the flanking, non-deleted borders to less than 3 kb. We then used primers placed on the non-deleted borders to amplify a fragment of about 2000 bp across the deletion breakpoint. In the normal chromosomes, these two primers are separated by about 16 kb.

Replacing the reverse primer with another primer located 250 bases upstream yielded a novel fragment of about 1750 bp in the same individuals, confirming the specificity of the reaction and the presence of the deletion (data not shown). Cloning of the deletion breakpoint allowed us to test for the presence of the mutation in both heterozygous or homozygous state using PCR.

Relationships between *PARK6* and *PARK7*

A different locus for autosomal recessive early onset parkinsonism (*PARK6*) has been localised 25 cM centromeric of PARK7 on chromosome 1p35-p36, in a single large pedigree.[14] Given the similarities between *PARK6*- and *PARK7*-associated phenotypes, and the proximity of the two loci, it is possible that mutations in similar or functionally-related genes underlie both forms. As an alternative possibility, a large genomic rearrangement could account for both linkage reports. Linkage analysis of autosomal recessive parkinsonism is complicated by the small size of most recessive pedigrees, presence of further locus heterogeneity, proximity of the two loci on chr.1p. As a result, the assigment of additional smaller families to either *PARK6* or *PARK7* must be considered as provisional.[12,15]

Mutation analyses of *DJ-1* gene in smaller families and isolated early onset cases

Nine additional families with a diagnosis of clinically definite idiopathic parkinsonism in at least two siblings, possible autosomal recessive inheritance (affected sibs, unaffected parents), disease onset before age 50 in at least one sibling, exclusion of linkage to the *PARK2* locus, or *parkin* gene mutations excluded by sequencing and exon dosage, were available for analysis.

By haplotype analysis, four of these families were compatible with linkage to *PARK7*; three families to both *PARK7* and *PARK6*; and two families to *PARK6* only.[16] None of the above-mentioned families could be definitely linked to either of the two loci. Furthermore, 22 patients with idiopathic parkinsonism, negative family history and onset below age 40 were also studied. In nine cases mutations in *parkin* gene had been excluded by genomic sequencing and exon dosage.

Sequencing the exons and exon-intron boundaries of the *DJ-1* gene in the patients and families described above revealed no pathogenic mutations. Among the same patients and families, there were no carriers of the *DJ-1* deletion identified in the Dutch family. However, detection of compound heterozygosity for different genomic rearrangements requires gene dosage analysis, which is currently being developed and therefore will be the subject of future studies.

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CHAPTER 2.3

Clinical features and neuroimaging in *PARK7*-linked parkinsonism

Abstract

We recently reported linkage to chromosome 1p36 (the *PARK7* locus) in a family with early-onset parkinsonism. Linkage to this locus has since been confirmed in an independent data set. We here describe clinical and neuroimaging features of the four patients in the original *PARK7*-linked kindred. Ages at onset of parkinsonism varied from 27 to 40 years. Clinical progression was slow, and response to dopaminergic therapy good. The clinical spectrum ranged from mild hypokinesia and rigidity, to severe parkinsonism with levodopa-induced dyskinesias and motor fluctuation. Three of four patients with *PARK7*-linked parkinsonism exhibited psychiatric disturbances. Structural neuroimaging was unremarkable, but functional imaging of the brain, performed in three patients, showed significant evidence for a presynaptic dopamine deficit, and assessment of cerebral glucose metabolism, as performed in one patient, showed possible cerebellar involvement.

Introduction

The cause of Parkinson's disease (PD), one of the most common neurodegenerative diseases in the elderly, is largely unknown. In recent years, however, attempts to dissect the genetic background of familial, mostly early-onset, PD have been successful.[1] In PD with an autosomal dominant pattern of inheritance, five genes and/or loci have been identified.[2-6] For autosomal recessive early-onset PD, so far one gene and two loci have been reported. The *parkin* gene (chromosome 6q25.2-27)[7] encodes a ubiquitin-protein ligase and accounts for a large part of early-onset PD.[8] The range of ages at onset in patients with mutations in *parkin* is very wide, and the clinical picture varies from parkinsonism to levodopa-responsive dystonia, with varying severity. Response to levodopa therapy is good and progression of disease is slow. After administration of levodopa, motor fluctuations and dyskinesias frequently occur.[8] *PARK6* (chromosome 1p35-p36) is a second locus for autosomal-recessive parkinsonism, and is associated with sustained response to levodopa and slow progression of symptoms.[9] In *PARK6*-linked parkinsonism, symptoms often reported in patients with early-onset autosomal recessive parkinsonism, such as early dystonia and sleep benefit, were not observed.[10]

We recently mapped a third locus (*PARK7*) in a consanguineous pedigree with early-onset autosomal recessive parkinsonism.[11] The *PARK7* critical-region lies on chromosome 1p36, and is clearly separated from *PARK6* by 25 cM. Since, linkage to *PARK7* has been confirmed in an independent set of families.[12] In this paper, the clinical and neuroimaging features of the original *PARK7*-linked family are described and compared to other forms of autosomal recessive early-onset parkinsonism.

Methods

Patients

This study was performed within the framework of a larger research programme named Genetic Research in Isolated Populations, or GRIP. The scientific protocol of GRIP has been approved by the Medical Ethics Committee of the Erasmus MC. Genealogical information of the *PARK7*-linked family was obtained and extended by means of local municipal and church records, as well as computerised registers. Patients were ascertained through their general practitioners, who informed them about the study and requested their cooperation.

Neurological examination

A neurological examination in each patient and available relative was carried out by two neurologists, blinded to the genotype status. The clinical diagnosis of parkinsonism was established by the presence of at least two of the three cardinal signs (resting tremor, muscular rigidity and bradykinesia) without atypical features or signs of other neurological systems (autonomic, pyramidal and cerebellar) being involved. The extent of motor dysfunction was evaluated by Unified Parkinson's

Disease Rating Scale (UPDRS)[13] and Hoehn and Yahr scores.[14] One patient had previously undergone an extensive work-up for mitochondrial encephalomyopathy, dopamine-responsive dystonia and other metabolic disease (screen for mitochondrial-DNA point mutations, biopterine-excretion assessment, bone-marrow and muscle biopsies), which did not yield evidence for those disorders (unpublished data).

Neuroimaging

During the assessment of neuroimaging results, the researchers were unaware of the genetic status of the patients. Structural brain-imaging data were obtained and reviewed. Functional brain-imaging was performed in three out of four patients. In all three patients, the presynaptic nigrostriatal system was assessed. Furthermore was assessed the postsynaptic nigrostriatal system in two patients, and brain glucose-metabolism in one patient. The scans were made in three medical centres in The Netherlands over a six-year period, since patients were treated by different neurologists. For this reason, the methods of neuroimaging could vary from one patient to the next. One patient (patient VII-2) had a ^{123}I-N-omega-fluoropropyl-2beta-carbomethoxy-3beta-(4-iodophenyl) nortropane single-photon emission CT (FP-CIT SPECT) scan made. In another patient (patient VII-3), ^{123}I-iodobenzamide (IBZM) and FP-CIT SPECT scans were made. In the third patient (patient VII-7), IBZM, 2-beta-carboxymethoxy-3-beta-(4-iodophenyltropane (beta-CIT) SPECT, ^{18}F-deoxyglucose (FDG) and ^{18}F-dopa (F-DOPA) positron emission tomography (PET) scans were performed. [15-17]

Results

Patients

The *PARK7* kindred consisted of two nuclear families, indicated as family 1 and family 2.[11] A genealogical study linked the patients to one common ancestor six generations ago in a pedigree which contained several consanguineous loops (Figure 1). All four patients were homozygous, and two out of three unaffected siblings as well as the parents of patient VII-7 were heterozygous for the disease haplotype. The third unaffected sibling (VII-1) did not carry the disease haplotype. Patient VII-3 from family 1 and patient VII-7 from family 2 were receiving neurological treatment at the time of the study (pergolide in patient VII-3 and levodopa with entacapone in patient VII-7). Neurological examination of the rest of the sibship of patient VII-3 (consisting of five other presumedly unaffected siblings) revealed two more affected individuals not previously known to have parkinsonism (patients VII-2 and VII-6). The patient in the second sibship (patient VII-7, family 2) had no other affected close relatives.

Figure 1. Pedigree of the PARK7-kindred
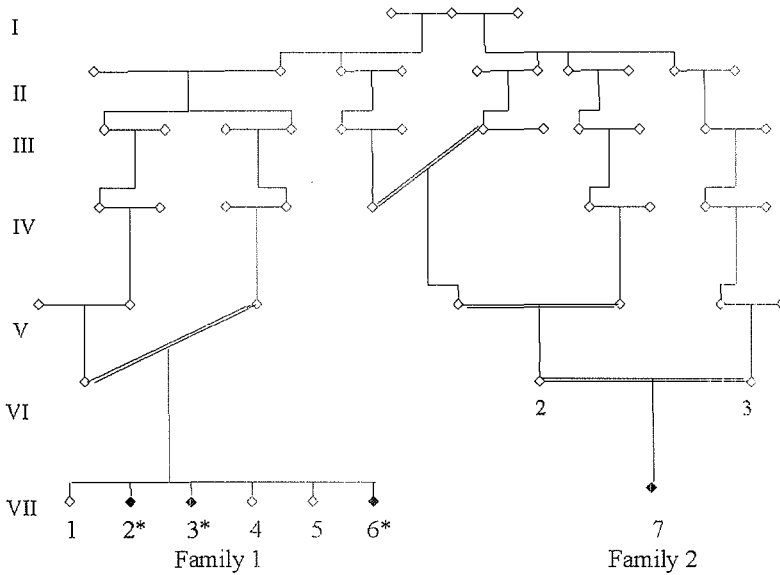
The symbol on top represents the common ancestor, and the symbols on the bottom row the examined individuals. Black symbols indicate affected individuals. Asterisks mark the presence of additional psychiatric features.

Neurological examination

Neurological examination in the parents and two siblings heterozygous for the disease haplotype, and in the sibling not carrying the haplotype yielded no evidence of neurological abnormalities. Clinical features of the four patients are listed in Table 1. Ages at onset of disease varied from 27 years to 40 years. Patient VII-6 was 40 years old at first neurological examination (which established the diagnosis of parkinsonism). All four patients showed the cardinal signs of parkinsonism without atypical (pyramidal, cerebellar, autonomic) features. Two patients had signs of focal dystonia: blepharospasm occurred in patient VII-3, and laterocollis and foot dystonia in patient VII-7, the latter worsening in the off-phase of medication   Patient VII-3 could not indicate whether the blepharospasm preceded therapy for parkinsonism, or only arose afterwards. In patient VII-7 the laterocollis occurred within a month of initiation of treatment. Patients VII-3 and VII-7 were treated with pergolide and levodopa with entacapone, respectively, and newly diagnosed patients VII-2 and VII-6 did not receive any dopaminergic medication. Patient VII-7 developed dyskinesias and dystonias within one month from levodopa treatment. In addition to parkinsonism, all three patients VII-2, VII-3

Table 1. Clinical features of *PARK7*-linked parkinsonism

Patient	VII-2	VII-3	VII-6	VII-7
Age at examination (yrs)	50	48	40	38
Age at onset (yrs)	40	31	not known	27
Resting Tremor	-	+	-	-
Rigidity	+	+	+	+
Bradykinesia	+	+	+	+
Postural Instability	+	+	+	+
Asymmetry at onset	+	+	-	+
Dystonia at examination	-	blepharospasm	-	laterocollis; foot dystonia
Hyperreflexia	-	+	-	+
Dopaminergic drugs (daily dosage in mg)	-	pergolide (1.5)	-	l-dopa (+ IDD); entacapone (187.5/600)
Side-effects levodopa	n.a.	n.a.	n.a.	dyskinesias/dystonias; motor fluctuations
UPDRS score (on/off phase)	29 (n.a.)	57 (on)	22 (n.a.)	25 (on)
Hoehn and Yahr stage (on/off phase)	III (n.a.)	III (on)	III (n.a.)	III (on)
Psychiatric symptoms	anxiety; psychotic episodes; paranoid delusions	anxiety	anxiety	-
Psychotropic drugs (daily dosage in mg)	Diazepam(5); haloperidol[a]; olanzapine[a]	temazepam (10)	diazepam (5)	-

UPDRS = Unified Parkinson's Disease Rating Scale; IDD = dopa-decarboxylase inhibitor

[a] *Intermittent usage, last time of administration at least six months before neurological examination*

and VII-6 in family 1 exhibited varying degrees of anxiety, which was reported to have existed for years before onset of parkinsonism. Patient VII-2 was also known to have suffered from recurrent psychotic episodes with paranoid delusions, for which treatment was often declined. In patient VII-7 of family 2, no such psychiatric or behavioural symptoms were noted.

Neuroimaging
During the assessment of neuroimaging results, the researchers were unaware of the genetic status of the patients. Structural brain-imaging data were obtained and reviewed. Functional brain-imaging was performed in three out of four patients. In all three patients, the presynaptic nigrostriatal system was assessed. Furthermore was assessed the postsynaptic nigrostriatal system in two patients, and brain glucose-metabolism in one patient. The scans were made in three medical centres in The Netherlands over a six-year period, since patients were treated by different neurologists. For this reason, the methods of neuroimaging could vary from one patient to the next. One patient (patient VII-2) had a ^{123}I-N-omega-fluoropropyl-2beta-carbomethoxy-3beta-(4-iodophenyl) nortropane single-photon emission CT (FP-CIT SPECT) scan made. In another patient (patient VII-3), ^{123}I-iodobenzamide (IBZM) and FP-CIT SPECT scans were made. In the third patient (patient VII-7), IBZM, 2-beta-carboxymethoxy-3-beta-(4-iodophenyltropane (beta-CIT) SPECT, ^{18}F-deoxyglucose (FDG) and ^{18}F-dopa (F-DOPA) positron emission tomography (PET) scans were performed. [15-17]

Table 2. Functional neuroimaging in *PARK7*-linked parkinsonism

| Patient	Scan performed	Results
VII-2	FP-CIT SPECT	uptake putamen and caudate bilaterally and symmetrically decreased
VII-3	FP-CIT SPECT,IBZM SPECT	uptake putamen and caudate bilaterally and symmetrically decreased; normal dopamine-D2 receptor binding
VII-6	-	
VII-7	beta-CIT SPECT,IBZM SPECT, F-DOPA PET,FDG PET	uptake putamen bilaterally decreased; uptake caudate unilaterally decreased; normal dopamine-D2 receptor binding; uptake putamen and caudate bilaterally decreased, more so contralateral to most severely affected body side; diffuse cerebellar hypometabolism

FP-CIT SPECT = ^{123}I-N-omega-fluoropropyl-2beta-carbomethoxy-3beta-(4-iodophenyl) nortropane single-photon emission CT; IBZM SPECT = ^{123}I-iodobenzamide SPECT; beta-CIT SPECT = 2-beta-

carboxymethoxy-3-beta-(4-iodophenyltropane) SPECT; F-DOPA PET = ^{18}F-dopa PET; FDG PET = ^{18}F-deoxyglucose PET

Neuroimaging

Structural brain imaging was available for patients VII-3 (computed tomography images) and VII-7 (magnetic resonance images), and was unremarkable. Functional brain-imaging was performed in three of four patients (VII-2, VII-3 and VII-7). Results are listed in table 2. The fourth patient (VII-6) has not undergone neuroimaging. On SPECT imaging, two patients (VII-2 and VII-3) had a significant, symmetrical presynaptic dopaminergic deficit, which was present bilaterally in putamen and caudate nucleus. The putamen was more severely affected than the caudate nucleus. Striatal dopamine-D2 receptor binding (assessed in patients VII-3 and VII-7) was normal on IBZM neuroimaging. Patient VII-7 showed a strongly reduced presynaptic dopaminergic neurotransmitter function on both sides in putamen and caudate nucleus on PET and SPECT imaging. Uptake was more reduced in the hemisphere contralateral to the clinically more affected body side, and the putamen was more severely affected than the caudate nucleus. Furthermore, the FDG PET scan performed in patient VII-7 showed decreased glucose metabolism in the cerebellum. ^{18}F-DOPA PET images of patient VII-7 and of an age-matched healthy control subject are shown in Figure 2.

Figure 2. ^{18}F-DOPA PET images of a healthy control subject and patient VII-7
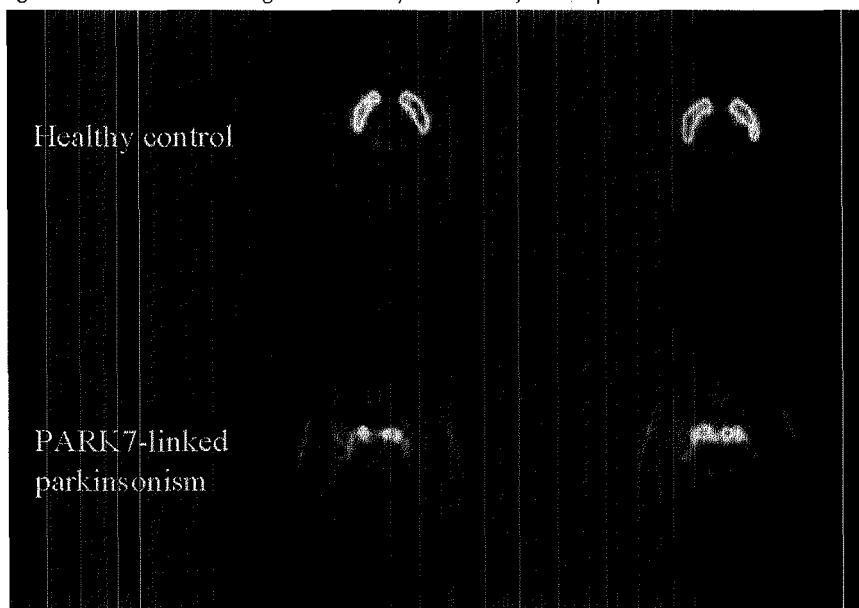
Two transaxial planes through the brain of an age-matched healthy control subject (upper row) and patient VII-7 (lower row). The subjects are viewed from the feet. Compared to the control

subject, uptake into putamen and caudate nucleus in patient VII-7 is markedly reduced on both sides.

Discussion

Based upon observations in this original kindred, *PARK7*-linked parkinsonism is of variable severity. Some of the patients had overt parkinsonism, receiving levodopa or dopaminergic therapy to which they responded well. Others had mild parkinsonism for which medical attention was never sought. Patients VII-3 and VII-7 had most pronounced motor dysfunction, and were on antiparkinson treatment. The latter two patients also had focal dystonia. Patient VII-7, who received levodopa, developed this within a month of starting dopamine therapy, as well as motor fluctuations, on-phase dyskinesias and off-phase dystonia. In all four patients, progression as observed over the 3-year period of our study was very slow. The features in early-onset parkinsonism mentioned above, such as the variable severity, the positive response to, with motor complications of, levodopa and slow progression of disease, are comparable to the phenotype of *parkin*-related[8] and *PARK6*-linked parkinsonism.[9] Similar observations were made in other *PARK7*-linked kindreds.[12] In families with *PARK6*-linked parkinsonism, however, focal dystonia was not observed and resting tremor prevailed.[10] Although the number of patients studied is too small to draw firm conclusions, *PARK6*- and *PARK7*-linked parkinsonism appear to be entities which are genetically as well as clinically distinct.

The siblings and parents who were heterozygous for the *PARK7*-linked haplotype showed no symptoms of parkinsonism at neurological examination. As for *parkin* and *PARK6*, being a heterozygous carrier of the *PARK7* disease-haplotype is probably not sufficient to confer clinical parkinsonism.

All patients in family 1 had symptoms of anxiety, for which they had been receiving benzodiazepine treatment for years. (Patient VII-3 had been using benzodiazepines even before parkinsonism was diagnosed and treated). Since the three siblings were treated by different general practitioners, this common medication cannot have been due to the propensity of one physician to prescribe benzodiazepines. In this family, complaints of anxiety may have been superimposed on, or confused with, initial symptoms of parkinsonism. In family 1, the three siblings who were unaffected with parkinsonism (VII-1, VII-4, VII-5) had no psychiatric symptoms either. These psychiatric symptoms may thus constitute part of the *PARK7* phenotype. In one patient of the independent family with significant linkage to *PARK7*,[12] psychiatric and behavioural problems at onset of disease were also present- aggression, irritability and obsessive-compulsive disorder.[18] In *parkin*-linked disease, psychiatric features, such as pronounced anxiety requiring treatment, have been reported occasionally,[19-21] and in classical, idiopathic PD, anxiety is also commonly observed.[22] Due to the consanguineous structure of our pedigree, however, psychiatric symptoms may form a 'private' trait in family 1 only, and be

unrelated to the *PARK7* phenotype. Additional families are therefore required to study psychopathological aspects of *PARK7*-linked parkinsonism.

On FP CIT and beta-CIT imaging in patients VII-2 and VII-3, there was significant evidence for a symmetrical presynaptic dopaminergic deficit. In patient VII-7, who underwent PET and SPECT neuroimaging, dopa uptake was greatly reduced bilaterally in both putamen and caudate nucleus. This was more pronounced in the cerebral hemisphere contralateral to the more severely affected body side. ^{18}F-DOPA PET-scan series performed in *parkin* patients and carriers[23-25] showed a bilaterally and symmetrically decreased dopa uptake, in which putamen as well as caudate nucleus were affected. Furthermore, asymptomatic *parkin*-heterozygotes showed a mild but significant decrease in dopa uptake, suggesting a sub-clinical disease process in heterozygotes.[24] In idiopathic, non-familial PD, dopa uptake is primarily asymmetrically decreased, affecting the putamen much more severely than the caudate nucleus.[26]

The dopa-uptake reduction is symmetrical in two patients in our study, but asymmetrical in a third patient, which does not permit straightforward comparison to dopa-uptake patterns in *parkin*-linked parkinsonism or to those in idiopathic PD. On the other hand, the presence of impaired uptake in putamen as well as in the caudate nucleus in our patients resembles *parkin*-linked parkinsonism more than idiopathic PD.[23-25] More data on *PARK7*-linked parkinsonism are therefore required to further delineate its functional-neuroimaging pattern.

The FDG PET image of patient VII-7 showed cerebellar hypometabolism of glucose. This may either be a primary phenomenon in the disease process, or occur secondary to striatal deafferentation. A decreased glucose metabolism in the cerebellum was also observed on FDG PET imaging in patients with a deletion in the *parkin* gene,[23] suggesting another feature that *PARK7*- and *parkin*-linked parkinsonism have in common. Furthermore, clinical cerebellar dysfunction and neuropathology have been described in Japanese and Dutch families with *parkin*-related parkinsonism.[27-28] In addition to the observations in *parkin*-related parkinsonism, a SPECT study reported symmetrical cerebellar hypoperfusion in an early-onset patient without *parkin* mutations.[29] Cerebellar involvement in autosomal recessive parkinsonism is still unclear and therefore requires further research.

In PET-neuroimaging studies on *parkin*-linked parkinsonism, ^{11}C-raclopride binding, assessing status of postsynaptic dopamine-D2 receptors, was shown to be significantly reduced.[24] In patient VII-7, a C-raclopride PET scan has unfortunately not been performed, but IBZM imaging in this patient and in patient VII-3 showed normal dopamine-D2 receptor binding. Finally, functional-neuroimaging studies of the *PARK7*-positive confirmation families and the *PARK6* families are not yet available, nor are neuropathological data.

The number of patients of this newly described form of parkinsonism is small, but nevertheless demands comparison to other forms of recessive

parkinsonism and idiopathic PD. Our first delineation of the *PARK7* phenotype can be refined as more *PARK7*-linked families become available.

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CHAPTER 2.4

**Brachydactyly and short stature in a kindred
with early-onset parkinsonism**

Abstract

In a consanguineous family from The Netherlands, we have identified a deletion of the DJ-1 gene leading to early-onset parkinsonism in those homozygous for the deletion. The function of DJ-1 is not known, although it is likely to play a role in the cellular response to oxidative stress. At clinical examination of these parkinsonism patients, we also observed short stature and brachydactyly. This prompted us to investigate body height and hand radiographs in patients and their relatives, as well as in a control group from the genetically isolated population the kindred originated from. Using the DJ-1 deletion as a marker for the 5.6-Mb homozygosity region on chromosome 1p36 (*PARK7*), we found a significant association of the deletion with body height, adjusting for age and gender (p = 0.005). This association suggests that either a gene or regulatory element elsewhere in linkage disequilibrium with DJ-1, or DJ-1 itself is implicated in short stature. Analysis of hand-bone length showed incomplete segregation of the deletion with brachydactyly, rendering it unlikely that the brachydactyly is fully explained by a gene in the DJ-1 region. Yet patients with parkinsonism, homozygous for the DJ-1 deletion, had a more pronounced overall bone-length reduction than relatives unaffected with parkinsonism, heterozygous for the deletion. Given the difference with the patients affected with parkinsonism, a modifier gene for brachydactyly may be present in the homozygous region containing the DJ-1 gene.

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Introduction

Recently, we identified mutations in the DJ-1 gene leading to early-onset parkinsonism with autosomal recessive inheritance in two consanguineous families from The Netherlands and Italy ([OMIM 606324], PARK7).[1,2] Unlike the other four PD genes identified to date, alpha-synuclein,[3] parkin,[4] UCH-L1[5] and NR4A2,[6] the DJ-1 protein possibly has a function in the defense against oxidative stress.[7] This indicates a novel pathogenetic mechanism for Parkinson's disease (PD). The ubiquitous DJ-1 protein is relatively small (189 amino acids) and has been highly conserved across species during evolution.[2] These characteristics suggest DJ-1 to have a relevant role in cell physiology. The exact function of the gene is, however, not yet elucidated. A comprehensive description of the phenotype associated with DJ-1 mutations may shed more light upon its function.

The PD patients in the originally reported Dutch kindred were homozygous for a region on chromosome 1p36 of 5.6 megabases (Mb), containing 90 genes. In the PD patients, a homozygous deletion was found involving five of the seven exons of the DJ-1 gene, including the start codon. Within the boundaries of the deletion, no other known genes or regulatory elements are contained. At clinical examination, the patients with parkinsonism in the Dutch kindred also had a proportionately short stature and remarkably small hands and feet. This raised the question whether a gene in the PARK7 homozygosity region or DJ-1 itself could account for the phenotype in the Dutch kindred. In order to assess the association of body height and segregation patterns of the hand phenotype with (the homozygosity region around) DJ-1, we carried out a systematic clinical-genetic and radiographic analysis of all patients with parkinsonism and their relatives in the original kindred, ascertained in a genetically isolated village. In order to assess the specificity of the traits, we also studied a control group derived from the same genetically isolated community.

Methods

Patients

The kindred in this study came to attention originally because of its four members suffering from early-onset parkinsonism.[1] Patients and relatives were ascertained as part of the research programme named Genetic Research in Isolated Populations (GRIP). The GRIP study takes place in a genetically isolated community of approximately 20,000 inhabitants in the Southwest of The Netherlands. All participants have provided informed consent prior to inclusion into the study. The scientific protocol of GRIP has obtained approval of the Medical Ethics Committee of the Erasmus MC.

The genetic analysis of the kindred described in this study has been described elsewhere.[1,2] The early-onset parkinsonism is explained by a recessive mutation in the DJ-1 gene at chromosome 1p36. In this kindred, it concerns a deletion affecting approximately 14 kilobases (kb), extending from 4 kb telomeric to

the start codon of the gene to 10 kb into the gene. Using the homozygosity mapping programme MAPMAKER-HOMOZ[8] in a genomic screen with an inter-marker distance of approximately 10 cM, four other regions of homozygosity could be detected, which were present in all parkinsonism patients and not in relatives unaffected with parkinsonism.[1] These regions on chromosomes 5, 11, 17 and 21, however, concerned alleles with a high allele frequency, which were also very common in a control group.

Figure 1. Pedigree of the kindred.
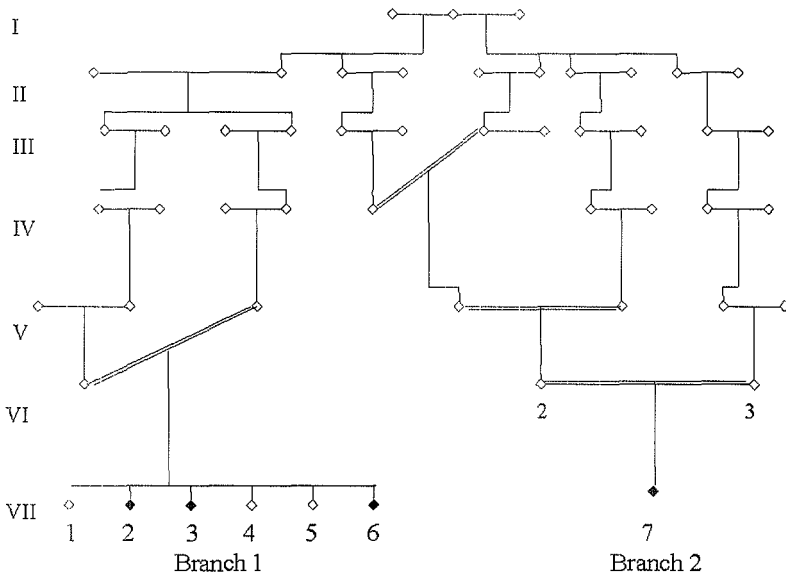
The symbol on top represents the common ancestor. Symbols on the bottom rows indicate the examined individuals by Arabic numerals. Black symbols indicate individuals affected with parkinsonism.

Initially, patients were ascertained in two nuclear families. Using a genealogical database, these families were connected to a common ancestor six generations ago. Figure 1 shows both branches of the pedigree and their common ancestor. All four patients with parkinsonism (VII-2, VII-3, VII-6 and VII-7) were homozygous for the deletion in the DJ-1 gene. Two of the three other siblings in Branch 1 (VII-4 and VII-5) were heterozygous for the deletion, and sibling VII-1 had the wild-type genotype (did not carry any deletion). The parents of patient VII-7 (Branch 2) were heterozygous for the deletion.[2] Individual genotypes are listed in Table 1.

Table 1. Genetic and clinical characteristics of the kindred

Individual	Gender	Genotype	Height (m)	Neurological examination
VI-2	m	del/nor	1.65	normal
VI-3	f	del/nor	1.60	normal
VII-1	m	nor/nor	1.60	normal
VII-2	f	del/del	1.50	parkinsonism; anxiety
VII-3	m	del/del	1.50	parkinsonism; psychosis
VII-4	m	del/nor	1.64	normal
VII-5	m	del/nor	1.62	normal
VII-6	m	del/del	1.58	parkinsonism; anxiety
VII-7	m	del/del	1.58	parkinsonism

[a] *Details of the neurological and psychiatric features in this kindred are provided elsewhere.*[1,9]
m = male, f = female, del = deletion of DJ-1 gene, nor = normal (no deletion)

Clinical examination and anthropometric assessment

Patients with parkinsonism and their relatives were ascertained as described previously.[1,9] The diagnosis of parkinsonism was verified by two independent neurologists. Researchers were blinded to the DJ-1 genotype at the time of examination. A clinical geneticist performed a systematic anthropometric and dysmorphologic assessment of all family members indicated by Arabic numerals in Figure 1. Body height and DJ-1 genotypes were also assessed in a control group derived from the same genetically isolated population within the framework of another study, consisting of 88 individuals. In order to exclude influence of a secular trend of increased body height in younger age groups, the age range of these control individuals closely approximated the age range of the individuals in the kindred. Furthermore, a manual for body height in the Dutch general population was used for reference.[10]

Radiographic analysis

For this study, radiographs of distal extremities were collected. From planar hand radiographs, metacarpal and phalangeal bone lengths were determined. Foot radiographs were used for visual inspection only, since standardised assessment of metatarsal/phalangeal bone lengths is not available. The analysis of hand structure was performed using the metacarpophalangeal profile (MCPP) program.[11] From bone lengths (in millimeters), Z-scores were calculated, representing the statistical difference (in standard deviations) of the mean bone length compared to a sample of control individuals. Parkinsonism patients and relatives in the kindred were firstly

compared to a baseline reference group, consisting of 44 individuals randomly drawn from the general (Dutch, caucasian) population.[11] Since the kindred originated from a genetically isolated population, a trait may not be specific to the kindred with DJ-1 deletions and early-onset parkinsonism, but rather represent a characteristic of the isolate. Therefore, in order to compare individuals from the DJ-1 kindred to other individuals from the same genetically isolated population (the GRIP population), we secondly studied MCPP profiles in 12 unaffected non-related controls from independent nuclear families segregating triphalangeal thumb ascertained in the same community (TPT).[12,13]

Statistical analysis
Body height was analysed as a continuous trait by multiple regression analysis adjusting for age and sex. The DJ-1 genotype was defined by the presence of deleted DJ-1 genes,[2] and was used as a marker for the 5.6-Mb homozygosity region on chromosome 1p36. Degrees of significance were expressed as two-sided p-values.

Results
Clinical examination
Clinical-genetic and anthropometric features are summarised in Table 1. On general clinical examination, all four patients with parkinsonism were found to have a proportionately short stature and small hands and feet. Figure 2 shows a photograph and a radiograph of one of the parkinsonism patients (VII-3).
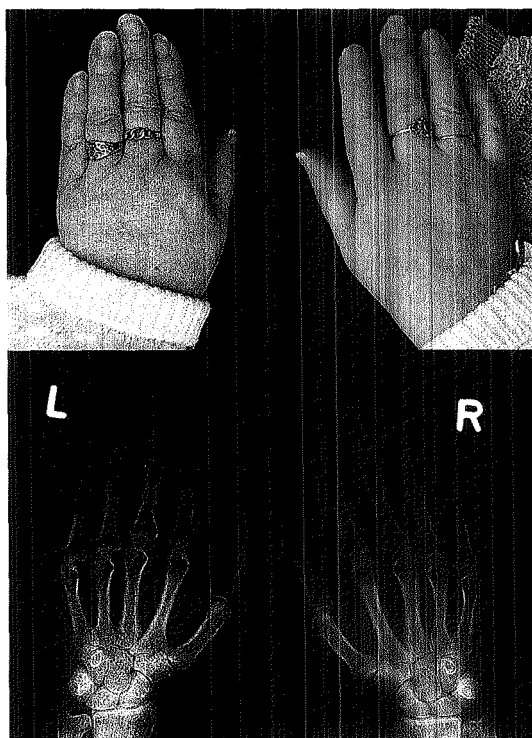
Figure 2.

At physical examination, the five relatives unaffected with parkinsonism (VII-1, VII-4, VII-5 in Branch 1, and VI-2, VI-3 in Branch 2) also had a degree of short stature, but not to the same extent as the patients. Body height of all individuals in the kindred was equal to or below the third percentile according to reference tables for the Dutch population.[10]

In the body-height control group, two individuals were heterozygous for the DJ-1 deletion (a female of 1.54 m and a male of 1.67 m). These two subjects were included the group of heterozygotes for the DJ-1 deletion. Figure 3 gives the mean height for homozygotes (n = 4), heterozygotes (n = 6) and non-carriers (wild-type genotype, n = 86), as measured in the pooled sample of the members of the kindred and controls. In this isolate-based pooled sample, the DJ-1 deletion was significantly associated with body height (p = 0.005, adjusted for age and sex). Compared to non-carriers, mean height was 13 cm reduced in homozygotes for the region containing the DJ-1 deletion. The mean body height in heterozygotes for the deletion was on average 5 cm reduced (p for trend = 0.006).

Figure 3. Association between the DJ-1 deletion and body height in the original kindred and population controls

| wild type (n=86) | 1 deletion (n=6) | 2 deletions (n=4) |
| 1.67 m (±0.10) | 1.62 m (±0.04) | 1.54 m (±0.05) |
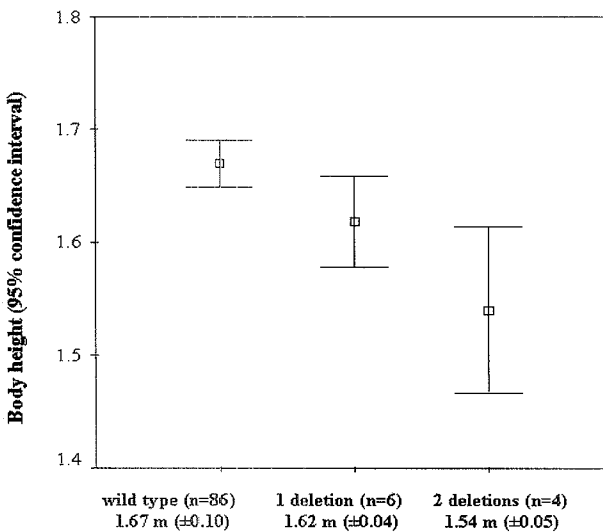
The number of deleted DJ-1 genes is plotted on the X-axis; mean body height (with 95% confidence interval) is plotted on the Y-axis.

Radiographic analysis
Hands
Z-plots of the MCPP profiles of individuals in Branch 1 and Branch 2 of the kindred are depicted in Figure 4a and 4b, respectively. Numbers VII-2, VII-3 and VII-6 (Branch 1, Figure 4a), and VII-7 (Branch 2, Figure 4b) represent the homozygous

85

patients. VII-4, VII-5 (Branch 1), VI-2 and VI-3 (Branch 2) are heterozygous for the deletion, and VII-1 (Branch 1) is not carrying the deletion. All parkinsonism patients had metacarpal and/or phalangeal bone lengths, which were at least two standard deviations shorter than the general-population mean, represented by the X-axis. Compared to the heterozygous/wild-type genotype relatives, they represent the shortest bone lengths overall in Figures 4a and 4b.

Figure 4. Z-scores of Branch 1 (a) and Branch 2 (b)

VII-1 ———, VII-2 ----, VII-3 ...., VII-4 — — —, VII-5 — · —, VII-6 — ·· — ··

VII-7 ———, VI-2 ...., VI-3 -----
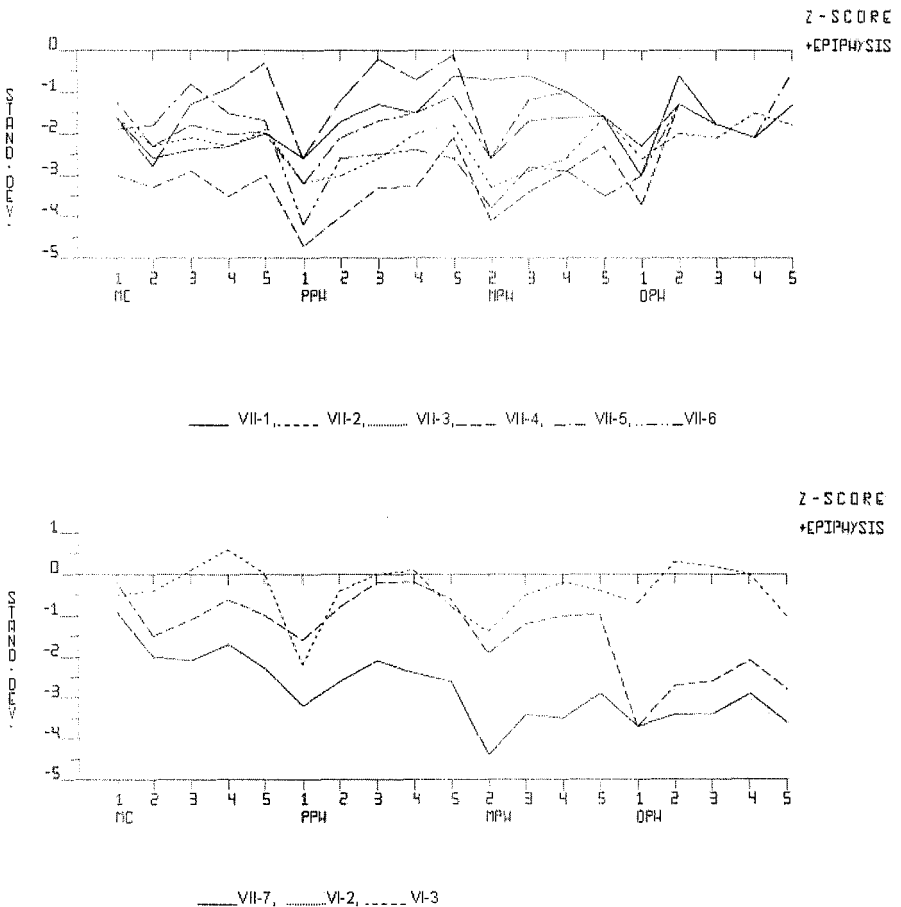
The numbers on the X-axis represent hand bones. They are listed as metacarpal bones 1 to 5 (MC 1-MC 5), proximal phalanges 1 to 5 (PPH 1-PPH 5), middle phalanges 2 to 5 (MPH 2-MPH 5) and distal phalanges 1 to 5 (DPH 1-DPH 5). The Y-axis represents the Z-scores. The zero line is the general-population mean.

MCPP profiles

In the MCPP profiles of all parkinsonism patients, nadirs were observed at the proximal phalanx of digit 1 (PPH1) and middle phalanx of digit 2 (MPH2), indicating reductions of three to four standard deviations from the general-population mean. These nadirs at PPH 1 and MPH2 were also present in the heterozygous relatives in both Branch 1 and 2, albeit to a lesser degree. The relative in Branch 1 who carried the wild-type genotype, however, also showed a nadir at PPH1. In Branch 1, for the middle phalanges 2 and 3, the heterozygotes displayed shorter bone lengths than the relative carrying the wild-type genotype. For all other metacarpals, proximal phalanges and middle phalanges, however, there was no clear-cut relation of bone length and DJ-1 genotype status (Figure 4a). In Branch 2, there is a conspicuous similarity between the profiles of both parents, which may be explained by the consanguinity (Figure 4b).

Controls

For comparison, Figure 5 contains a Z-plot of the MCPP profiles of a control group of 12 unrelated controls from the same genetic isolate. For better visibility MCPP plots only contain information of up to six curves.[11] Therefore, only six individuals from different nuclear families are shown. The MCPP profiles of the isolate-based controls cluster around the reference line, suggesting no significant difference from the randomly selected controls from the general population. Brachydactyly was not observed in any of the control individuals.

Figure 5. Z-plot of six individuals from independent nuclear families in the same genetically isolated population
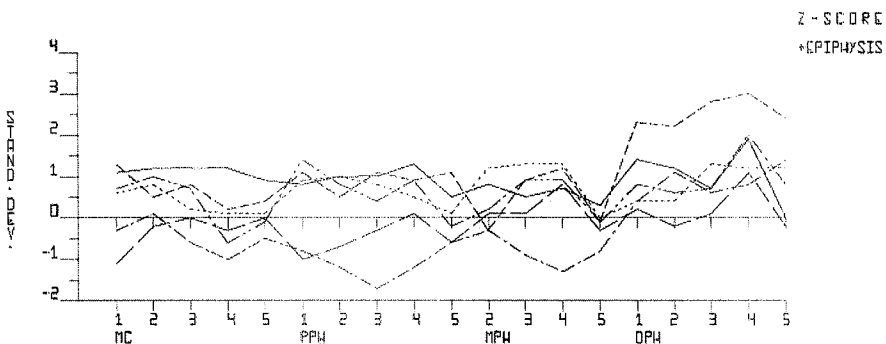
Feet

Bone length of the feet was not quantified since a standardized method was not available. On inspection of foot radiographs, short metatarsals were observed, most pronounced in the three patients from Branch 1. In these three patients, a *pes equinus*-like deformation was also present (data not shown). This deformity

rendered measurement of foot bones in a two-dimensional plane unreliable. The patient in Branch 2 also had short metatarsals, albeit to a lesser degree.

Discussion
Body-height phenotype

In this family with early-onset parkinsonism, we observed a proportionately short stature and brachydactyly. Short stature and brachydactyly can be found in a variety of syndromes,[14] but no known short-stature syndrome corresponds to the type of brachydactyly observed in our study.

All individuals in the kindred had some degree of short stature, but the four, homozygous, patients had the lowest body height of all. Several aspects of this finding need mentioning. Firstly, since short stature is genetically heterogeneous, the short stature observed in both branches in the kindred could be a familial trait. Regarding DJ-1 deletion status and body height overall (in individuals from the kindred and in controls from the isolate), an additive effect in the association with body height was noted. Yet on an individual level, the sibling carrying the wild-type genotype was not the tallest individual in Branch 1, which possibly indicates the presence of other genetic and/or environmental factors acting upon body height. Secondly, short stature may be more common in chronic disease with early onset, usually occurring secondary to the chronic nature of the disorder. Short stature, however, is not a feature of parkinsonism associated with mutations in parkin, the other gene for early-onset parkinsonism,[15] and therefore, parkinsonism *per se* is unlikely to be responsible for impaired growth since childhood. Thirdly, considering the genetic isolation of the GRIP population, reduced body height may be a local, genetically determined trait not specific to our kindred. Mean body height in controls derived from the same genetic isolate was significantly increased compared to members of our kindred. Furthermore, the finding of a DJ-1 deletion in two controls with a shorter stature than average (both $>$ 1 standard deviation below the gender-specific means) supports the, highly significant, association between the homozygosity region containing the DJ-1 deletion and short stature.

Brachydactyly phenotype

The brachydactyly, most pronounced in the proximal phalanx of the thumb and the middle phalanx of the index finger, was present in the parkinsonism patients (homozygous for the DJ-1 deletion), in the heterozygous relatives of Branch 1, but also in the relative with the wild-type genotype. Brachydactyly was neither observed in a control group from the general population, nor in a control group from the same isolated community. The parkinsonism patients showed a very pronounced reduction of the proximal and middle phalangeal bones. In the members of Branch 1, complete segregation with the DJ-1 deletion (a reduction of bone length by number of DJ-1 gene deletions) was observed for only 2 of the bones in the hand. In Branch 2, this cannot be fully assessed, since relatives with the wild-type genotype

were lacking. The inconsistent relation of brachydactyly with the DJ-1 deletion may therefore indicate brachydactyly to be genetically different from the body height and the parkinsonism phenotypes.

Brachydactyly is often present in upper and lower distal extremities.[16] A standardised method to quantify foot-bone length such as the MCPP profile method is not available. Short metatarsals were evident, but a *pes equinus*-like deformation in the feet of all three individuals from Branch 1 of the kindred rendered measurement of bone lengths based on a planar radiograph unreliable.

Genetics

It is not evident which gene or genes are responsible for the short stature and for the brachydactyly in this kindred. Firstly, a genomic screen with 10-cM marker spacing has been performed on all but three individuals (VII-1, VII-4, VII-5) of the kindred.[1] The individuals included in the genomic screen shared other homozygous regions, but these concerned very common alleles. Yet individuals not included in the genomic screen, as well as the limited inter-marker distance, may have caused another homozygosity region to remain undetected. Furthermore, this approach assumes the mode of inheritance to be recessive. Since the observed phenotype (parkinsonism, short stature, brachydactyly) possibly consists of genetically independent phenotypes, modelling a trait as recessive may not apply to phenotypes such as brachydactyly and height.

The *PARK7* homozygosity region,[1] later shown to contain a deleted DJ-1 gene, spans about 5.6 Mb and harbours approximately 90 genes.[2] The breakpoints of the deletion in this kindred have been cloned; the deletion does not affect other known genes or regulatory elements. A gene located elsewhere in the homozygous region, however, could account for the association with body height. Therefore, the DJ-1 deletion can presently only be considered a 'marker' for short stature and brachydactyly. A search for genes in the *PARK7* candidate-gene interval with a likely function in growth, however, has yielded no obvious candidates so far (data not shown).

The observation that four patients from distantly related nuclear families with a rare form of early-onset parkinsonism due to homozygosity to the DJ-1 region showed a similar degree in reduction in hand-bone length is, however, remarkable from a clinical and genetic perspective. Regarding the fact that bone-length reduction was less pronounced in heterozygotes, this raises the question whether there may be a gene in the DJ-1 region that modifies bone length.

About the other originally reported family with early-onset parkinsonism and a DJ-1 mutation, the L166P point mutation,[2] no detailed anthropometric information is available to date. To our knowledge, however, body height and hand size in patients and relatives was unremarkable (Bonifati, personal communication). It therefore remains to be determined whether short hands and stature are also present in families segregating other DJ-1 mutations, thus supporting the presence of a

monogenic trait. More families with mutations in DJ-1 will help delineate its phenotype.

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CHAPTER 2.5

PET neuroimaging in a kindred with DJ-1 associated parkinsonism

Abstract

Mutations in the DJ-1 gene lead to autosomal recessive early-onset parkinsonism. In parkin and *PARK6* associated parkinsonism, heterozygotes were shown to have mild yet statistically significant dopaminergic dysfunction on functional neuroimaging, suggesting the involved gene to be haploinsufficient. In order to assess the effect of one or more DJ-1 mutations upon dopaminergic metabolism, we performed 6-[^{18}F]-fluoro-L-3,4-dihydroxyphenylalanine (F-DOPA) and [^{18}F]-fluorodeoxyglucose (FDG) PET neuroimaging in 2 homozygotes (parkinsonism patients), 3 heterozygotes for a DJ-1 mutation and a non-carrier (clinically unaffected). Both homozygous patients with parkinsonism had, regardless of the degree of clinical severity, significantly reduced F-DOPA uptake concordant with typical PD, and putaminal FDG hypermetabolism. In all heterozygotes for a DJ-1 mutation and a relative not carrying a deletion, F-DOPA metabolism was unremarkable. From F-DOPA PET imaging in heterozygotes, a DJ-1 dosage effect appears to be absent. Due to the small number of participants in this study, however, dopaminergic function must be assessed in more individuals with DJ-1 mutations when they become available.

Introduction

Mutations in the DJ-1 gene (*PARK7*) are associated with early-onset autosomal recessive parkinsonism.[1] DJ-1 was suggested to be part of the response to oxidative stress,[2,3] supporting the long-standing yet still unproven hypothesis of oxidative damage in the aetiology of typical Parkinson's disease (PD).[4] The exact properties of DJ-1, however, remain to be determined. Since neuropathology of individuals with DJ-1 mutations is not yet available, functional neuroimaging may provide important pathophysiological clues about DJ-1 associated parkinsonism *in vivo*. Some patients in the originally described kindred with DJ-1 mutations from The Netherlands[1,5] previously underwent PET and SPECT neuroimaging.[6] These patients had a dopaminergic deficit typical for PD, but a more pronounced bilaterality and involvement of the putamen as well as the caudate nucleus distinguished their pattern from typical PD.[6,7] Furthermore, the patient who underwent PET neuroimaging displayed diffuse cerebellar hypometabolism of glucose.[6]

6-[^{18}F]-fluoro-L-3,4-dihydroxyphenylalanine (F-DOPA) PET imaging performed in heterozygotes for parkin mutations and the *PARK6* haplotype, two other forms of recessive parkinsonism,[8,9] showed a modest yet significant reduction in dopa uptake on F-DOPA PET imaging, indicating a mild form of preclinical disease in heterozygous individuals.[10,11] Neuroimaging studies in heterozygotes for DJ-1 mutations have not been performed before. In order to assess striatal dopamine decarboxylase capacity and cerebral energy metabolism, we carried out an F-DOPA and and [^{18}F]-fluorodeoxyglucose (FDG) PET study in homozygotes, heterozygotes and a non-carrier for DJ-1 mutations from the original Dutch kindred.

Methods

Participants

Patients with parkinsonism and their relatives were ascertained within the framework of an ongoing study on the genetic determinants of chronic disease, called Genetic Research in Isolated Populations (GRIP). Two patients in two nuclear families came to attention because of their early-onset parkinsonism. Upon clinical examination, two more siblings in the first family were diagnosed with parkinsonism. By means of genealogical research, both nuclear families were shown to have a common ancestor six generations ago.[5] The genetic analysis of the participants to this study is specified elsewhere.[1] Briefly, all parkinsonism patients were homozygous for a deletion of 5.6-kilobase (kb) in the DJ-1 gene, which renders the gene product absent. Apart from the DJ-1 gene, no other known genes or regulatory elements are contained within the deletion. Because this study could possibly concern preclinical pathology, data on age and gender of the clinically asymptomatic participants are not given. This PET-scan study has obtained approval of the Medical Ethics Committee of the Erasmus MC. Participants in this study have provided informed consent in both stages of the study, and have, upon their request, remained unaware of their genetic status.

Clinical examination

Two independent neurologists specialised in movement disorders examined the participants in this study. The diagnosis of PD was based on presence of the cardinal signs (bradykinesia, tremor and rigidity) and clinical improvement upon dopaminergic treatment if applicable. Neurological examination was performed blinded to genotype status.

Neuroimaging

F-DOPA and FDG PET scans were performed at the PET Centre of the University Hospital Groningen. Patients were scanned on a Siemens ECAT Exact HR+ scanner (Siemens, Munich/Erlangen, Germany). F-DOPA and FDG scans were performed consecutively over a two-day period. One hour prior to F-DOPA administration, participants were given 2 mg/kg carbidopa orally in order to block peripheral decarboxylation. A bolus injection of approximately 200 MBq was given intravenously one hour later. Ninety minutes after injection, '3D' PET scans were made of the brain in static mode. Scanning time was 6 minutes. Attenuation correction was obtained by means of a standard mathematical algorithm. Regions of interest (ROI) were localised and analysed by means of a ROI template. The ROI analysis was performed blinded to the genotype status of the participants. The striato-occipital uptake ratio index was calculated (striatal uptake minus occipital uptake, divided by occipital uptake) by estimation on a pixel-by-pixel basis. For reference were used F-DOPA scans of 10 patients with idiopathic PD who had undergone F-DOPA PET scans twice: the first time when newly diagnosed (*de novo*), and the second time two years later. For FDG-scanning, approximately 180 MBq FDG was injected intravenously 30 minutes prior to a performing a '3D' static-emission scan over 30 minutes. Attenuation correction was obtained via the standard mathematical algorithm. In the FDG PET images, anatomical ROI were selected from a larger series of ROI in Talairach stereotactic space using a standard template (thalamus; putamen; caudate nucleus; cerebellum; various regions in frontal, temporal, occipital and parietal cortex). For each participant, ratios were calculated comparing the standard FDG uptake value for each individual ROI to the overall-ROI mean of that individual. Reference values for FDG uptake were derived from a control group of 13 healthy individuals of approximately the same age range.

Results
Clinical examination

Six members of the originally described kindred with DJ-1-associated parkinsonism[1,5] participated in this study. Two participants with parkinsonism (individuals VII-6 and VII-7) were homozygous for the deletion. On first neurological examination two years previously, individual VII-6 had no complaints concordant with parkinsonism, but displayed bradykinesia, rigidity and was diagnosed with parkinsonism. Upon examination for this study, patient VII-6 had

also developed a tremor. The UPDRS-III score was 22. Dopaminergic medication was never required but for symptoms of anxiety, benzodiazepine treatment was prescribed (diazepam 5 mg daily). Patient VII-7 has been clinically described elsewhere.[6] In this patient, disease duration was at least 15 years. Briefly, patient VII-7 displayed signs and symptoms of parkinsonism and a good, sustained response to levodopa yet complicated by dyskinesias and dystonia. The UPDRS-III score on medication was 25. Three other members from the original kindred were heterozygous for the DJ-1 deletion, and one member had a normal genotype without deletions. In the latter participants, clinical examination was unremarkable.

Figure 1. F-DOPA PET imaging
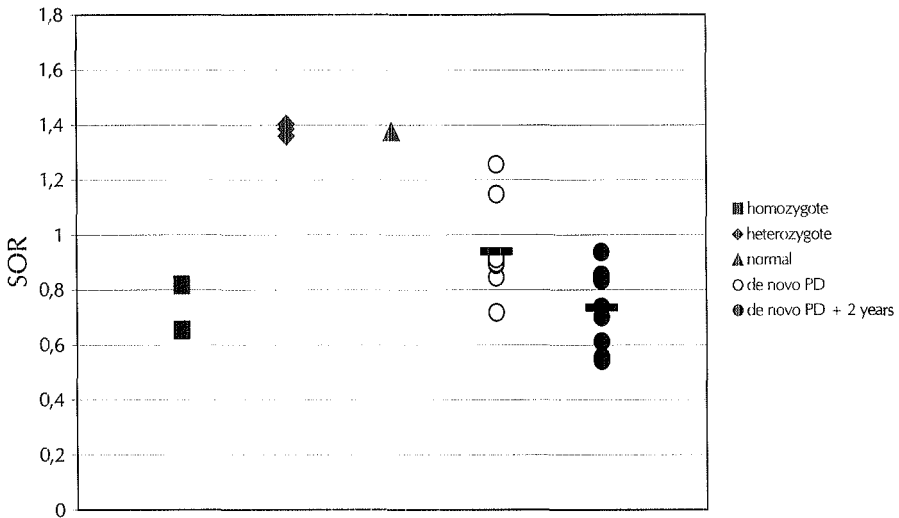
Striato-occipital uptake ratio index in putamen (average left-right) in the kindred with DJ-1 deletions, and in de novo PD patients, scanned twice over 2 years (n = 10).

Neuroimaging

F-DOPA and FDG PET results are depicted in Figures 1 and 2. One of the heterozygotes did undergo the F-DOPA scan but subsequently declined the FDG scan. The striato-occipital ratio index of F-DOPA uptake in putamen and caudate nucleus in the two patients (VII-6 and VII-7) was significantly decreased (0.82 and 0.65 for putamen and 1.04 and 0.90 for caudate nucleus, respectively). Compared to a group of typical-PD patients, their values were concordant with a disease duration of 2 years (Figure 1). In the heterozygous relatives as well as the relative with the wild-type genotype, individual values for the uptake ratio index were within the normal range.

Both parkinsonism patients displayed FDG hypermetabolism in the putamen and to a lesser degree in the caudate nucleus (Figure 2a). In two first-degree relatives, a homozygote (patient) and a heterozygote, significant glucose hypometabolism was observed in the cerebellar, parietal and occipital regions (fourth and fifth bars from the left in Figure 2b). FDG uptake ratios in the other ROI were unremarkable in all participants (homozygous, heterozygous, non-carrier).

Figure 2. Individual FDG uptake ratios by ROI.
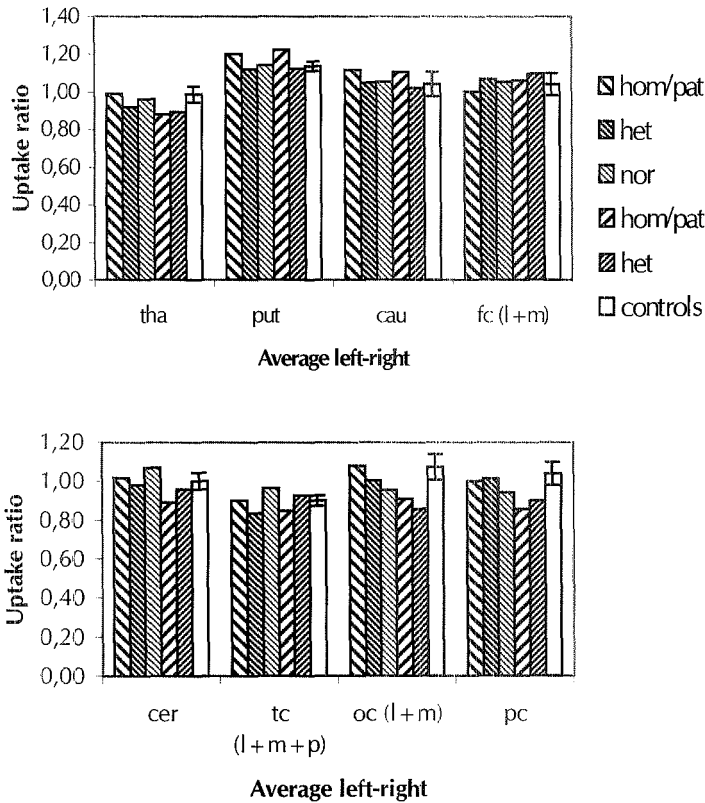
Participants are listed by individual genotypes, but have been anonymised. The rightmost bars represent the control group (n = 13). a) thalamus; putamen; caudate nucleus; frontal cortex b) cerebellum; temporal cortex; occipital cortex; parietal cortex.

hom/pat = homozygous parkinsonism patients; het = heterozygote; nor = relative with normal genotype (non-carrier); tha = thalamus; put = putamen; cau = caudate nucleus; fc = frontal cortex; l = lateral; m = medial; cer = cerebellum; tc = temporal cortex; p = pole; oc = occipital cortex

Discussion

In this parkinsonism kindred with mutations in the DJ-1 gene, we have visualised striatal dopaminergic and cerebral glucose metabolism by PET imaging. This is the first report on functional neuroimaging characteristics in asymptomatic carriers of a mutation in the DJ-1 gene.

In all clinically unaffected relatives heterozygous for DJ-1 deletions and the relative without a DJ-1 deletion, F-DOPA PET imaging was unremarkable. This suggests that heterozygosity for a DJ-1 mutation is compatible with a normal presynaptic dopaminergic neurotransmitter system in the striatum, and consequently a truly recessive property of the DJ-1 gene deletion. Mild yet significant dopaminergic dysfunction was reported in dynamic PET imaging of small groups of heterozygotes (n = 5 and n = 3) for parkin and *PARK6*-linked parkinsonism.[10,11] The subtle decrease observed in these other forms of recessive parkinsonism indicates that the F-DOPA PET results obtained in our study may require confirmation in larger patient series. The differences between results in the aforementioned studies[10,11] and ours may also be due to differences in scanning techniques (dynamic[10,11] versus static) and concordant analyses. A recent study, however, showed no clear advantage of the dynamic approach over the static approach.[12] Moreover, the former, dynamic, scanning method is a larger burden to patients.

A further question to be answered is whether heterozygosity for a recessive mutation would predispose to typical PD at later age. Ideally, the heterozygous individuals in our study should be followed up by neuroimaging in order to detect any decrease in dopaminergic function in the future. In a group of late-onset PD patients from the same genetically isolated population, however, no DJ-1 mutations were found,[1] arguing against a role for a single DJ-1 mutation as a susceptibility factor in typical PD.

The two parkinsonism patients (homozygous for a DJ-1 deletion) had significantly decreased F-DOPA uptake concordant with observations in typical PD[7] and increased FDG uptake in the putamen. The two patients had different degrees of clinical severity and different durations of disease (> 15 years in VII-7 versus 2 years in VII-6[6]), but both fell within the range of patients with typical PD of a duration of 2 years (Figure 1). The discrepancy between striatal abnormalities on neuroimaging and the clinical phenotype suggests the existence of some unknown compensatory mechanism in the basal ganglia, bypassing the circuitry that lacks the DJ-1 protein. Similar degrees of dysfunction are also observed in other mendelian forms,[10,11] suggesting a common property of inherited forms of parkinsonism. The possibility remains, however, that it is a phenomenon secondary to a long-standing degenerative process and reflects the duration of disease.

On FDG PET imaging, in one of the two parkinsonism patients and a heterozygous first-degree relative, uptake ratios were decreased over cerebellum and parietal and occipital cortex (Figure 2b). The remarkable similarity between these close relatives

suggests another neurological abnormality, which is present in one particular branch of the kindred, and not in the other branch. This implies that cerebellar hypometabolism[6] may not be part of DJ-1 associated parkinsonism *per se* but rather have resulted from another (most likely genetic) defect. The high degree of consanguinity in this family could indicate that another gene in the *PARK7* region or elsewhere is responsible for these abnormalities. Unfortunately, no FDG information was available for the third heterozygotes for a DJ-1 deletion.

In conclusion, our study suggests that dopaminergic function agrees with typical PD in patients homozygous for the DJ-1 deletion, and is normal in heterozygotes. More families with DJ-1 mutations are now required for further study.

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CHAPTER 3

SPORADIC PARKINSON'S DISEASE

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CHAPTER 3.1

Genetic epidemiology of neurological disease

Abstract

Genetic epidemiology is a young but rapidly developing discipline. Although its early years were largely dedicated to family-based research in monogenic disorders, genetic-epidemiologic research today increasingly focuses on complex, multifactorial disorders. Along with the development of the human genome map and advances in molecular technology grows the importance of genetic-epidemiologic applications. Large-scale population-based studies, requiring close integration of genetic and epidemiologic research, determine future research in the field. In this chapter, we review the basic principles underlying genetic-epidemiologic research, such as molecular genetics and familial aggregation of disease, as well as the typical study approaches of genome screening and candidate-gene studies.

Introduction

Genetic epidemiology is a discipline that covers a broad spectrum of research, ranging from studies of disease aggregation in families to studies of the specific molecular origin of a disorder. Many developments have contributed to the rapid growth of genetic epidemiology in the past decade, including the sequencing of the human genome, the identification of common genetic variants, and advances in the vast armamentarium of high-throughput methods for studying the human genome. In recent years, research interest has shifted from genetic disorders that are caused by a single gene (e.g., Huntington's disease) to common multifactorial disorders or complex diseases such as Alzheimer's disease, which are likely to result from the interaction of genes and the environment.

This chapter will begin with a discussion of mendelian versus complex (non-mendelian) neurological disorders, followed by an overview of the structure of DNA and the molecular basis of disease. We will review the methods of genetic epidemiology, differentiating family-based studies from population-based studies of unaffected individuals. The options for genetic-epidemiologic study designs will be presented, with a discussion of strengths and limitations of each approach. Finally, we will summarise recent technological developments, and outline important ethical and social implications of genetic-epidemiologic research for neurological disorders.

Genetic transmission of disease

Mendelian versus non-mendelian (complex) diseases

As a first step in examining the genetic transmission of a disease, classical genetic approaches are used to estimate disease heritability. Heritability is the proportion of the total variance in a trait (or percentage of disease) that can be explained by the additive effects of genes. Twin studies are the classic method for estimating heritability, and studies of the degree of clustering or familial aggregation of disease are also valuable. A distinction is often made between mendelian and non-mendelian traits. Mendelian disorders are usually single-gene disorders caused by either a single dominant or two recessive autosomal mutations. Autosomal disease genes are located on one of the 22 autosomes, whereas sex-linked genes are on either the X or Y sex chromosomes.

For a mendelian disorder, examining the transmission patterns of the disease through a family may yield clues to the nature of the mutation involved in the disease. If a disorder has an autosomal dominant pattern of inheritance, the disease is expected to be present in multiple generations, because having one copy of the mutation is sufficient to lead to disease pathology. Since subjects carrying two dominant mutations (homozygotes) are rare and often incompatible with life, most patients carry only one copy of the mutation (heterozygotes). For a dominant mutation, disease risk is thus conferred when an individual receives one mutant form of a gene from either parent.[1] Affected parents will pass on disease to approximately 50% of the offspring, since the

probability that the mutant or normal gene is transmitted to offspring is equal. Huntington's disease is an example of an autosomal dominant disorder.

If two defective copies of an autosomal gene are needed to develop disease, a mutation is referred to as autosomal recessive. Parents of patients with a recessive disorder are most often heterozygous and not affected. Recessive disorders typically emerge in consanguineous matings, since these matings increase the probability that both parents are carriers of the same mutation. An example of an autosomal recessive neurological disorder is parkinsonism associated with mutations in the DJ-1 gene.[2]

Genetic disorders can also be linked to the sex chromosomes. In particular, since males only possess one X-chromosome, a recessive mutation may lead to disease when only one copy is present whereas in women two defective copies are needed. Duchenne muscular dystrophy is a sex-linked recessive disease that shows a typical clustering of disease in males, with no male-to-male transmission.

A problem in examining mendelian transmission of disease is that mutations may not always lead to disease. The cumulative incidence of disease in a mutation carrier (penetrance, or the probability that the person develops the disease during life) may depend on age, sex and other factors. For many mendelian diseases, the reason for reduced penetrance is unknown, but may be related to other genetic or environmental factors. The presence of a number of dominant or recessive mutations with reduced age- or sex-related penetrance may result in a disorder that appears to be non-mendelian.

In contrast to the mendelian inheritance patterns discussed thus far, the inheritance of a non-mendelian or multifactorial (complex) disorder is more difficult to investigate. Complex diseases, which comprise the majority of neurological conditions, are likely to result from several low-penetrance susceptibility genes, each of which constitutes a minor contribution to pathogenesis. The relationship between a given genotype and phenotype (the observable trait) is not straightforward. Complex disorders are likely explained by the interaction between multiple susceptibility genes (gene-gene interaction), or by the combination of one more susceptibility genes with environmental factors (gene-environment interaction). Most common neurological disorders such as Alzheimer's disease, epilepsy, Parkinson's disease and multiple sclerosis exhibit complex inheritance patterns, which may be the result of expression of different genes in combination with exogenous (environmental) exposures.

Assessment of familial aggregation is the first step in dissecting the genetic components of a neurological disorder. Knowledge of the transmission of disease (mendelian versus non-mendelian) is crucial for determining the most powerful strategy to study the molecular basis of disease. For most complex neurological disorders, patients with mendelian disease are relatively rare. Familial aggregation rarely comprises more than 10-15 percent of a neurological disorder, and the remaining individuals affected are sporadic in nature, with no apparent genetic or environmental

causes. The disease aetiology of sporadic cases is likely to be a complex combination of genetic and environmental factors.

Molecular basis of neurological disease

Genetic information is stored as deoxyribonucleic acid (DNA). At the molecular level, DNA is made up of a sugar, a phosphate and a base. The DNA sequence is described by the order of the bases (adenine, guanine, cytosine, thymine), represented by their initials A, G, C, and T. Three-base nucleotides form a codon, which codes from one of the 20 amino acids that can be synthesized in the human body. In a process called transcription, a string of codons is joined together and copied into single-stranded ribonucleic acid (RNA), which is then processed into protein. Proteins are the building blocks of the body's structures such as bone, muscle, vasculature, neurons, enzymes, hormones and neurotransmitters.

In the 19th century, without knowledge of underlying molecular biology, Mendel introduced the term "gene" as the fundamental unit that transmits traits from parents to offspring.[1] The most straightforward definition of a gene is the part of DNA that encodes for a protein. Genes actually comprise no more than five percent of human chromosomes; [3] the large majority of DNA (over 90 percent) does not code for proteins. Exons are the parts of a gene that are translated into proteins. DNA in the area flanking the exons is involved in the regulation and transmission of gene expression. Between exons, non-coding regions called introns occur, and are later removed (spliced out) when messenger RNA (mRNA) translates the coding regions (codons) to protein. The largest quantity of genomic material lies between genes (intergenic) and the DNA in this region has presently unknown function.

At present, the human genome is estimated to contain 30,000 genes. Mutations in exons leading to a change in amino acid in a protein may be pathogenic due to loss or gain in the function of the protein. Although introns do not encode for protein, mutations in the intronic regions may affect gene splicing and may subsequently change the structure or synthesis of the protein. Nucleic acid changes in the promoter region that regulate gene expression can change the level, location or timing of gene expression.

The genetic code is degenerate, meaning that several triplets can code for the same amino acid (e.g., the codons A-C-T and A-G-T both code for the same amino acid: threonine). Hence point mutations do not always result in changes at the level of the amino acid; these silent mutations may be dispersed throughout the population. Thus, at one particular locus in the human genome, several forms of the same gene (alleles) may exist. These genetic variants are called alleles, and when a variant occurs in more than 1-5 % of the population, it is called a polymorphism. Table 1 provides a summary of the location of variants in the genome and the expected effects of mutations depending on their location on a chromosome.

Table 1. Predicted relative risks for SNPs in candidate-gene association studies according to type, location and functional effect of genetic variant

Type of genetic variant	Location	Functional effect	Frequency in genome	Predictive relative risk
Nonsense	coding sequence (exon)	premature termination of amino-acid sequence	very low	very strong
Missense/ non-synonymous	coding sequence (exon)	changes in amino acid in protein to one with similar properties	low	weak to very strong depending on location
Insertion/deletions (frameshift)	coding sequence (exon)	changes the frame of the protein-coding region, usually with very negative consequences for the protein	low	very strong, depending on location
Insertion/deletions (in frame)	coding or non-coding	changes amino-acid sequence	low	weak to very strong
Sense/ synonymous	coding sequence (exon)	does not change the amino acid sequence in the protein – but can alter splicing	medium	weak to strong
Promoter/ regulatory region	promoter, untranslated regions	does not change the amino acid but, can affect the level, location or timing of gene expression	low to medium	weak to strong
Splice site/ intron-exon boundary	within 10 base pairs of exon	might change the splicing pattern or efficiency of introns	low	weak to strong
Intronic	deep within introns	no known function, but might might affect expression or mRNA stability	medium	weak
Intergenic	non-coding regions between genes	no known function, but might might affect expression through enhancer or other mechanisms	high	very weak

Adapted with permission from Tabor et al. [33]

A distinction commonly encountered in literature is that between mutations and polymorphisms. At a molecular level, however, this distinction is not clear-cut. Mutations have low frequency (generally less than one percent, as high as five percent) but are thought to be highly penetrant, resulting in disease pathogenesis in a majority of carriers. For example, in virtually all carriers, mutations of the presenilin 1 (PSEN1) gene lead to Alzheimer's disease with an onset before 55 years of age.[4] In contrast to disease-causing mutations, polymorphisms may not be related to the risk of disease or may be associated with only a modest increase in disease risk. They are said to be disease-associated rather than disease-causing variants.

The number of affected nucleotides in a genetic variant can range from a single base pair to thousands of base pairs such as the Huntington's disease gene. There are several types of polymorphisms including nucleotide insertions and deletions and nucleotide repeat polymorphisms, but most polymorphisms in the genome are single nucleotide polymorphisms (SNPs). SNPs are increasingly utilised in genetic research. Table 1 provides a summary of the predicted relative risks for SNPs in candidate-gene association studies according to type and location of genetic variant. This Table illustrates how polymorphisms can be prioritised for study based on the nature of a variant and the predicted functional effect on its protein product.

Example 1. *An example of a disease-associated polymorphism is the apolipoprotein ε4-allele (APOE*4), which has an allele frequency of approximately 17% in Caucasians. The risk of developing Alzheimer's disease for carriers of the APOE*4 allele is 1.5-2.5 fold increased.[5] Nevertheless, APOE*4 is neither necessary nor sufficient to develop Alzheimer's disease.[6]*

Methods in genetic epidemiology of neurological disease

The primary goals of genetic epidemiology are to identify specific genes that contribute to the pathogenesis of neurological disorders, and to quantify the impact of a given gene or genetic variant on the occurrence of a disease in the general population. The strength of the association between a genetic variant and disease may be obtained either from a cohort study that compares disease incidence in carriers to that in non-carriers, or from a case-control study that contrasts the frequency of the genetic variant among cases and controls.[7] In this section, we describe two different strategies for discovering susceptibility genes: genome screening and candidate gene studies.

Genome screening

A genomic screen may be conducted to partially or completely search the genome for genes involved in a disorder. This approach often begins with no *a priori* knowledge of the genes that are involved. In some cases, however, linkage studies can have provided some information that certain regions on certain chromosomes are more promising than others. A genomic screen is carried out by genotyping a set of polymorphic markers that are distributed across the genome. Usually, these markers cover all

chromosomes and are approximately equally distributed across the genome. In some studies, however, markers are distributed more densely in gene-rich regions of certain chromosomes or in regions where previous studies have indicated the likely presence of a disease-associated gene. These markers are not necessarily located in a gene, but often are located in non-coding areas not involved in any biological process.

The rationale behind genome screening is that a causally related mutation or polymorphism should be found more often in patients than in controls. However, given that the genome contains over 3 billion base pairs,[8] the probability that a random marker is located at a disease mutation by chance is virtually zero. The basic principle underlying genome screening is that our genetic information is linearly arranged in chromosomes. Loci physically close together on a chromosome are likely to be transmitted together from parent to offspring. Therefore, patients who inherit a disease gene from a common ancestor often not only receive the disease mutation, but also adjacent parts of the chromosome. Any marker that is physically located near a causal mutation should be present more often in cases than in unaffected relatives or unrelated controls, and can serve to flag the mutation. Using this principle, disease genes can be identified by screening the genome of patients by means of a limited number of markers covering the genome.

Example 2. *A recent study used pooled DNA samples to screen the whole genome for disease-associated markers in a study of multiple sclerosis (MS) patients and controls.[9] The genome scan was conducted with microsatellite markers spaced 0.5 centiMorgans apart using DNA pools (i.e., combined DNA samples) from 216 MS cases, 219 controls and trio families (745 affected individuals and their 1490 parents). Ten markers were associated with MS, including three markers previously identified within the HLA region on chromosome 6p (D6S1615, D6S2444 and TNFa), four markers from regions previously identified in linkage studies, and three markers from novel sites not yet found with linkage analysis (D1S1590 at 1q; D2S2739 at 2p; and D4S416 at 4q). This study provided evidence to support MS-linked regions on chromosomes 6p, 17q, 19q and 1p.*

Candidate-gene studies

Candidate-gene studies offer an alternative to genome screening. Investigators who use this strategy begin with knowledge of disease pathogenesis and identify promising candidate genes. These are genes that may be expected to play a role in disease pathogenesis based on the gene product, the protein, or homology to a gene that is known to be involved in the disease. Candidate genes chosen by this method are then sequenced from DNA obtained from a number of individuals, with the purpose of identifying specific mutations or polymorphisms of a nucleic acid that differ from the normal (wild-type) sequence. If a gene has two or more variants (alleles), then a group of cases with a neurological disease can be compared with a control group to determine whether the allele occurs more frequently in one group than the other (i.e., an allelic association). Gene-disease associations can also be examined according to

the *genotype* of an individual. The genotype depends on whether she or he carries one normal allele and one variant allele (heterozygote), or two variant alleles (homozygote), using the group with two normal alleles (wild type) as the comparison group. A dose-response relationship is said to occur if the relative risk for homozygotes (2 variant alleles) is higher than the relative risk for heterozygotes (1 variant allele), and this finding strengthens the evidence of a causal relationship between the marker and disease risk.

A major challenge of the candidate gene approach is that *a priori* knowledge of the pathogenesis of the disease is required, and biological evidence must argue for a role for a given protein in the pathogenic process before the large expense of genotyping assays are justified. For a large number of neurological disorders, knowledge of proteins involved in the aetiology is limited or absent. For example, before cloning of the PSEN genes involved in early-onset Alzheimer's disease, the presenilin protein and its function were unknown.[4]

Another problem in studies of candidate genes is that having a large number of candidate genes may create a multiple testing problem because a number of associations, even those that are not true, will arise on the basis of chance alone. For genomic screens in families with multiple affected generations, established criteria are available to adjust for multiple testing based on the number of tests that can be made given the size of the genome and the linkage between regions. In the context of candidate gene studies, however, adjustment for the multiple-comparison problem is not as straightforward: a large number of candidate genes, each with several polymorphisms of interest, may quickly accumulate a large number of associations that require statistical tests of significance.

Example 3. *A great number of proteins may be hypothetically involved in diseases such as Parkinson's disease, based on proteins detected post-mortem in brain tissue, or on the large number of dopamine-regulating proteins in the substantia nigra and striatum such as the dopamine transporters, dopamine receptors, and the enzymes monoamine oxidase-B and tyrosine hydroxylase. Given the large number of genes that can be tested in this context, adjustment of statistical significance levels for multiple testing is necessary. If hundreds of tests are performed, testing using a significance level of 0.05 will yield a large number of false-positive findings (approximately 5 false positive findings for every 100 genetic markers tested). For candidate-gene studies, the debate on how to adjust for multiple testing is ongoing since standard corrections for multiple comparisons such as the Bonferoni method are too conservative.[10,11] Although adjustment for multiple testing is necessary in candidate-gene studies, the need for replication of findings in different populations is even more important. False-negative findings may also pose problems for candidate-gene association studies, especially when the frequency of the variant alleles is less than 5-10%. In this case, only large association studies will have enough statistical power to detect associations. It may be argued, however, that it is most important to identify polymorphic genes of*

*relatively high frequency (e.g., APOE*4, 17%) because a greater percentage of the population risk will be attributable to such genes.*

Figure 1. Linkage analysis to localize a mutation transmitted through generations
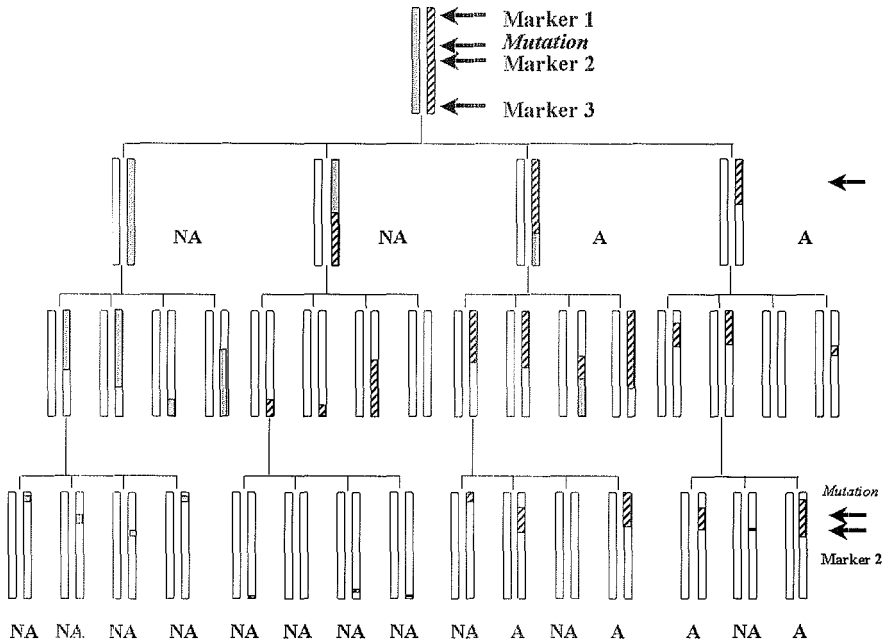
This figure depicts two homologous chromosomes of a founding parent (dashed and grey). In one of them (indicated by the dashed chromosome), a mutation occurred, which will be passed down to 50 % of the progeny. Each carrier receiving the mutation may pass it down to offspring with a 50% probability. Along with the mutation, an amount of flanking DNA is transmitted. Due to recombination, the piece of DNA shared by patients consecutively becomes smaller over generations. In the figure three markers (1,2,3) flanking the mutation are shown. In a genomic search, patients in the third generation may no longer share marker 1 and 2 of the mutated chromosome, but marker 2 still flags the mutation.

Study designs in genetic epidemiology
Family-based study designs
Family-based study designs are of great importance to the identification of new genes and have been the traditional backbone of genetic-epidemiologic research. Several reviews of study design options in genetic epidemiology, including family-based designs, are available.[12,13] Using linkage studies of extended pedigrees from affected families, remarkable progress has been made in unravelling the aetiology of several

monogenetic neurological disorders in which there is a clear-cut relation between a genetic factor and occurrence of disease (e.g., Huntington's disease, Duchenne muscular dystrophy).

The objective of a linkage study is to find markers of alleles that are preferentially transmitted to affected individuals within a family. Linkage is based on the concept that relatives who develop a disease due to the same mutation are expected to share alleles on DNA markers that flank the disease mutation. Linkage analysis uses the principle of recombination to localise the disease mutation transmitted in a family that confers the disease risk. During the process of meiosis, homologous pieces of chromosomes may cross over, which results in an exchange or recombination of the genetic information encoded on the two chromosomes. Two loci close together on a chromosome will more frequently be transmitted together, whereas for two loci located far apart, recombination is likely to occur. The closer two loci are, the less likely it is that recombination will occur between them, and as a result, the two loci are linked to each other. By using genetic markers spaced throughout the genome, linkage analysis capitalises this closeness, or linking, to find disease-associated regions by identifying markers that are shared by family members affected with the disease.

Detailed statistical aspects of linkage studies are beyond the scope of this chapter. Basically, in a linkage analysis the number of recombinations between disease status and a marker allele (observed in a family) is compared to the expected number of recombinations under the null hypothesis. The test statistic for linkage is the log odds (LOD) score. The LOD score is the log of the likelihood ratio of linkage of the disease to the studied marker, versus the likelihood of no linkage. By convention, significant evidence for linkage is found when LOD scores exceed 3, whereas LOD scores below -2 imply exclusion of the region.

As it is often impossible to clinically distinguish between patients who developed the disease due to a specific mutation and those who have a different etiology, recombination between the disease and marker may be falsely inferred; therefore the power of linkage analysis in complex disorders is low. Family members share environment in addition to genes, and the polymorphism-associated risk may depend heavily on presence of other genetic or environmental risk factors, a situation making linkage studies unfeasible. In this situation, linkage studies are not informative regarding the cause of disease.

An alternative approach to linkage studies for studying complex disorders is a family-based study using affected sib-pairs. Siblings share a high proportion (50%) of their genetic material including large segments of DNA. The *a priori* probability of a patient sharing 0 alleles with any other sib is 25%; 1 allele is 50%; 2 alleles is again 25%. For markers located close to the disease mutation, affected sibs are expected to share more alleles than the average of 1 allele. The test statistic for the analysis is based on counting alleles shared by a pair of affected siblings. Counts exceeding the

expected value under the null hypothesis (1 allele shared) are compatible with a disease locus nearby the marker examined.

An advantage of the sib-pair design is that two siblings with the same common disease are more likely to have developed the disease due to the same mutation than two distantly related subjects. Furthermore, siblings not only share a high proportion of DNA, but also large chromosomal regions. In principle, the disease gene may thus be detected with a limited number of markers. The statistical power of sib-pair studies is limited, particularly if multiple genes are involved,[10] and detecting linkage in such disorders will require astronomically large numbers of (affected) sibling-pairs.

The choice of the most powerful study design (linkage versus sib-pair approach) requires studies on clustering of disease in families in order to determine whether the disorder is segregated as a mendelian or non-mendelian trait. The most powerful design for a mendelian disorder is linkage analysis, whereas for a non-mendelian trait, sib-pair analysis is more suitable. Among patients with a common non-mendelian disorder, however, subgroups with distinctly mendelian segregation may be identified. For example, linkage analysis has successfully at identified single- gene mutations for early-onset forms of Alzheimer's disease and Parkinson's disease.[2,4] Although these traits are rare and may only explain a minor fraction of disease in the population, knowledge of the molecular genetic origin of the disease in these early-onset cases may yield clues toward key proteins involved in pathogenesis. These proteins may serve as targets in development of therapy for early-onset as well as late-onset forms.

When examining familial aggregation, clustering of a disease may not only be due to genetic factors, but also environmental factors. For instance, nutritional habits cluster in families and may explain familial aggregation of disease, as might other characteristics such as cigarette smoking and occupational exposures. Hypertension, a major risk factor for stroke, is a complex condition showing strong familial aggregation and high heritability. Salt sensitivity is known to be a genetically determined risk factor for hypertension. For example, in a population with a uniformly low salt consumption, genetic contribution to the incidence of hypertension will appear to be low because the trait will be less likely to be expressed, whereas in populations with larger variation in salt intake the contribution of genetically-conferred salt sensitivity may be considerable. For complex disorders, familial aggregation is therefore not a fixed property of a trait but can vary with the environmental determinants of disease risk.

Estimating the risk of a disease mutation in families requires ascertainment of a random group of unrelated families. Such studies are expensive and time-consuming, and this study design is rarely used to estimate gene-associated risk. For mutations that are extremely rare in the population, such as the mutations involved in early-onset Alzheimer's or early onset Parkinson's disease,[2,4] astronomical sample sizes would be needed to observe enough cases of disease. Studies of genetic and environmental factors that modify the penetrance of disease may only be feasible in those few families segregating the rare diseases.

Figure 2. Expected allele sharing in affected and unaffected siblings
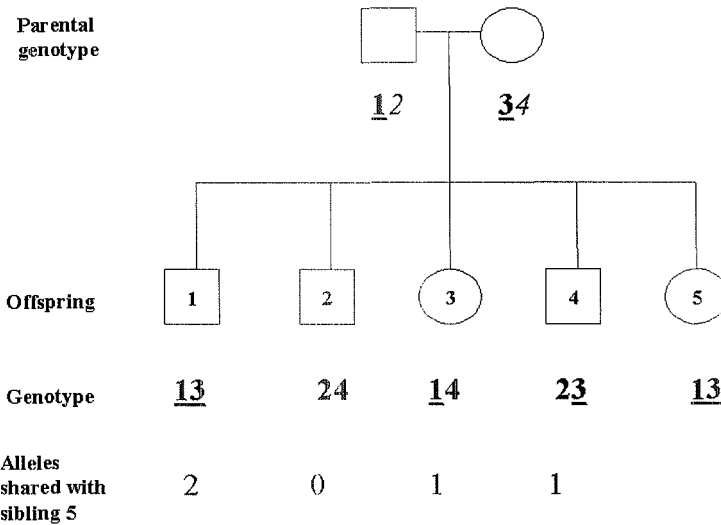
This figure shows a pedigree of 2 parents and 5 children. Squares indicate males and circles females. Since parents pass 1 of 2 alleles to offspring with equal probability, siblings share 50% of DNA on average. On average, two siblings share 1 allele.

Population-based studies

Family-based studies constitute the classic approach to determine the genetic aetiology of a trait, however, only in monogenic or oligogenic (small number of genes) mendelian disorders is this approach feasible. With the exception of rare and conspicuous phenotypes, a family-based approach rarely yields sufficient power to detect a genetic cause for common disorders. The affected sib-pair design might seem to provide an alternative for genetic linkage analysis, but large numbers of affected sib-pairs are required to gain sufficient statistical power in order to detect genes involved. For disorders with a high mortality and/or late onset, affected sib-pairs are difficult to trace, which further limits feasibility. For these reasons, much of the current focus of neurogenetic research is on individuals without affected relatives (i.e., sporadic). Migraine, epilepsy, Alzheimer's disease, and Parkinson's disease are among the diseases in this category.

Recent years have seen the growth of population-based gene association studies because some of the limitations of family based-designs can be avoided through the use of population-based study designs. The rationale behind population-based studies is similar to family-based studies because the DNA of the disease-associated gene is flanked by DNA that is passed to the next generation, and is thus dispersed throughout the population. As a result, a mutation related to disease risk can be ascertained in a

genomic screen by identifying chromosomal regions shared by patients (Figure 1). This unique alignment of genes along the chromosome is called a haplotype.

In the general population, it is difficult to perform a genomic screen for markers in linkage disequilibrium with a disease because there is only a small probability that any two patients with a common complex disorder have inherited a gene from a common ancestor. People with a common trait, randomly derived from the general population, are expected to be only very distantly related, and as a result, any two people only share a small amount of DNA. As shown in Figure 2, the amount of DNA shared progressively diminishes over generations. Thus, in order for a disease-associated marker to identify a region that may contain a disease-associated gene, the marker and disease locus must be very close together. A large number of markers with dense spacing and an extensive number of patients are therefore needed for whole genome screens to be successful.[14]

Samples derived from the general population are very suitable for candidate gene studies, yet candidate gene studies have been widely criticised because of the repeated failure to replicate results.

Example 4. *Extensive candidate studies have been conducted on Parkinson's disease. Impairment of enzyme detoxification capacity has long been thought to account for an increased susceptibility to Parkinson's disease,[15] possibly due to an impaired ability to handle environmental neurotoxins. Two genes encoding detoxification enzymes, the CYP2D6 gene (encoding for debrisoquine 4-hydroxylase cytochrome P450) and the NAT2 gene (encoding for N-acetyltransferase 2) have been studied extensively. Due to their function in detoxification, these genes were studied as obvious candidates. Although initial findings were positive, they could neither be replicated in individual studies nor in a pooled re-analysis of study data.[16] Candidate-gene studies have sometimes proved more successful when used as follow-up studies after linkage or sib-pair studies identify a chromosomal region that may contain a disease-associated variant.*

Example 5. *APOE*4, the most common genetic factor implicated in Alzheimer's disease, was primarily discovered by means of the candidate-gene approach after linkage analysis had suggested an Alzheimer's disease gene on chromosome 19.[17] APOE was considered as a positional candidate, because its gene product, apolipoprotein E, was found to be associated with senile plaques in brains of patients with Alzheimer's disease.*

The importance of population-based genetic-epidemiologic studies

Population-based studies play a pivotal role in the assessment of risks associated with genetic factors. Population studies are needed to assess the frequencies of disease-causing mutations and disease-associated polymorphisms so that the proportion of disease attributable to genetic factors can be estimated. Furthermore, population-based association studies are of critical importance for estimating the strength of the association between a given genetic variant and the occurrence of a neurological

disorder (i.e., the odds ratio or relative risk associated with a given disease-associated polymorphism), and for determining whether environmental or lifestyle factors interact with the disease-associated genotype to increase the risk of the disease.

Risk estimation for genetic factors follows the classical approach of epidemiologic studies. Relative risks of disease may be derived from studies comparing risk of disease in carriers to that in non-carriers. Alternatively, relative risks may be estimated by obtaining odds ratios for the disease-associated genotypes in case-control studies using incident patients derived from a single study base. To obtain absolute measures of risk, cohort studies are needed to estimate the difference in disease incidence between carriers and non-carriers of a disease-associated variant.[7]

Example 6. *Studies aiming to quantify the incidence of disease associated with common polymorphisms are lagging behind developments in molecular genetics. Few studies have addressed the question whether the incidence of Alzheimer's disease is associated with the APOE*4 allele. Upon its identification in 1991, most studies have used prevalent AD patients from clinic-based series,[6] and no population-based studies have been done. For clinical as well as public health purposes, unbiased risk estimates are essential. To date, few follow-up studies have been conducted in order to assess absolute and relative risks of disease for carriers of the APOE*4 allele.[18]*

The importance of assessing gene-environment interactions

For complex genetic disorders where multiple genetic and environmental factors contribute to the development of the disease, it is unlikely that a gene effect is independent of that of other risk factors. A key aim of neuroepidemiologic studies is to examine how environmental factors act against certain genetic backgrounds to increase the risk of developing a neurological disorder. It is believed that many complex neurological disorders result from one or more environmental factors acting on a background of (or in the presence of) common disease-associated genetic variants. Large-scale epidemiologic studies are therefore needed that collect data on genes and environmental factors, in order to dissect the complex aetiology of complex neurological diseases.

Challenges in the conduct of genetic association studies

Genetic association studies often face the same challenges as other epidemiologic studies and may fail to identify true associations due to factors such as small (underpowered) studies, poor choice of control group in case-control studies, over-interpretation of study results, and unwarranted conclusions that a disease-associated genetic variant is a causal factor in disease prior to study replication.[19] In addition to the usual study challenges, population-based genetic association studies may be influenced by a problem called population admixture or population stratification. This problem may occur when two or more distinct genetic subgroups are included in a

study. For example, the odds ratio from a case-control study can be biased if the frequency of the disease-associated genetic variant is more common in certain ethnic subgroups than other and if the case and control groups have different ethnic compositions. Bias due to population admixture may occur in any genetic association study, whether it is a cohort (follow-up) study or a case-control study. In a case-control study, cases and controls may be drawn from different subpopulations.

Population-stratification bias can occur in any study where cases and controls are not matched for their genealogical history, because many genetic variants differ in frequency between individuals from different ancestral backgrounds. Several methods have been proposed for minimising or controlling for population stratification bias, including firstly statistical methods to induce comparability, secondly the use of family controls from the same genetic background as cases, thirdly the use of unlinked genetic markers to determine whether cases and controls have a similar genetic background (genomic controls), and finally conducting studies in genetically isolated populations where the genetic background of the residents is homogeneous.

Addressing the admixture problem by using statistical control for ethnicity
There has been criticism on of population-based genetic association studies conducted in heterogeneous ethnic populations suggesting that population stratification (the mixture of individuals from heterogeneous genetic ancestries) undermines the validity of these studies. Many epidemiologists believe that the problem of admixture is more of a theoretical concern and does not introduce material biases into a study in most real-life circumstances. They propose that population stratification can be adequately addressed by collecting information on the genetic (ancestral) background of cases and controls in the study, and controlling for this potential confounder in the same way that other confounding variables are treated in epidemiologic studies.

Example 7. *Using empirical data on the frequency of N-acetyltransferase (NAT2) slow acetylation genotypes and incidence rates of bladder cancer among U.S. Caucasians from eight different European ancestries, Wacholder et al. showed that the relative risk for the NAT2 – bladder cancer association was minimally biased if the subjects' ethnicities were not adjusted in the statistical analysis.[20] The investigators concluded that U.S. studies restricted to non-Hispanic U.S. Caucasians of European origin are unlikely to be significantly biased, even when statistical methods that adjust for different European ancestral backgrounds are not applied. Despite this empirical evidence, many believe the statistical control method is inadequate for controlling for the diverse genetic backgrounds of subjects who participate in a study, and other approaches are proposed, such as the use of family controls.*

Addressing the admixture problem by using family controls
In order to overcome the problem of population admixture, the transmission disequilibrium test (TDT) can be used. Originally, the TDT was used in family-based

studies, but it has been adapted for use in population-based studies.[21] Rather than ascertaining a control group, alleles of parents not transmitted to the patients can be used to construct a virtual control genotype.[22] A disease-associated allele will more frequently than not be transmitted to the affected individual. The TDT approach requires ascertainment of DNA from the parental generation. For late-onset disorders, this approach is of limited value due to the fact that parents are often deceased. Although variations of the TDT for sibling controls based on siblings have been developed to overcome this problem, the power of the sib-TDT is significantly lower than that of a case-control approach.[23]

Addressing the admixture problem by using genomic controls

Another method for dealing with the problem of population admixture is to use genetic markers that differ in frequency according to a person's genetic ancestry, but are unlinked (not associated) with the disease of interest.[24] While the genomic control method can identify situations in which the diseased and non-diseased groups are similar or dissimilar with respect to population admixture, there is currently no method whereby the use of unlinked markers allows an investigator to statistically adjust for differences in population admixture between groups.

Addressing the admixture problem by studying genetic isolates

Another way of circumventing distortions due to admixture is to select genetically isolated populations for study. The population of a genetically isolated community originates from a limited number of ancestors (founders). Such a founder population limits the degree of genetic diversity introduced, leading to a more homogeneous population. Genetic drift, a random process occurring in small populations, further reduces the number of putative susceptibility genes in these populations. Studies of genetic isolates also merit discussion because of their potential value in finding disease susceptibility genes.

Studies of genetic isolates

In genetically isolated populations, there is a higher probability that patients have developed the disease due to a mutation inherited from a common ancestor. Finland is a prototypic population that is widely studied as a genetic isolate because it has experienced isolation for over 100 generations, and expanded from a small group of founders into the 5 million inhabitants of today, resulting in a genetically homogeneous population.[10] Another example of a genetic isolate is Iceland.[25] In contrast to studies in the general population, genome screens have proven to be useful in genetic isolates.[10]

In addition to studies of populations of prolonged isolation, some studies in more recently isolated populations have also been successful. In these populations, the founder effect is the major determinant of the limited genetic variation.[26] Using

populations isolated for as little as 300-400 years (i.e., up to 20 generations), genetic loci associated with genetically complex disease have been identified, including genetic loci associated with manic depression in Costa Rica and susceptibility loci for mycobacterial infection in Malta.[27, 28] A method called haplotype sharing, as depicted in Figure 3, has been applied with success in studies of recent genetic isolates.[26]

Figure 3. Allele sharing in a genetically isolated population

Chromosomal region shared in offspring:
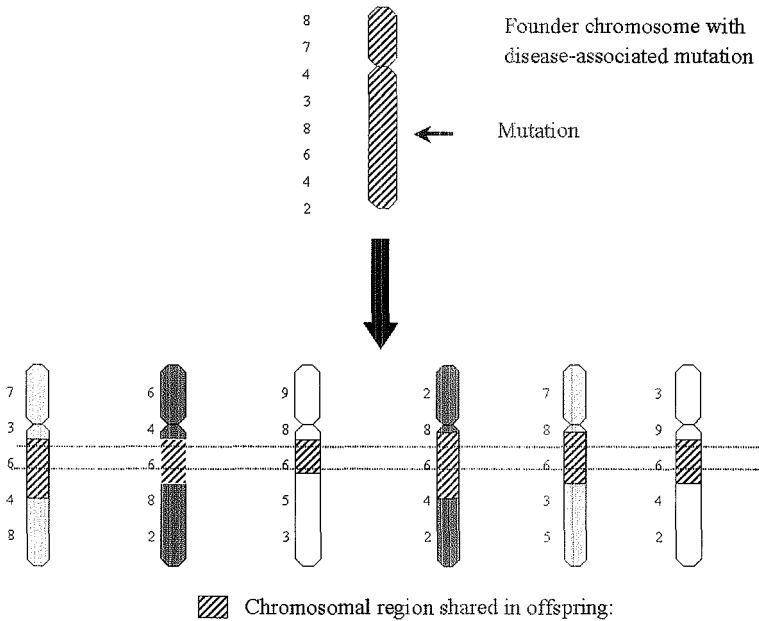
Above: Founder chromosome with disease-associated mutation
Below: Region surrounding the disease locus, shared by patients with the same phenotype. These affected individuals are all descendants from a common ancestor.

One drawback of studying genetically isolated populations is the limited value of extrapolation of study results from genetic isolates to other populations. Isolation that spans over 100 generations may have caused a population like Finland to have obtained a more or less "private" make up of the genome.[29] An advantage of studies in populations of more recent isolation is that the genetic make-up of the isolated population may more closely resemble that of the general population. However, it remains to be determined whether disease-related mutations or polymorphisms detected in an isolated population will also be present in the general population.

On a population level, studies of rare phenotypes caused by mendelian genes have made only a limited contribution to our understanding of the occurrence of disease in the general population. The challenge for the future for genetic-

epidemiologic research will be the identification of genes involved in the aetiology of common sporadic neurological disorders. With the shift in genetic-epidemiologic research from monogenetic to complex disorders, its study designs will change dramatically. As discussed earlier, the study of complex disorders will see a shift from family-based designs to towards sib-pair and population-based studies. This implies a trend of data collection towards ascertainment of large series of patients and affected siblings in order to reach sufficient statistical power in a study.[14] Such studies require large-scale genotyping, and therefore, developments in genetic epidemiology will depend heavily on advances in high-throughput molecular genotyping techniques.

Impact of the Human Genome Project on neuroepidemiology

One of the most important developments in molecular genetics that will boost genetic epidemiologic research is the recent completion of the draft of the Human Genome Project. This accomplishment has resulted in an enormous amount of information on genes and genetic variation that will greatly benefit genetic research on complex diseases. Along with growing insight into biology of neurological disease, the Human Genome Project will create opportunities for candidate genes studies. If major improvement of the design of studies can be achieved, then candidate gene studies may capture a prominent position in genetic-epidemiologic research. Another possibility created by the Human Genome Project is that of including markers for all human genes in a genome screen. This approach differs from a classical genome screen because it emphasizes the use of markers that are more likely to have a functional effect on the protein (approximately five percent of DNA).[14]

A technical development important for the feasibility of large-scale genetic-epidemiologic research is the introduction of microarrays that include (binary) information on the presence or absence of polymorphism in a gene, enabling thousands of SNPs to be tested for association with a neurological condition. These technical devices will create the opportunity to rapidly screen for DNA mutations or variations in large series of affected individuals.[30, 31] In this respect, identification of single-nucleotide polymorphism maps (SNPs) throughout the human genome is crucial (Table 1).[15] Major progress is anticipated in this field within the next decade.

Ethical, legal and social issues in genetic epidemiology studies

The ethics of research involving human participants are continuously evolving as society changes and as technology advances. Genetics, more than any other field, has undergone a technological explosion leading to greater understanding of disease etiology, but also has challenged existing concepts of informed consent and confidentiality. Investigators involved in genetics research should be familiar with published guidelines on the ethical use of collected samples for DNA studies. The *American Society of Human Genetics* published a report in 1996 on informed consent

for genetic research.[32] This statement provides recommendations for use of newly collected samples for genetic research as well as for use of existing samples.

Future directions and conclusion
Although the early years of genetic epidemiology have been largely dedicated to family-based research in monogenetic disorders, research in the future will increasingly focus on complex multifactorial disorders and large-scale population-based studies. Perhaps the most dramatic aspects of genetic-epidemiologic research arising from the switch of emphasis from mendelian disorders to complex disorders are the implications for clinical medicine and public health. In contrast to the limited number of subjects at risk for monogenic disorders such as Huntington's disease, clinical and public health implications in studies of complex genetic disorders are relevant for a large number of subjects. Future studies on the genetics of multifactorial disease will therefore require close integration of genetic and epidemiologic research. Hitherto unknown proteins involved in disease pathogenesis will be identified and enable development of diagnostic tests for neurological disorders and investigation of protein targets for drug development to benefit patients.

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CHAPTER 3.2

A clinical-genetic study of Parkinson's disease
in a genetically isolated community

Abstract

The role of genetic factors in idiopathic, late-onset Parkinson's disease (PD) remains unclear, in spite of the recent advances in the genetics of early-onset forms of familial parkinsonism. There is increasing interest in using genetically isolated populations to unravel the genetics of complex diseases such as late-onset PD. We have studied genetic and clinical features of 109 patients with parkinsonism from an area comprising a genetically isolated population in the South-West of The Netherlands. Of the 109 patients with parkinsonism, 41 patients with PD could be linked to a common founder 14 generations ago. The distribution of ages at onset of PD in the genetically isolated population was significantly bimodal (one peak with a mean at age 67 years and another with a mean at 44 years, the young peak being significantly larger than that in a population-based study, the Rotterdam Study). For other clinical features, the only statistically significant difference between early-onset and late-onset PD was decreased motor and cognitive function in the patients with late-onset PD. Involvement of other PD genes including DJ-1, a gene implicated in a kindred with early-onset parkinsonism from the same genetic isolate, was excluded in other PD patients in the population. The finding of a common ancestor in 41 idiopathic-PD patients along with the exclusion of known PD genes and loci suggests the presence of at least one other, yet unknown, susceptibility gene involved in PD in this population.

Introduction

A genetic contribution to Parkinson's disease (PD) has been suspected since the late 19th century.[1] At present, four genes have been identified in familial parkinsonism. Mutations in the alpha-synuclein gene (chromosome 4q22) are implicated in autosomal dominant parkinsonism,[2] and a mutation in the UCH-L1 gene (chromosome 4p14) was found in a family with autosomal dominant parkinsonism.[3] The most common cause of early-onset parkinsonism with autosomal recessive inheritance consists of mutations in the parkin gene (chromosome 6q25.2-q27).[4] Another gene for autosomal recessive parkinsonism, DJ-1 (chromosome 1p36),[5] is implicated in, amongst others, a kindred from the genetically isolated population in which the current study was performed. Other loci for familial parkinsonism were reported on chromosomes 1p36,[6,7] 2p13,[8] 4p14-16.3[9] and 12p11.2-q13.1.[10] Finally, a susceptibility gene for late-onset PD in the Icelandic population was mapped on chromosome 1p32.[11] In spite of these promising developments, the cause of PD in the majority of patients is still unknown. A heritability study in twins suggested that genetic factors may play a key role in early-onset, but less so in late-onset PD.[12]A major obstacle in dissecting the genetics of late-onset idiopathic PD is the weak familial aggregation, which may only become evident when studying distant relatives of an affected individual. This is possible when extensive genealogical data of the study population are available, as is the case in Iceland. Icelandic PD patients, randomly ascertained and previously thought to be largely unrelated, could be traced back to a common ancestor by using a genealogic database.[11,13] The Icelandic population has, however, been isolated over a large number of generations, such that findings may be of limited relevance to other populations.

We performed a pedigree study of idiopathic-PD patients in a young genetically isolated population in The Netherlands in order to evaluate the extent of relatedness among PD patients. PD-candidate genes and loci were assessed on a possible contribution to PD in our study population. To test whether the phenotype of these patients was representative of PD in the general population, we compared clinical characteristics of PD to those in the Rotterdam Study, a population-based study in The Netherlands of 7,983 subjects aged 55 years and over.[14]

Methods

Patients

Patients were derived from a genetically isolated community in the Southwest of The Netherlands. The study is part of a larger research programme named Genetic Research in Isolated Populations (GRIP), which is ongoing in this population. Around 1750, the population consisted of a mere 150 individuals. Demographically, the GRIP population is characterised by minimal inward migration and rapid population growth over the last two centuries. Descendants of this population, an estimated number of 20,000 individuals, are now scattered over eight adjacent villages. Church and

municipal registers are readily available and date back to the 1800s. Genealogical history up to 1600 has been computerised to a large extent. The Medical Ethics Committee of the Erasmus MC approved of the scientific protocol of GRIP.

Patients with parkinsonism were traced through registered general practitioners, neurologists and nursing-home physicians, whom were asked about all of their patients with parkinsonism. The catchment area consisted of the genetically isolated area and its surroundings. An extension to a 20-km radius was made because several PD patients, born and raised in the GRIP area, had been admitted to nursing homes in nearby communities. According to the GRIP research-protocol, patients were invited by their treating physician to participate in the study. All patients provided informed consent, firstly to their own physician and secondly to the research physician. Their medical records, and, if available, CT and/or nuclear magnetic resonance (NMR) scans of the brain were obtained and reviewed. In all patients, we carried out a questionnaire on age at onset, presenting and subsequent symptoms (tremor, rigidity, bradykinesia and asymmetry), medical history, medication and exposure to putative risk factors for PD.

Clinical examination
The research physician performed a neurological examination in each patient, including motor assessment according to the Unified Parkinson's Disease Rating Scale (UPDRS). Parkinsonism was diagnosed when at least two out of three cardinal signs (bradykinesia; rigidity; resting tremor), as well as clinical improvement on dopamine or dopaminergic therapy were present. The diagnosis of idiopathic PD was established after exclusion of other causes, and was verified by two independent neurologists, according to criteria of EUROPARKINSON as applied in the Rotterdam Study.[15] Age at onset was defined as the age at which the diagnosis of PD was established. UPDRS,[16] Hoehn and Yahr[17] and Mini Mental State Exam (MMSE)[18] scores were obtained as described elsewhere.[15] For the diagnosis of dementia in PD, onset of PD had to clearly precede cognitive decline. Medical records were therefore reviewed in order to assess onset, degree and rate of cognitive decline in all patients with an MMSE score below 24 points. In PD patients with cognitive or physical conditions hampering reliable neurological examination and UPDRS assessment, UPDRS scores were excluded from the statistical analysis (n = 10). The MMSE was not performed in patients declining the test (n = 3) and in patients with profound dementia (n = 6). Basic clinical characteristics such as age at onset of PD were also assessed in patients derived from the baseline study population at the start of the Rotterdam Study, a population-based study in The Netherlands of 7,983 subjects aged 55 years and over.[14]

Genealogy
Data on presence of PD, essential tremor and dementia in first-, second- and third-degree relatives were collected by a family-history questionnaire. Genealogical

information such as name, date and place of birth of parents and grandparents was obtained from the participants. By means of local municipal, church and computerised registers, this information was extended up to 16 generations. In order to detect any subclinical or untreated parkinsonism, first-degree relatives of patients underwent neurological examination.

Genetics

From each patient, 20 ml of venous blood was obtained for DNA isolation. Blood was also collected from relatives including parents (if alive), spouses, children and sibs. In all PD patients the DJ-1 gene was screened for mutations by means of a PCR protocol specific for the deletion observed in the community. The parkin gene was screened for mutations by direct sequencing in 6 out of 7 patients with early onset of PD.[4,5] All other known PD loci and genes were studied in a subset of distantly related patients (n = 20). Only distantly related patients were selected, since the inclusion of closely related patients would in that respect lead to dependency amongst genetic observations. Genetic markers flanking all known PD genes and loci[2-11] were therefore studied in 3 of 7 early-onset distantly related PD patients, and in 17 of 34 late-onset PD patients, also distantly related. Only a subset of patients was studied due to a transient delay in data collection at the time of the candidate-gene study. At chromosome 4q22 the markers D4S414 and D4S1572 were assessed (alpha-synuclein, *PARK1*); at chromosome 6q25.2-q27 markers D6S1581 and D6S264 (parkin, *PARK2*); at chromosome 2p13 markers D2S337 and D2S2368 (*PARK3*); at chromosome 4p14-16.3 markers D4S419 and D4S1592 (*PARK4*); at chromosome 4p14 markers D4S419 and D4S405 (UCH-L1, *PARK5*); at chromosome 1p35-36 markers D1S199 and D1S234 (*PARK6*); at chromosome 1p36 markers D1S468 and D1S214 (DJ-1; *PARK7*); at chromosome 12p11.2-q13.1 markers D12S85 and D12S345 (*PARK8*), at chromosome 1p36 markers D1S234, D1S199 and D1S2697 (*PARK9*), and at chromosome 1p32 markers D1S2797, D1S2890 and D1S230 (*PARK10*).

Statistical methods

Statistical analysis of clinical data was performed using Mann-Whitney and Fisher's exact tests, with degrees of significance expressed in (two-sided) p-values. For the age-at-onset graph, in order to assess modality (i.e., the presence of one versus that of multiple distributions), the distribution of age at onset of PD was plotted as the natural logarithm of onset age versus frequency of onset age amongst patients.[19] Statistical evidence for linkage of the aforementioned genetic markers to PD was assessed using the DISLAMB programme.[20]

Table 1. Clinical features in 7 early-onset (< 55 yrs) and 34 late-onset (> 55 yrs) patients with idiopathic Parkinson's disease from the genetically isolated population

	Early-onset PD (< 55 years)	Late-onset PD (≥ 55 years)	
	n = 7	n = 34	
Women (number)	3 (43%)	24 (71%)	n.s.
Mean age at onset (years)	44.6 (±4.8)	67.1 (±6.5)	p < 0.0001
Mean duration of illness (years)	7.0 (±7.1)	5.6 (±4.3)	n.s.
Levodopa treatment	6 (86%)	31 (91%)	n.s.
daily dosage (mg/day)	497 (±411)	541 (±470)	n.s.
dyskinesia/dystonia (number)	3 (43%)	7 (21%)	n.s.
Clinical symptoms at onset (number)			
tremor	7 (100%)	25 (74%)	n.s.
bradykinesia	3 (43%)	23 (68%)	n.s.
asymmetry[a]	7 (100%)	22 (73%)	n.s.
Clinical signs at examination (number)			
rigidity[b]	6 (100%)	24 (96%)	n.s.
Bradykinesia[b]	6 (100%)	23 (92%)	n.s.
resting tremor[b]	6 (100%)	17 (68%)	n.s.
Mean Hoehn and Yahr score at exam	2.3 (±0.9)	2.8 (±1.1)	n.s.
Mean UDPRS score at examination[b]	17 (±5)	29 (±11)	P < 0.05
Mean MMSE score at examination[c]	29 (±3)	25 (±4)	P < 0.05
MMSE score < 24[c] (number)	1 (14%)	15 (44%)	n.s.
Family history[d] (number)			
Parkinson's disease	0	3 (9%)	n.s.
Essential tremor	0	3 (9%)	n.s.
Dementia	2 (29%)	6 (15%)	n.s.

[a] About laterality at onset, information was not available in four late-onset patients. [b] Due to dementia, or due to motor impairment in physical conditions, no reliable neurological examination could be performed in 9 late-onset patients and one early-onset patient. These patients were excluded from the UPDRS scores. [c] Nine late-onset patients did not take the Mini Mental State Exam (three declining cooperation; six due to profound dementia). [d] In 1st-degree relatives. UPDRS = Unified Parkinson's Disease Rating Scale; MMSE = Mini Mental State Exam; n.s. = not significant

Results

Patients

Of 113 individuals with parkinsonism ascertained from the genetically isolated population and its surroundings (Figure 1), 109 subjects (44 men, 65 women) were willing to participate in the study. After clinical and genealogical work-up, 52 of 109 parkinsonism patients had no ancestors from the GRIP region. The remaining 57 (52 percent) were linked to one common ancestor 14 generations ago. Of the 57 patients from the genetically isolated population, 16 were excluded from the present study of idiopathic PD. Of these 16 patients, 11 had other causes of parkinsonism (i.e., previous stroke in two patients, non-progressive symptoms in five patients and suspicion of Lewy Body disease in four patients), four early-onset patients were shown to have DJ-1-linked parkinsonism[5,21] and one had early-onset parkinsonism with additional neurological and dysmorphic symptoms suggestive of a different aetiology. In the remaining 41 patients, the clinical features were compatible with idiopathic PD.

Figure 1. Ascertainment of 41 idiopathic-PD patients in the genetically isolated population
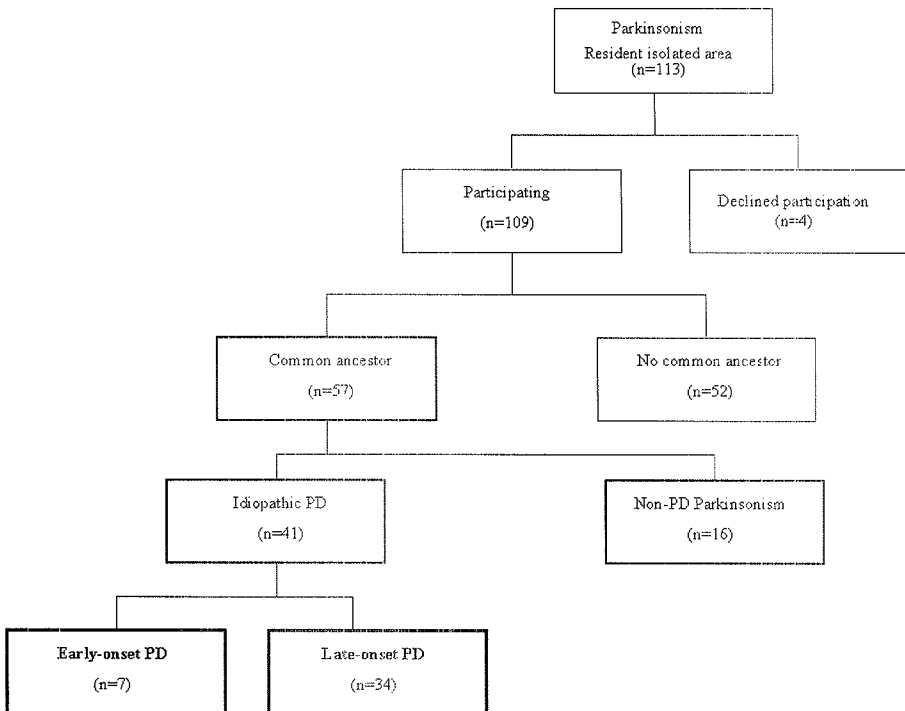
Clinical examination

In the study population at large, the average age at onset of idiopathic PD was 63.3 years. When plotting ages at onset (Figure 2), the distribution was found to be bimodal.

Assuming a log-normal distribution with equal variances, the hypothesis of a unimodal distribution was rejected in favour of the bimodal distribution (p = 0.0001). Based on peaks in this bimodal distribution, the geometrical mean in the age-at-onset distribution was 44.3 years in early-onset PD, and 66.8 years in late-onset PD. Using a cut-off point of 55 years, as in the Rotterdam Study[15], seven patients (17 percent) had a disease onset below age 55 years (early-onset PD), and 34 patients (83 percent) an onset at or over age 55 years (late-onset PD).

Figure 2. Distribution of age at onset of idiopathic PD in the genetically isolated population
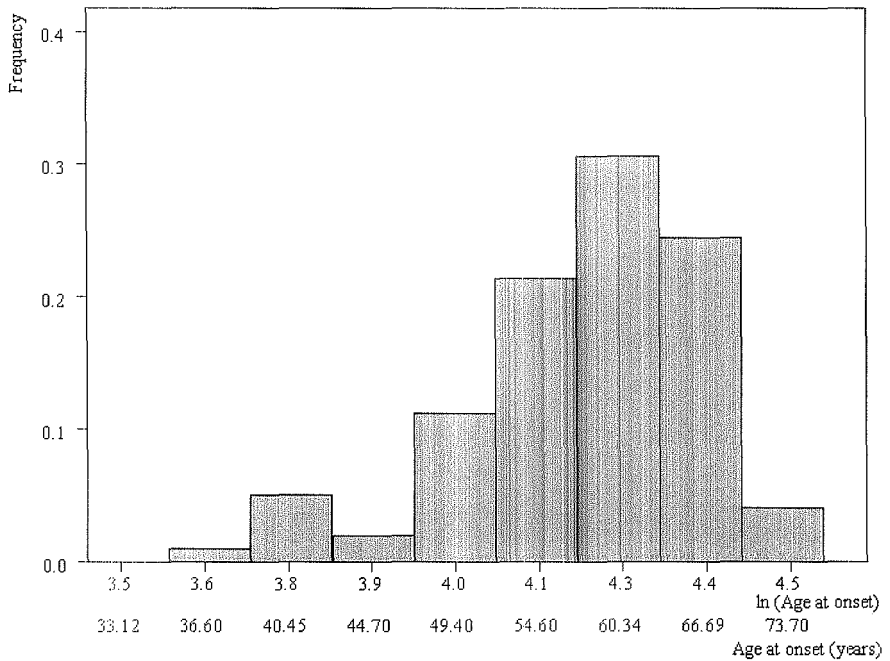
Clinical features of the seven early-onset and 34 late-onset patients with PD are compared in Table 1. The duration of illness was similar in both groups (7.0 years in the early-onset group compared to 5.6 years in the late-onset group). Levodopa treatment had been administered in 37 patients, whereas four patients had received amantadine or dopamine agonists only. Drug-induced dystonia and/or dyskinesias were reported in 43 percent of early-onset and in 21 percent of late-onset PD patients, but this difference was not statistically significant. All patients with early-onset PD had resting tremor and an asymmetric pattern as presenting symptoms at disease onset. These symptoms were less frequent, although not significantly so, in the late-onset patients (74 percent for resting tremor, and 73 percent for asymmetry). At neurological examination, the early-onset and late-onset patients did not differ significantly with

respect to rigidity, bradykinesia or resting tremor. The UPDRS score of the early-onset patients (17±5), however, was significantly better than that of the late-onset patients (29±11,p=0.012). Furthermore, patients with early-onset PD had significantly better MMSE scores than those with late-onset PD (p=0.025). Neuroimaging, available in 26 out of 41 patients, showed age-concordant cerebral atrophy in seven patients and a lacunar infarct in three patients. In the remaining patients, neuroimaging was unremarkable.

Figure 3. Pedigree of 41 idiopathic-PD patients from the genetically isolated population
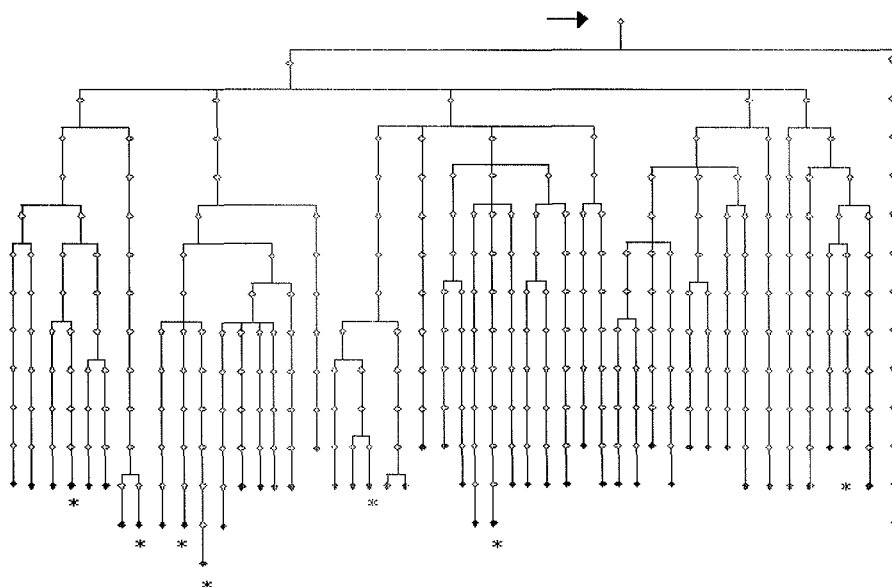
The filled symbols denote PD patients, and those marked with an asterisk early-onset PD patients. The arrow indicates the common ancestor.

Genealogy

Few patients had a positive family history for PD, essential tremor and dementia in first-degree relatives (see Table 1). Although only three patients reported a positive family history for PD in first-degree relatives, a large pedigree could be constructed (Figure 3) in which the seven early-onset patients (filled symbols with asterisks) and the 34 late-onset PD patients (filled symbols) were all connected to one common ancestor. The entire pedigree contains 601 individuals spanning 14 generations. The pedigree depicted in Figure 3 is constructed in such a way that the largest possible number of patients could be linked to the closest common ancestor. Multiple links between patients exist, but are not shown in this figure. Some patients could not be linked to another patient in a shorter way than through common ancestors up to 14 generations

ago. Eighty-three percent (34/41) of patients, however, could be connected to another patient within at most eight generations. When comparing separate pedigrees of the late-onset and early-onset groups of PD patients, patients with early-onset PD were not more closely related to one another than patients with late-onset PD. The pedigrees of early-onset and late-onset PD patients could thus be merged to make one large pedigree.

Genetics
None of the 41 PD patients in this study carried mutations in the DJ-1 gene. No parkin mutations were detected in the tested early-onset patients. Other known PD genes and loci (studied in 3 out of 7 early-onset patients and in 17 out of 34 late-onset PD patients) showed no common haplotype for markers flanking candidate genes. Furthermore there was no evidence for association to the disease with any of the markers surrounding the genes and loci on chromosomes 4q22, 6q25.2-q27, 2p13, 4p14-16.3, 4p14, 1p35-36, 1p36, 12p11.2-q13.1 and 1p32.

Discussion
In this study, we show that out of 109 patients with parkinsonism, a group of 41 PD patients could be linked to one common ancestor 14 generations ago. Between early-onset and late onset PD, few features differed significantly. Involvement of all known PD genes could be excluded.

In genetically isolated populations, disorders may present with a different phenotype compared to the general population. The size of the group of patients we studied was limited by the fact that they were derived from a small community and therefore warrants caution in the interpretation of differences between sub-groups. However, in terms of presenting and subsequent symptoms, PD patients in our study resembled PD patients from the Rotterdam Study.[15]

The earlier onset of PD in our study was nevertheless remarkable. In our study population overall, the average age at onset of idiopathic PD was 63.3 years, which is lower than the average age at onset observed in prevalent PD-patients included at baseline in the Rotterdam Study (71 years),[15] and than that observed in a large European study (72 years).[22] This could partly be explained by a high proportion of early-onset idiopathic PD in the genetically isolated population (17 percent), which was higher than that observed in PD patients at baseline in the Rotterdam Study (five percent).[15] The Rotterdam Study, however, only included in its follow-up those subjects surviving to be 55 years and older. Excluding from our study group all early-onset patients, the mean age at onset in the idiopathic-PD patients from the genetically isolated population increased to 67.1 years, which is still significantly lower than the mean age at onset in the Rotterdam Study ($p < 0.001$).

One explanation for the early age at onset of disease in our patients may be selection bias in favour of patients with early onset of disease. Our patients were,

however, ascertained through general practitioners based on the diagnosis of PD and use of dopamine and dopaminergic medication. Previously was shown that in The Netherlands, over 88 percent of PD patients can be ascertained in this way, and health-care facilities in the GRIP population closely resemble those elsewhere in the country.[15] Moreover, the overall participation rate in our study was high (109 out of 113 patients). Participation bias in early-onset patients (due to a greater disease awareness in individuals affected at a younger-than-average age) was therefore less likely to occur. The proportion of women in the late-onset PD sample from the genetically isolated population (71 percent) approximates the 65 percent female PD-patients observed in the Rotterdam Study,[15] which suggests that our ascertainment procedure led to a series of patients similar to that obtained in a door-to-door survey such as performed in the Rotterdam Study. In the early-onset PD patients in our study, the proportion of women is 43 percent, which agrees with the approximately equal sex ratio reported in another study of early-onset PD patients.[23] These findings make a high percentage of early-onset PD by biased ascertainment less likely. Furthermore, the gene implicated in early-onset parkinsonism in the local population[5] may represent a locally larger genetic burden in PD, resulting in more early-onset disease.[12]

Bimodality in idiopathic-PD onset-age distribution was present in PD patients in our population (p < 0.0001), but also in the Rotterdam Study, albeit of lesser significance (p = 0.03). Both geometrical means of the peaks in the Rotterdam Study were, however, positioned in the late-onset range of onset over 55 years (57 and 76 years).[15] This phenomenon could therefore be an artefact caused by a population-dynamic bottleneck in the general population (with a subsequent 'baby-boom'). From general demographic and genealogical data in the GRIP population and the Rotterdam Study, however, no such bimodality in age distribution could be noted (unpublished material). This suggests that the bimodal pattern is a PD-specific entity in our genetic isolate. The earlier-positioned age peak for PD in the GRIP population compared to the Rotterdam Study along with the finding of a larger genetic contribution in early-onset PD[12] points to another genetic factor for PD in our genetically isolated population.

As also appears from other neurodegenerative diseases such as Alzheimer's disease, neurological signs and symptoms show few differences between early-onset and late-onset PD.[15,23] Motor and cognitive performance scores were significantly worse in late-onset PD compared to early-onset PD, while the duration of disease was similar in patients with late and early onset of PD. Furthermore, PD-related cognitive decline occurred nearly exclusively in the late-onset patients. These are significant differences between early-onset and late-onset PD, but may not necessarily represent distinct clinical phenotypes or genetic causes.

The genealogical studies linking 41 patients with idiopathic PD to a common ancestor show that familial aggregation of PD may become evident by availability of extensive genealogical information.[13] The possibility cannot be excluded that the familial aggregation of PD patients is a chance finding. Unfortunately, no control

population was available from the same community to test for excess familial aggregation amongst PD patients. Even without a reference population, however, the common ancestor in these patients may indicate that PD patients in our genetic isolate can share a genetic risk factor. This risk factor is likely to only have low penetrance, since no consistent inheritance pattern for PD could be deduced from pedigree or family-history information on patients in our study.

The candidate-gene study did not show association of PD with presently known genes. Mutations in DJ-1, a gene for early-onset, autosomal recessive parkinsonism recently identified within the framework of our study, are frequent in the GRIP population, having a carrier frequency of almost one percent.[5] On the contrary, no mutations in DJ-1 were found in 380 control samples from the directly surrounding area and from elsewhere in The Netherlands. Although this finding supports the presence of a strong degree of genetic isolation of the GRIP population, none of the other, mostly late-onset, PD patients in this study carried mutations in DJ-1. Mutations in another gene, parkin, being the most important cause of disease in patients with early-onset parkinsonism,[23,24] did not account either for the excess of early-onset PD in the genetic isolate. In this population, yet another genetic factor may therefore be involved in PD.

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CHAPTER 3.3

Parkinson's disease in a genetically isolated population: results from a genomic screen

Abstract

Apart from some rare mendelian forms, the genetic basis of Parkinson's disease (PD) is not known. In contrast to PD in the general population, genetically isolated populations with a limited number of founders are expected to harbour a genetically more homogeneous variant of PD. We performed a genomic screen in PD patients from a genetic isolate, results of which are given here. A second genomic screen in a larger series of patients is ongoing. Two peaks were identified on chromosomes 19 and 10. Upon follow-up analysis of the positive findings, the association for chromosome 19 appeared to be false positive. The association for chromosome 10 was further examined by saturating the region with a denser set of markers. Unfortunately, haplotype construction was not possible due to an ambiguous marker order. Furthermore, more than one allele was found to be associated. Analysis using the CLUMP programme, which merges alleles to give the highest chi-square statistic, yielded significant results for two markers in the fine-typed region on chromosome 10. The first concerned the originally associated marker on chromosome 10, of which two alleles gave a significant result. One of these was the originally associated allele in the first genomic screen. A second marker on chromosome 10 had a single associated allele. The marker order now needs to be determined, and the finding replicated in an independent data set.

Introduction

Parkinson's disease (PD) [MIM 168600] is a late-onset neurodegenerative disorder characterised by bradykinesia, resting tremor and rigidity. The cerebral pathology includes loss of dopaminergic neurons, particularly in the substantia nigra, and cytoplasmatic eosinophilic inclusions called Lewy bodies. In the majority of patients the cause of PD is unknown, and currently available therapy neither halts nor cures the disease. The role of genetic factors in the origin of PD with mendelian inheritance is being rapidly elucidated, now that 10 recessive and dominant forms have been identified.[1-10] The genetic basis of the, usually late-onset, form of PD with a complex inheritance pattern, however, is less evident. Although twin studies showed the genetic component of late-onset PD to be much smaller than that of early-onset PD,[11] the risk in 1st-degree relatives of PD patients to develop the disorder is still 2-14 times the risk in 1st- degree relatives of unaffected family members, arguing in favour of a genetic component.[12] In Icelandic PD patients who were at first sight unrelated, familial aggregation of the disorder was demonstrated by a genealogical search upon which a locus for late-onset PD, PARK10, could be mapped to chromosome 1p32.[13,14]

Linkage disequilibrium (LD) mapping of disease loci is based on the identification of marker alleles or haplotypes, which are more frequent in patients than in controls. The general population is assumed to be too genetically heterogeneous for such an approach. In such populations, genome-wide association studies using LD mapping would require very dense maps of polymorphic markers or Single Nucleotide Polymorphisms (SNPs) in large case-control series, which is currently not yet feasible.

On the contrary, genetically isolated populations may be more homogeneous due to a small number of founders. Furthermore, LD might be maintained over sizeable regions around disease genes in recently isolated populations. Coarser marker maps of highly informative single tandem repeat (STR) markers might therefore be sufficient to successfully map disease genes in isolated populations,[15] as was illustrated by the recent discovery of the PARK10 locus.[14] Kruglyak has shown that factors of importance for successful LD mapping in isolated populations are a small founder population and subsequent exponential population growth with only limited inward migration.[16]

We studied 42 PD patients with ancestry from a genetically isolated population in The Netherlands. The aim of this study was to find a gene involved in the late-onset form of PD. An analysis of the positive findings in a genomic screen with 21 PD patients in this genetically isolated population is reported here. A second genomic screen of all 42 patients and in-depth analysis of the data is ongoing.

Methods

Patients

This study is part of a research program named Genetic Research in Isolated Populations (GRIP).[7,17-19] The scientific protocol of GRIP has obtained approval of the

Medical Ethics Committee of the Erasmus MC. The GRIP population is a genetically isolated community in the Southwest of The Netherlands. Around 1750, this population counted approximately 150 individuals, followed by minimal inward migration and considerable population growth.[20] An estimated 20,000 descendants of this population are now scattered over eight adjacent villages. Church and municipal registers are readily available and date back to the 1800s. Genealogical history up to 1600 has been computerised to a large extent, holding information on approximately 60,000 individuals.

Patients with parkinsonism were traced through local general practitioners, neurologists and nursing-home physicians as described in *Chapter 3.2*. All patients provided informed consent to participate in the study. Parkinsonism was diagnosed when at least two out of three cardinal symptoms (bradykinesia; rigidity; resting tremor), as well as clinical improvement on dopaminergic therapy were present. The diagnosis of idiopathic PD was established after exclusion of other causes of parkinsonism and was verified by two independent neurologists according to criteria of EUROPARKINSON.[21] Data on presence of PD, essential tremor and dementia in first-, second- and third-degree relatives were collected by means of a family-history questionnaire. In order to detect any subclinical or untreated parkinsonism, first-degree relatives of patients also underwent neurological examination. Finally, genealogical information obtained from participants and local municipal, church and computerised registers was extended up to 16 generations.

The initially ascertained data set used for the first genomic screen contained 21 PD patients and their relatives. In order to increase sample size for the follow-up analysis of our positive findings, this initial group was extended with another 21 PD patients, their relatives and 88 control individuals, all from the GRIP population. The latter series were ascertained later on. Regarding clinical and genealogical (kinship) characteristics, there were no significant differences between the first and the second group. The second, ongoing, genomic screen includes all 42 patients, their relatives and 88 controls.

Genetics

For all patients and available first-degree relatives, genomic DNA was isolated from peripheral blood as described elsewhere.[22] For the systematic genome scan were used short tandem repeat polymorphisms (STRPs) from the ABI PRISM® Linkage Mapping Set MD-10 (Applied Biosystems). Additional markers for fine mapping were obtained from the Genethon and Marshfield genetic marker sets. Information about marker order and distances were obtained from the Marshfield integrated linkage map and Celera human genome database. Genomic DNA (20 ng) was amplified in a 7,5 µl PCR reactions containing 1X GeneAmp PCR Gold Buffer; 1.5 mM $MgCl_2$; 10 pmol of each primer (forward primer labeled with FAM, TET or HEX), 250 uM dNTPs and 0.4 units of AmpliTaq Gold DNA polymerase. Initial denaturation was 15' at 95 °C followed by

32 cycles of 30'' denaturation at 95°C, 30'' annealing at 55 °C and 90'' extension at 72 °C. Reactions were prepared using a Beckman Biomek 2000 robot system and performed in 384-well plates covered with sealing lids (Costar 6557; 6555). Amplification was done using a dual 384-wells equipped Perkin Elmer GeneAmp PCR System 9700. PCR products were pooled and loaded on an ABI377 (filterset D; 6.25% denaturing FMC LongRanger acrylamide gel) or ABI3100 automated sequencer, data were analyzed using ABI GeneScan 3.1 and ABI Genotyper 2.5 or Genemapper software from Applied Biosystems. As reported in Chapter 2.2 and 3.2, all patients in this study screened negative for mutations in the DJ-1 gene (which were identified in a family with early-onset autosomal recessive parkinsonism from the same community). Furthermore, 3 of 7 early-onset, and 18 of 35 late-onset PD patients were studied for genetic markers flanking PD genes, [1-10,14] but showed no association.

For chromosome 10, saturation mapping with additional markers (D10S1651, D10S217, D10S1676, D10S1439, D10S1655, D10S1248, D10S169, D10S1770, D10S555, D10S1675, D10S590, D10S1711 and D10S1700) was done in all 42 patients and in a control group of 88 healthy individuals from several other studies in the GRIP population. For chromosome 19, marker D19S418 was typed in additional sets of healthy controls (n = 92), Alzheimer's disease (AD) patients (n = 19), diabetes mellitus (DM) patients (n = 60) from the GRIP population, as well as in a group of randomly selected individuals from the Rotterdam Study, a population-based cohort study[23] (n = 352).

Statistical analysis
Marker data from the genomic screen were analysed using a test for combined linkage and association. This test is a family-based adaptation of the method described by Terwilliger and assumes that, when many of these chromosomes descend from a single ancestor carrying the disease mutation, marker alleles will be over-represented on chromosomes with the disease mutation.[24,25] The proportion of increase of the ancestral allele in disease chromosomes is represented by the parameter lambda (λ), which is estimated by maximizing the log-likelihood function. Here, the likelihood is the weighted sum over potential founder-alleles of conditional likelihoods given an ancestral allele. The weights are equal to the a priori probability of an ancestral allele, which are estimated from the allele frequencies in controls (in this situation the spouses of the 21 patients). For the linkage component of the analysis, a dominant genetic model with a penetrance of 40 % was assumed. The disease-gene frequency was kept constant at 0.1 % and the recombination frequency at 0.01.

For the saturation-mapping analysis, phases were set using GENEHUNTER[26], yet haplotype construction was not possible due to an ambiguous order of markers on the physical map. Moreover, with the assumption of a single allele being associated, no haplotypes extending beyond two markers were possible. We then decided to perform single-point analysis of each marker separately, and allowing for more than

one associated allele per locus. To this end, the CLUMP programme was used. This method clumps alleles together into a 2-by-2 table in such a way that the highest chi-square statistic is obtained.[27] Its corresponding p-value is obtained by means of Monte Carlo simulations. By doing so, more than one ancestral haplotype in patients is allowed for because of marker mutations, early recombinations or multiple ancestors introducing different alleles. For the associated markers, lambdas are estimated for risk alleles.

Results

Forty-one patients with idiopathic PD could be connected to a common ancestor 14 generations ago. A simplified version of the pedigree of 41 patients is depicted in Figure 3 of *Chapter 3.2*, leaving out the multiple links amongst individual patients to obtain better visibility. A forty-second patient with ancestry from the GRIP population was added to this series in a later stage. The first genomic screen, which comprised the series of 21 PD patients and their 37 first-degree relatives who were ascertained initially, yielded significant evidence for combined linkage and association for a marker on chromosome 10qter, the 211-base pair (bp) allele of D10S1651 ($\lambda = 0.47$, p = 0.009). A second positive finding concerned the 96-bp allele of marker D19S418 ($\lambda = 0.99$, p = 0.02).

The lambda value for the associated allele of D19S418 was exceptionally high because all patients carried at least one 96-bp allele and 7 patients were homozygous. Since the method used for this analysis[24,25] derived allele frequencies from the control group, we extended our original sample to more groups in order to obtain better estimates of the control allele frequency for this marker. Therefore, D19S418 was also typed in series of individuals from GRIP (92 healthy controls, 19 AD patients, 60 DM patients) and from the Rotterdam Study (352 randomly selected individuals), and information on CEPH families was obtained. Upon extension to larger control groups, it appeared that the 96-bp allele is not only by far the most frequent allele amongst PD patients, but also amongst all other groups. The peak for this marker was therefore assumed to be false positive, probably due to the small sample size of control alleles used in the genomic screen, or to a minimally informative marker.

The lambda value for marker D10S1651 was also high ($\lambda = 0.47$) and was therefore tested in a larger control group in order to obtain a better estimate of the allele frequency. Moreover, the marker flanking D10S1651, D10S217, was weakly positive in the first genomic screen, which raised the question whether there could be a haplotype of two adjacent markers. Twelve additional markers on chromosome 10 around D10S1651 (D10S217, D10S1676, D10S1439, D10S1655, D10S1248, D10S169, D10S1770, D10S555, D10S1675, D10S590, D10S1711 and D10S1700) were subsequently tested in the case-control series of 42 patients and 88 controls from the GRIP region. Attempts were made to construct haplotypes by using the originally associated 211-bp allele as a starting point, but only by assuming early recombinations

or mutations could any haplotype be extended beyond two markers. Moreover, the order of the markers in this telomeric region of chromosome 10q proved to be unreliable: publically available maps through the NCBI showed conflicting positions, which could not be omitted by consulting the existing physical maps at NCBI or Celera as large parts of this region consist of draft and unfinished sequence. Therefore, the CLUMP method was used.[27]

By using CLUMP, two of 13 markers appeared to be significant. All other markers had p-values > 0.30. In Table 1, the risk alleles of the two associated markers are given. One of these markers was the original marker D10S1651, for which two risk alleles were identified (p = 0.049). The original 211-bp allele has a lambda of 0.12, and the other allele, the 229-bp allele, a lambda of 0.08. The other marker was D10S1711 with only one risk allele, the 177-bp allele (p = 0.006, λ = 0.21).

Table 1. Association of marker D10S1651 and D10S1711 with PD (42 patients; 88 controls)

Marker	Frequency cases	Frequency controls	λ	p-value
D10S1651[a]				0.049[c]
211-bp allele[b]	0.17	0.10	0.12	
229-bp allele	0.27	0.17	0.08	
D10S1711				0.006[c]
177-bp allele	0.32	0.14	0.21	

[a] Original peak from genome scan; [b] originally associated allele; [c] two-sided p-value obtained by simulation (n = 10,000), taking into account that 2-by-2 table is formed in order to obtain the highest chi-square statistic

Discussion

We here report significant evidence for linkage and association for a marker on chromosome 10q26.3-qter in a group of 21 patients with typical PD from a genetically isolated population in The Netherlands who share an ancestor 14 generations ago. Upon saturating this region with a denser set of markers in an augmented patient sample (42 patients as well as 88 controls from the GRIP population), the control allele frequencies could be estimated more accurately and the significance of the association with the original marker was maintained. Haplotype analysis was not possible, firstly because the assumption of association of a single allele did not hold and secondly because of an unknown marker order. Our initial approach, used for analysis of the genomic screen,[24,25] assumes a single ancestral allele and hence lacks power in situations such as reported here.

When more than one risk allele can be expected (due to early recombinations dispersed throughout the population or multiple founders) clustering alleles into one group would increase the power to detect these variant alleles. The CLUMP programme allows for multiple alleles to be merged into one virtual risk-allele.[27] Using CLUMP, two alleles of D10S1651 together gave a significant over-representation by patients compared to controls.

When identified risk alleles only slightly differ in length (2-4 bp), early marker mutations may have occurred. The two alleles of D10S1651 identified by CLUMP in our study, however, differed by 18 bp. The explanation of marker mutations is therefore biologically implausible, leaving multiple founders or early recombinations as a possible reason. Although the originally associated risk allele maintained a significant association in the follow-up analysis in a larger series of patients, a marker order could not be determined with sufficient certainty to construct haplotypes. This currently limits the interpretation of the findings reported here. To enable haplotype analysis, the marker order will hopefully be ascertained on a physical map or on a newly constructed genetic map. Finally, independent and larger patient series must be tested to confirm these findings.

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CHAPTER 3.4

Mutations in the haemochromatosis gene (HFE),

Parkinson's disease and parkinsonism

Abstract

Iron overload increases oxidative stress and can lead to neurodegenerative diseases like PD. We therefore studied the role of mutations in the haemochromatosis gene HFE in PD and parkinsonism. Two population-based case series were studied- the first counting 137 patients with PD and 184 with parkinsonism, and the second of 60 patients with PD and 85 with parkinsonism. In the first series, PD patients were significantly more often homozygous for the C282Y mutation than controls (p = 0.03), and in the second, parkinsonism patients were more often homozygous for the C282Y mutation than controls. Although hampered by small numbers, our data suggest that homozygosity for the C282Y mutation in the HFE gene increases risk of PD, and possibly of parkinsonism overall. Given the rarity of this genotype, large series of patients are required to prove this hypothesis.

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In as early as the nineteen twenties, iron was suggested to be involved in the pathogenesis of Parkinson's disease (PD).[1] Increased iron concentrations in the substantia nigra are seen in brains of PD patients on magnetic resonance imaging and at postmortem examination.[2,3] It has been postulated that excessive iron deposition in the brain causes overproduction of free radicals, giving rise to parkinsonism.[4] The recent implication of DJ-1 in autosomal-recessive parkinsonism, a gene involved in the response to oxidative stress,[5] raises the question which is the contribution of iron-related oxidative stress to PD and parkinsonism in the general population.

Haemochromatosis is an iron overload disorder with increased absorption and progressive storage of iron in several tissues of the body. Two mutations in the HFE gene account for most cases of hereditary haemochromatosis.[6,7] The C282Y mutation is highly penetrant, leading to intracellular sequestration of iron.[8] The H63D mutation is much less penetrant and is, although biological evidence for abnormal iron processing is not conclusive, a susceptibility factor for haemochromatosis.[9] In haemochromatosis, iron deposits were observed in the basal ganglia, causing clinical parkinsonism.[10] Its role in iron metabolism makes HFE a potential candidate gene for PD.

Results of studies on the role of HFE mutations in PD have not been consistent and vary from an unexpected protective effect of C282Y heterozygosity,[11] to no effect of either mutation.[12] Problems in the interpretation of the previous studies are the highly selected patient series (with a likely mendelian origin of disease) the studies were based upon, as well as the various sources from which the control series were derived. We therefore studied mutations in the HFE gene in two series of PD patients and in controls, all derived from population-based samples.

HFE mutations may not necessarily lead to classical, idiopathic PD, but rather to parkinsonism which does not entirely meet criteria for PD (bradykinesia, rigidity and tremor, and improvement on dopaminergic substitution).[10] We therefore also assessed presence of HFE mutations in all patients with parkinsonism (consisting of PD plus all other syndromes, regardless of pathogenesis, of which clinical signs resemble PD) in the two population-based series.

The first series of 137 patients with PD, 47 patients with parkinsonism by other causes and 2914 controls was derived from the Rotterdam Study, a large cohort study in an elderly population (aged 55 years and over) in suburban Rotterdam, The Netherlands. A detailed description of this cohort of 7,983 individuals is given elsewhere.[13] The second series comprised another 60 patients with PD and 25 patients with parkinsonism by other causes and was derived from the Southwest of The Netherlands. The control group from the Rotterdam Study was also used for the second series. The protocols of both studies have obtained approval of the Medical Ethics Committee of the Erasmus MC. Before being entered into the study, all participants provided their informed consent. Information on past and present health status was obtained by means of a standardised questionnaire. Participants were screened for and eventually diagnosed with PD or parkinsonism according to the EUROPARKINSON

protocol.[14] Control individuals with a history of PD, parkinsonism or other neurodegenerative disease were excluded.[15] DNA was isolated from whole blood using the salting-out procedure,[16] and the samples were genotyped for the HFE mutations C282Y and H63D as described previously.[7] Chi-square tests, Fisher exact tests and regression analysis were performed with two-sided significance thresholds of 0.05.

In the two series of cases and in the controls, there were no significant deviations from Hardy-Weinberg equilibrium proportions. The first series of PD patients derived from the Rotterdam Study consisted of 137 individuals (41 percent males and 59 percent females) with an average age of 75.8 ± 8.1 years. The second series of PD patients from the Southwest of The Netherlands consisted of 60 individuals (37 percent males and 63 percent females) with an average age of 68.2 ± 10.0 years. The control group of 2914 individuals from the Rotterdam Study (50 percent males and 50 percent females) was on average significantly younger (66.8 ± 7.5 years) than the case series from the Rotterdam Study, but not from the series from the Southwestern Netherlands. Genotype and allele frequencies in PD and parkinsonism cases and controls are given in Table 1. For the C282Y mutation, the proportion of C282Y homozygotes amongst PD patients from the Rotterdam Study was higher than in controls (OR 5.4; CI 1.1-25.6, p = 0.03). A C282Y allele-dosage effect (an increase in frequency of C282Y heterozygotes) was not observed. Of PD patients from the Southwest of The Netherlands, none were homozygous for the C282Y mutation. In patients with parkinsonism from the Rotterdam Study, the C282Y-homozygosity excess was accounted for by the aforementioned typical-PD patients. From the smaller series from the Southwest of The Netherlands, one patient with parkinsonism proved to be homozygous for the C282Y mutation, but the excess of C282Y homozygotes did not reach significance. There was no evidence for a difference in frequency of any of the other genotypes in the two series. In particular, the frequency of heterozygosity for C282Y was not significantly reduced in patients with patients with PD or parkinsonism.[11]

Our study showed an increased prevalence of C282Y homozygotes amongst PD patients in the Rotterdam Study. Two more case-control studies on the relation between C282Y and PD have been conducted.[11,12] One study reported a protective effect for C282Y carriers.[11] These findings are, however, difficult to interpret as the control group was composed of, amongst others, siblings of patients, thereby possibly diluting the effect of genetic risk factors such as the C282Y mutation. The other study [12] reported no association of HFE mutations with PD. In the patient series in this study, the proportion with a positive family history for the disease was high (52.3 percent[12]). In comparison, the proportion of patients with a positive family history for PD was 4.9 percent in the Rotterdam Study[17] and 7 percent in the Southwest of The Netherlands (data not shown). Possibly, genes other than HFE play a predominant role in PD

Table 1. Distribution of the HFE genotypes and alleles in Parkinson's disease (PD) and parkinsonism (PS) patients from the Rotterdam Study and from the Southwest ern Netherlands, and in controls

	Rotterdam Study			Southwestern Netherlands			Controls (%)
	All PS (%)	PD (%)	other PS (%)	All PS (%)	PD (%)	other PS (%)	
	n = 184	n = 137	n = 47	n = 85	n = 60	n = 25	n = 2914
Genotype							
wt^a/wt	117 (63.6)	89 (65.0)	28 (59.6)	51 (60.0)	38 (63.3)	13 (52.0)	1838 (63.1)
wt/H63D	38 (20.7)	31 (22.6)	7 (14.9)	20 (23.5)	16 (26.7)	4 (16.0)	661 (22.7)
H63D/H63D	2 (1.1)	2 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	68 (2.3)
wt/C282Y	20 (10.9)	10 (7.3)	10 (21.3)	12 (14.1)	6 (10.0)	6 (24.0)	290 (10.0)
H63D/C282Y	5 (2.7)	3 (2.2)	2 (4.2)	1 (1.2)	0 (0)	1 (4.0)	49 (1.7)
C282Y/C282Y	2 (1.1)	2 (1.5) [b]	0 (0)	1 (1.2)	0 (0)	1 (4.0)	8 (0.3)
Allele frequencies							
wt	79.7 %	80.6 %	77.2 %	77.5 %	79.6 %	72.1 %	79.4 %
H63D	12.7 %	13.8 %	9.1 %	13.9 %	15.3 %	10.4 %	14.5 %
C282Y	7.6 %	5.6 %	13.7 %	8.6 %	5.1 %	17.5 %	6.1 %

[a] wt = wild-type allele; [b] p = 0.03

patients with a positive family history for the disease.[12] Furthermore, the mean age at onset of PD in the latter study was comparatively low (52.3 years), which may also indicate a, different, genetic contribution to PD in this particular study population.

Here we analysed two separate groups of PD cases with respect to a large control group. The first series of patients and the controls were derived from the same strictly defined source of the Rotterdam Study,[13] reducing a potential selection bias. In order to minimise any underestimation of the effect of the HFE mutations on PD or parkinsonism, we excluded patients with neurodegenerative disease in the selection of control subjects. The genotypes in the PD patients and controls from the Rotterdam Study and the PD patients from the Southwest of The Netherlands were in Hardy-Weinberg equilibrium proportions, suggesting no major selection against C282Y homozygotes. A question arising when studying a gene such as HFE in a late-onset disorder such as PD, is whether C282Y homozygotes live long enough to develop PD. They are at increased risk of liver pathology, diabetes and vascular disease.[18] Interestingly, both C282Y-homozygous PD patients in our study were female (data not shown), and women are known to have an increased survival in haemochromatosis.[19] Probably due to regular blood loss during menstruation, female C282Y homozygotes are at lower risk for clinical symptoms of iron overload than male C282Y homozygotes.

Our study shows an increase in frequency of PD in individuals homozygous for the C282Y mutation, and is in line with the hypothesis that, through iron deposition, C282Y homozygosity increases the risk of PD and possibly of parkinsonism overall. An important implication of our findings is that the protective effect of the C282Y mutation[11] is not supported by our data. The number of C282Y homozygotes in our study, however, was small, and our findings remain to be confirmed. Further study in larger populations is needed to assess the role of C282Y homozygotes in PD.

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CHAPTER 4

GENERAL DISCUSSION
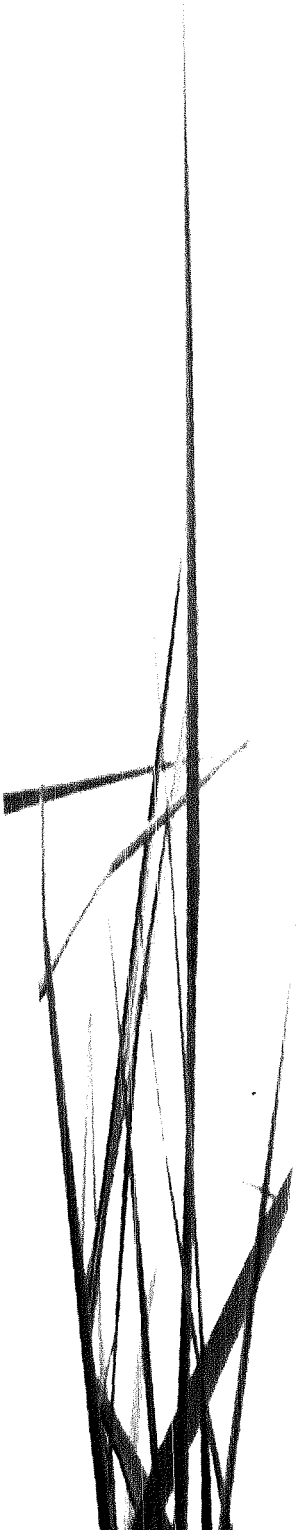
4.1   Introduction

Parkinson's disease (PD), one of the most frequent movement disorders of old age, has raised many a debate about its cause. The first known descriptions of PD come from ancient India and date back approximately 4,000 years.[1] Lead by the cardinal symptoms bradykinesia, rigidity and tremor, it was probably the Greek physician Galen (175 A.D.) who introduced the descriptive term 'shaking palsy', which was in use up to the 19th century. Yet it was James Parkinson, an English physician, paleontologist and politician, who in 1817 provided a modern and detailed account of the disorder.[2] His name has been associated with the condition since.

After these two centuries, treatment of PD is still based on symptom relief. As dopamine deficiency is largely responsible for the motor disturbances in PD, the therapeutic regimen is primarily formed by dopamine substitution. Medicinal extracts of cowage or velvet bean (*Mucuna pruriens*), the seeds of which contain large amounts of levodopa,[3] have been used to alleviate symptoms of PD for thousands of years. In 1961, a synthetic analog of levodopa was manufactured, which formed a milestone in the development of PD therapy. The American Food and Drug Agency (FDA) registered synthetic levodopa for clinical applications nine years later, upon which it became the mainstay of PD treatment.[4] Dopamine suppletion can, however, neither cure nor halt the disease process.[1] Furthermore, although not confirmed *in vivo*, some experimental evidence suggests that therapeutic dosages of levodopa accelerate nigral decline *in vitro* by the production of free radicals.[5]

Prevention or cure of PD will become feasible only when causal mechanisms in PD are clear. In recent years, rare mendelian forms of PD have been unraveled (see *Chapter 2* on familial parkinsonism).[6-15] The bulk of PD, however, is the common, sporadic form; mendelian variants form the tip of the iceberg. *Chapter 1.1* has already reviewed general aspects of the genetics of PD. This chapter will address methodological aspects, implications and forthcoming lines of research of the studies conducted within the framework of this thesis.

4.2   Familial parkinsonism

4.2.1   *Methodological aspects*

Mapping and significance of PD genes

However rare single-gene variants of PD may be (see *Chapter 1.1*), they have lifted a tip of the veil covering the causal mechanism(s) in PD. Ten mendelian forms identified to date represent the growing body of evidence for genetic heterogeneity in PD.[6-15]

Three of five PD genes, alpha-synuclein, parkin and DJ-1, were identified by positional cloning.[6,7,12] Their biological properties have provided the first important clues about pathways leading to PD. Alpha-synuclein and parkin[6,7] were the first two genes discovered and are, in terms of biology and neuropathology, characterised extensively, leaving little if any doubt about their causal role in the corresponding

forms of PD.[16-19] About DJ-1,[12] no details about function or neuropathology are known yet.

The remaining two PD genes UCH-L1 and NR4A2 were candidate genes for a role in PD based on their function, and large series of familial-PD patients were screened for mutations in these genes.[10,15] UCH-L1 is an enzyme in the ubiquitin-mediated protein degradation pathway and NR4A2 plays a role in the genesis and maintenance of mesencephalic dopaminergic neurons.[10,20] One mutation in the UCH-L1 gene in a single family, and two mutations in a non-coding exon of the NR4A2 gene in 10 families were thus identified.[10,15] In UCH-L1, the mutation was associated with a decreased enzymatic activity *in vitro*, and in NR4A2, the two exonic mutations were associated with decreased mRNA transcription (by an unknown mechanism).[10,15] In both cases it concerned, in terms of PD neuropathology, biologically plausible genes. Also, the mutations had functional consequences. Nevertheless, regarding the fact that the latter two genes were studied based on an a *priori* hypothesis on their role in the pathogenesis in PD, they raised the question whether the observed mutations could be chance findings due to large-scale screening, and in fact be rare polymorphic variants.[21,22] Extensive efforts have been made to confirm the role of UCH-L1 mutations in familial PD, yet in the five years since the original report, no confirmation in other families has become available.[10,23,24] The possibility remains, however, that the originally reported mutation[10] is a valid finding but mutations in UCH-L1 are simply excessively rare. Preliminary confirmation studies on the role of NR4A2 in other families have been negative,[22,25] but due to its only recent discovery, more studies can be expected.

Research in genetic isolates
Genetically isolated populations represent an important and powerful tool in genetically mapping inherited disorders. Populations can develop into genetic isolates due to a variety of reasons (Table 1).[26-33] As a result of a limited number of founders and genetic drift, such isolated populations are less genetically heterogeneous than the general population. Within a genetically isolated population, one can assess genetic traits on a family basis, but also on a population basis. Patients from an isolate who have a common ancestor are expected to share stretches of DNA flanking a disease gene and thus exhibit a larger extent of linkage disequilibrium than individuals from the general population. This property may facilitate detection of disease genes. Homozygosity mapping, the method used in *Chapter 2.1*, enables localisation of homozygous chromosomal regions that are identical by descent (IBD). In consanguineous families showing a pattern of disease inheritance compatible with a recessively acting gene defect, this is a suitable approach to map a disease gene. Although homozygosity mapping can be useful to examine a distinct trait in a consanguineous pedigree, caution is warranted when several traits co-exist in one family. The exact segregation pattern should be determined for each trait

independently, since one would be inclined to analyse these in terms of a syndrome (with the underlying assumption of a single genetic or genomic cause). Even in an inbred family with a rare disease, the degree of consanguinity can be larger than anticipated, such that coexistence of genetically independent phenotypes (caused by other genes elsewhere, and/or in associated homozygosity regions) must be considered, an issue also addressed in *Chapter 2.4*. Furthermore, the assumption of recessive inheritance does not necessarily apply to all of the observed traits, since some may in fact be inherited in a dominant or other fashion.

Table 1. Genetically isolated populations worldwide

Population	Nature of the isolation			Reference
	Religious	*Geographical*	*Sociocultural*	*Reference*
Amish (N.-America)	X			Kelley et al.,2002 [26]
Costa Rica		X		Freimer et al.,1996 [27]
Iceland		X		Gulcher et al.,2001 [28]
Finland		X		Kallinen et al.,2001 [29]
GRIP population (Netherlands)			X	This thesis
Hutterites	X			Robertson et al.,1999[30]
Mennonites (America)[a]	X			Morton et al.,2002 [31]
Palau (Micronesia)		X		Kruglyak,1999 [32]
Sardinia		X		Parratt et al.,2002 [33]

[a] *Groups of the Mennonite People (originally Swiss/German Dutch immigrants to the Eastern US) are found from Canada to Argentina*

Three of the four recessive PD genes and loci, *PARK6*, DJ-1 and *PARK9*, could be mapped to chromosome 1p36 by means of homozygosity mapping in consanguineous families.[11,14,34] In families with many consanguineous loops, homozygosity can be ample, leading to false-positive association with disease for a certain locus.[35] Furthermore, telomeric regions (such as chromosome 1p36) possibly have higher recombination rates, requiring a denser map of markers to detect linkage disequilibrium. A chance finding becomes unlikely when mutations (or linkage to the

same region) are found in independent families, suggesting independent mutational events. To date, mutations in the DJ-1 gene have been found in families of several ethnic origins across the world (Bonifati personal communication). Although support for linkage to PARK6[11] was obtained in eight families in different European countries,[36] this must be interpreted with caution, since it concerns linkage results from small families not showing statistically significant linkage by themselves, and largely based on the presence of compound heterozygotes. The causative gene within PARK6 remains to be identified. Finally, PARK9, the third locus on chromosome 1p36,[14] was identified in a single consanguineous Jordanian family. Linkage to PARK9 has not been confirmed elsewhere, but the locus is tentatively close to the DJ-1 gene and the PARK6 locus. This vicinity suggests some genomic rearrangement (e.g., inversions) or overlap for the PARK6- and PARK9-linked families. DJ-1 mutation analysis in the PARK9 and PARK6 families is necessary to rule-out involvement of DJ-1 in these closely adjacent PD-related loci.

Genealogical tools
The usefulness of community-derived patient series from a genetic isolate primarily comes with the availability of genealogical information. Nowadays, databases can contain genealogical relationships of an entire community over several generations. Since the early days of clinical genetics, detailed genealogical records have been indispensable for the assessment of family structures such as in the genetically isolated Amish and Mennonite demes of the United States of America. The availability of extended pedigrees facilitated the systematic clinical-genetic descriptions by McKusick, whose pioneering work in the Amish formed the basis of Mendelian Inheritance in Man (URL: http://www.ncbi.nlm.nih.gov/omim/). Furthermore, the Icelandic population recently passed a bill allowing their inclusion into a nationwide database used for genetic research in the deCODE project.[28] Similarly, the GRIP project, the framework within which research for this thesis was conducted, has a community-wide genealogical database, reaching back until the end of the sixteenth century and containing information about 60,000 individuals. Results of high-capacity genealogical searches can be impressive, linking all patients with a certain trait to a common ancestor many generations ago. Yet originating from a genetically isolated population *per se* is unspecific: finding a gene requires familial aggregation of a certain trait. Familial aggregation of a disease must always be viewed relative to a, preferably disease-free, control group from the same population. If this reference group is not available, the assumption that the selection of patients may have inherited a common susceptibility factor for a disease can then only be made with the greatest caution. A better approach is comparing a family history for PD amongst PD patients versus a family history for PD amongst controls.[37] If there are more observed than expected PD cases among patients' relatives than among the controls' relatives, there is familial aggregation. Information can still be unbalanced, however, as there is likely to be some

degree of recollection bias amongst patients (more inclined to remember having relatives with PD). Furthermore, the possibility that shared environmental factors also play a role in the clustering of traits within particular families cannot be excluded, such that exposure to putative risk factors should also be registered.

4.2.2 Implications and suggestions for further research

We ascertained a consanguineous family with early-onset parkinsonism suggesting an autosomal recessive pattern of inheritance in a genetically isolated community in the Southwest of The Netherlands. By means of homozygosity mapping and linkage analysis, a new locus for familial PD was identified on chromosome 1p36: *PARK7* (OMIM 606324) (*Chapter 2.1*). Since the identification of mutations in the alpha-synuclein gene in 1997,[6] this was the 7th monogenic form of parkinsonism identified worldwide. After an extensive search for the responsible gene in the 16-cM wide candidate-gene interval, all parkinsonism patients were shown to have a homozygous deletion of the DJ-1 gene (*Chapter 2.2*). In the other (Italian) family with significant linkage to *PARK7*, a point mutation in the DJ-1 gene was identified.

The function of the DJ-1 gene is unknown. *In vitro* studies on the DJ-1 protein and exposure to e.g., hydrogen peroxide or the neurotoxic pesticide component paraquat suggested involvement in the response against oxidative stress.[38,39] Another, more recent, study suggested the *S. cerevisiae* DJ-1 ortholog to assume chaperone-like properties in response to the presence of misfolded proteins.[40] This property, albeit observed in yeast, could be a tentative link to the postulated mechanisms of neurodegeneration in alpha-synuclein and parkin-linked parkinsonism.[16-19]

The identification of mutations in DJ-1 in familial parkinsonism will have impact on views of the pathogenesis of PD and according research. Interactions of parkin and alpha-synuclein are multiple,[18,19,41] but the common feature in all three previously identified PD genes alpha-synuclein, parkin and UCH-L1 is their involvement in ubiquitin metabolism. Anti-oxidant properties and/or chaperone-like properties of DJ-1 could all make DJ-1 a suitable candidate for a role in neurodegeneration.

The hypothesis of oxidative damage in PD has been long-standing but also much debated. Conclusive evidence for a role of oxidative damage in common PD is still lacking (see *Paragraph 4.3.1*). The discovery of DJ-1 therefore sheds new light upon an old hypothesis, nourishing further research. Since its identification in 2002, other mutations have been identified worldwide, making DJ-1 mutations the second-most common genetic cause of PD after parkin (Bonifati, personal communication). Yet it raises more questions than can be currently answered.

Genetics and molecular biology

By the time of the identification of DJ-1 mutations in parkinsonism, the available functional studies suggested it to be an mRNA transcription factor,[42] and to have

oncogenic properties.[43] Furthermore, DJ-1 was shown to be involved in male infertility in the rat, and, finally, in the response to oxidative stress.[38,39,44,45] The variety of suggested functions along with the ubiquitous presence and the strong conservation across species[46] suggest that DJ-1 has an important function. Which exactly, remains to be clarified in studies elaborating upon the association to neurodegeneration. Computer simulations have predicted the DJ-1 protein configuration to be changed by the L166P point mutation (identified in an Italian family, *Chapter 2.2*). The properties of the mutant DJ-1 protein and the neurodegenerative potential resulting from that abnormal protein configuration will have to be studied in more detail. With the identification of more mutations it will also be interesting to assess the similarities and differences of the impact upon protein configuration and function. In parkinsonism associated with mutations in alpha-synuclein, the severity of the clinical phenotype was correlated with the degree of disruption of the normal protein and its subsequent tendency to form aggregates.[46] Of the two parkinsonism kindreds with DJ-1 mutations in *Chapter 2.2*,[46] the Italian patients (in whom mutant DJ-1 protein was co-localised with mitochondrial structures in the cell) appear to be more severely affected than the Dutch patients (in whom the DJ-1 protein is absent) (Bonifati, personal communication). In order to obtain an accurate impression of the genotype/phenotype relation, more families with DJ-1 mutations are needed.

The function of the DJ-1 protein could be intertwined with other PD-gene products (e.g., parkin and alpha-synuclein) via a common pathway or mechanism. Therefore, any interactions between the DJ-1, parkin and alpha-synuclein proteins will yield a more complete picture of pathways to PD. Furthermore the co-localisation of the L166P mutant DJ-1 to mitochondria in an immunofluorescence study (*Chapter 2.2*) is intriguing and still unexplained. Firstly, the exact position of the precipitated protein will have to be determined, since it is not clear whether the immunofluorescent layer (containing the L166P mutant DJ-1 protein) coats the outer mitochondrial membrane, or in fact sits inside the mitochondrial organelle. Secondly, a routine screen for metabolic disease in one parkinsonism patient with DJ-1 deletions in the Dutch kindred revealed abnormal but not classifiable results for some aspects of ATP metabolism (Horstink, unpublished data). A systematic evaluation of the possible influence of the DJ-1 mutation on basal energy metabolism would be helpful to identify the pathogenic effects of mutant DJ-1.

Variations in a gene implicated in familial PD may also play a role in idiopathic PD. Polymorphisms in the DJ-1 gene were recently identified (Bonifati, personal communication). If these polymorphisms prove to be sufficiently informative, the involvement of DJ-1 polymorphisms on a population level could be assessed in series of PD patients and controls (e.g., population-based series such as the Rotterdam Study).

Finally, as long as autopsy material of patients (and clinically unaffected carriers) with DJ-1 mutations is not available for study, animal models homozygous or heterozygous for DJ-1 mutations may provide clues about the exact patterns of

degeneration in the brain and, if any, pleiotropic expression of the gene elsewhere in the body. An important issue to be addressed in this respect is the presence of Lewy bodies, a neuropathological key feature in PD,[47] which are usually absent in some mendelian forms of parkinsonism.[13,48] Furthermore, if other protein inclusions or aggregates reminiscent of other neurodegenerative disorders are observed, they could provide more clues about the mechanism of neurodegeneration. It will then be important to find out whether any such neurodegenerative features preferentially affect the substantia nigra or also other regions of the brain, such as the cerebellum and cortical areas (*Chapters 2.3* and *2.5*).

Clinical-genetic studies

In *Chapters 2.3*, *2.4* and *2.5*, various aspects of the phenotype of the patients with early-onset parkinsonism associated with deletions in the DJ-1 gene are described. Due to the small number of patients and relatives in this original kindred, conclusions are drawn with caution. The clinical presentation of parkinsonism and their neuroimaging features are similar to other recessive forms (parkin and *PARK6*-linked parkinsonism[7,11]) (*Chapters 2.3* and *2.5*). Distinctive clinical features in parkinsonism associated with DJ-1 mutations are psychiatric symptoms, which were not only present in all three patients of the first branch of the kindred, but also in one patient in the Italian family with a mutations in the DJ-1 gene. Furthermore, apart from a dopaminergic deficit typical for PD, functional neuroimaging as performed in one of the patients revealed cerebellar hypometabolism of glucose. Although this was also observed in parkin-associated parkinsonism, the hypometabolism could as well represent another, independent, familial trait (*Chapter 2.5*). Contrary to parkin- and *PARK6*-associated parkinsonism, heterozygotes for the DJ-1 mutation had normal presynaptic dopaminergic function on F-DOPA PET imaging (*Chapter 2.5*).[49,50] Due to the small number studied here, however, dopaminergic function will have to be assessed in larger series of patients and carriers. A detailed description of the original parkinsonism kindred with DJ-1 mutations was important to provide a first delineation of the associated phenotype. The more families are described in terms of clinical presentation, the sharper the delineation of the associated phenotype can become. The question whether, for instance, psychiatric features form a part of the parkinsonism phenotype or are a private trait in the Dutch and in the Italian patients (*Chapter 2.3*), can then be addressed. If compound heterozygous patients can be identified in whom psychiatric features do not or only rarely occur, this may indicate that these features are in fact caused by another gene in the *PARK7* homozygosity region.

When examining the patients from the Dutch kindred, a striking short stature and brachydactyly was noted, which formed a rather unusual combination with parkinsonism (*Chapter 2.4*). In order to assess segregation patterns of these traits we studied body height and hand-bone length in all patients and relatives in the kindred as well as in a control group from the same community. A significant association of the

DJ-1 deletion with body height was found, but for the brachydactyly segregation with the DJ-1 deletion was incomplete. This suggests that short stature is possibly caused by a gene or regulatory element within the region surrounding the DJ-1 gene, or even by DJ-1 itself. Brachydactyly, on the other hand, may be caused by yet another gene, and/or modified by a gene present in the *PARK7* homozygosity region. As mentioned in *Paragraph 4.2.1*, in families with a high degree of consanguinity with more conspicuous traits such as this kindred, disentangling the influence of DJ-1 gene mutations from the influence of other genes can be difficult, more so since the function of DJ-1 is unknown. In order to obtain a valid impression of the effect of the respective gene(s) involved, however, more families segregating this DJ-1 deletion, but also those segregating other DJ-1 mutations will have to be studied. Equally important in this respect are any relatives of DJ-1 deletion carriers who do not have DJ-1 deletions (wild-type genotype). In the kindred studied in this thesis, except for one sibling with the wild-type genotype, no other relatives with the wild-type genotype were available. This makes neither confirmation nor exclusion of a relation between the body-height and hand traits with the DJ-1 deletion possible (let alone a valid impression of the penetrance of the genotype).

The report on DJ-1 mutations in parkinsonism is the first to implicate DJ-1 in human disease. In animal studies, however, DJ-1 was shown to be associated with infertility in male rats.[45] Of the patients in the Dutch kindred homozygous for the deletion in DJ-1, three are male and one is female. The female patient has had one child, but none of the male patients have children. Any infertility, however, cannot be viewed independently of the psychiatric symptoms (present in all but one patient). In a genealogical assessment of all known heterozygous deletion carriers and generation-matched controls in the GRIP population (unpublished data), however, the number of offspring in male or female heterozygotes for the deletion was not smaller than that in the controls, which argues against fertility problems in at least the heterozygous deletion carriers. In this respect, in a work-up of novel families with DJ-1 mutations, the issue of fertility should be kept in mind.

Genetic counseling

When attempting to qualify a genetic study, implications for the patients involved are regularly speculated upon but often do not have to be dealt with all at once. When pathogenic mutations are indeed identified, communicating the findings to patients and their close relatives is very important. Depending on whether and how much the patient wishes to know about the genetic origin of his disorder, genetic counseling can be offered to patients and their relatives. In the case of early-onset disease (such as applies here), the choice whether or not to have children could depend on the outcome of genetic counseling. In rare recessive conditions occurring in the general population, this usually means that the affected parent may have physical and emotional limitations due to his disease, but that the *a priori* risk of the disease in

offspring is low, since the partner only has a small chance to also carry a mutation in the same gene. In genetically isolated populations, however, a priori probabilities can be quite different, aptly demonstrated by the fact that the carrier frequency of the DJ-1 deletion in the GRIP population estimated from a sample of village controls was almost one percent (Chapter 2.2). This frequency is higher than the frequency of 0.004 assumed in the linkage model described in Chapter 2.1. This might mean that genetic counseling and testing for this mutation in patients and partners from the GRIP population who (plan to) have children has certain 'high-risk' aspects, since that partner would also have the risk of almost one percent to carry a DJ-1 deletion. In this respect, the GRIP population would be comparable to e.g., Ashkenazi Jew, Amish and Mennonite populations with considerable degrees of consanguinity and high carrier frequencies for certain mutations. Ethically, counseling and testing must in this case only concern those individuals affected by parkinsonism and their relatives. Offering genetic counseling to asymptomatic heterozygotes from the community would be rather questionable, as it may generate more anxiety than it provides reassurance. Moreover, problems are the variable penetrance of the mutation in DJ-1 (Chapter 2.3) and the current lack of any effective long-term treatment for parkinsonism. Yet in this community with a high degree of consanguinity, based on allele-frequency calculations more homozygotes for the DJ-1 deletion are expected than the four individuals with parkinsonism detected in our survey (Table 2).

Table 2. Expected number of individuals for the DJ-1 deletion in the GRIP population.

Core population GRIP area	~ 8,000 inhabitants
Allele frequency in community control group	0.009
Probability of couples of heterozygous carriers	$(0.009)^2 = 0.0081$
Probability of homozygous offspring	$0.0081*0.25 = 0.002025$
Expected number of homozygous offspring[a]	$0.002025*8,000 \sim 16$

[a] p = 0.0008 versus observed number (4 homozygotes)

Population-genetic aspects of DJ-1

The GRIP population (which the originally described family originates from) provides a unique opportunity to study some population-genetic aspects of the DJ-1 deletion and its phenotype. By means of a genealogical study (Figure 1), all presently identified homozygous and heterozygous deletion carriers could be connected to a common ancestral couple seven generations ago (second half of the 18th century). The pedigree contained various consanguineous loops running between carriers, but these were left out of the Figure for better visibility. Furthermore, individuals in the pedigree have been masked for gender to protect confidentiality. The wife from the ancestral couple

(marked by the arrow) had children from two different men. The fact that deletion carriers are observed in the lineages from offspring of *both her* husbands suggests that she has introduced the mutation into the pedigree.

Calculations based on the allele frequency of 0.009 (Table 2) indicate that the statistically expected number of homozygotes for the DJ-1 deletion is fourfold the observed number (16 versus 4 individuals, respectively, $p = 0.0008$). Furthermore, the estimated number of expected homozygotes may be even higher due to considerable inbreeding in some sub-groups of the GRIP population. Several explanations for this discrepancy are possible.

Figure 1. Pedigree of DJ-1 deletion carriers in the GRIP population

The likely founder is marked with an arrow. For reasons of confidentiality, carrier gender has been masked. Filled diamonds indicate individuals homozygous for the DJ-1 deletion, and semi-filled diamonds heterozygotes.

Firstly, the assumed allele frequency of 0.009 (4 heterozygotes / 440) may not be representative of the true frequency due to a biased selection of controls. The control individuals, however, consisted of patients as well as healthy individuals derived from various other population-based studies in the GRIP population, and, to our knowledge, had no overrepresentation of particular diseases, family history for diseases or other features (unpublished data). Secondly, if the statistically expected number holds true, then the question arises what has become of the other 12

homozygotes. Any (combination) of the following explanations could account for these 'missing cases'. Since even amongst the four known parkinsonism patients in this isolate the severity of the observed phenotype is extremely variable (supported by the mild phenotype in two of the homozygotes who were newly diagnosed with parkinsonism in the framework of our research, *Chapter 2.3*), the other homozygotes may simply go unnoticed because their mild symptoms do not require medical attention. Furthermore, psychiatric features are a common feature of this and the Italian kindred, suggesting an association with mutations in DJ-1. In some homozygotes for the DJ-1 deletion, prominent psychiatric symptoms may thus be masking the motor symptoms of parkinsonism. If the psychiatric pathology in these individuals also requires admission to a psychiatric hospital, they reside outside the catchment area of the isolate and may not easily be ascertained. A survey for DJ-1 deletion mutations amongst psychiatric patients who originate from the genetically isolated population would clarify this, yet is hard to justify.

Finally, as mentioned in the section on clinical-genetic studies of DJ-1, male homozygotes for the deletion might be infertile and thus would not transmit the mutation due to 'reduced fitness'. One could further hypothesise that the DJ-1 protein, highly conserved during evolution (*Chapter 2.2*), is important during embryogenesis, such that offspring homozygous for the deletion are less viable and die more frequently *in utero*. In the parents of the Dutch parkinsonism patients, however, no history of (frequent) miscarriage or stillbirth was reported.

Therapeutic intervention ?

As stated in the introductory section of this chapter, developing a causal therapy for PD is within reach when at least some of the pathogenic mechanisms are clear. If the mechanism for DJ-1 associated parkinsonism concerns an impaired response to oxidative stress, three approaches are theoretically possible. The first to mention but the last in terms of (short-term) feasibility is gene therapy. Since in patients in this kindred the DJ-1 gene is not expressed, viral-vector mediated DJ-1 incorporated in the patient genome may (partially) reverse symptoms. Unfortunately, current developments in gene therapy have not yet reached a level of practical applicability. A second approach and within the range of feasibility is putting patients on a simple regimen of dietary compounds, which can 'buffer' reactive oxygen species. Examples are vitamin E (tocopherol, a component of vitamin E, traps free radicals), vitamin C (ascorbic acid, an electron buffer) and coenzyme Q_{10} (or ubiquinone, a key enzyme in oxidative phosphorylation and electron buffer). All three compounds have been studied with respect to PD before.[51-53]

Ascorbic acid has interactions with levodopa. It has been shown to protect against levodopa-induced toxicity *in vitro*, suggesting a means of slowing progression in PD.[51] A trial on vitamin E in PD showed no effect on symptom severity or progression, but coenzyme Q_{10} was recently shown to significantly ameliorate PD

symptoms.[52,53] The latter study, however, was conducted in a small sample (80 patients), and will need confirmation in larger patient series. Since parkinsonism associated with defects in DJ-1 may pathophysiologically differ from PD, and be targeted along a (presently unknown) pharmacological route, some of the compounds may be beneficial in this rare variant. Although the efficacy of this simple interventional approach is unknown, conventional dosages probably have no harmful adverse effects. Furthermore, they could easily be combined with any existing therapeutic regimen. Regarding the long preclinical period of parkinsonism,[54] and the long-standing disease in most of the currently known patients with DJ-1 associated parkinsonism, however, any interventions aiming to reduce oxidative stress in a progressed stage of neurodegeneration (in the case of the studied kindred disease durations up to 20 years) probably have little to no impact anymore. Therefore, thirdly and equally feasible, patients (or prospectively: DJ-1 deletion homozygotes at risk of/with early-stage parkinsonism) can be instructed to adapt a lifestyle and professional choice minimising environmental exposure to possible oxidative agents, such as heavy metals and pesticides. Smoking, although shown to be protective for common PD through an unknown mechanism,[55,56] should also be avoided due to the damage it may inflict upon these patients who are possibly more susceptible to oxidative stress.

4.3 Sporadic Parkinson's disease

The majority of patients with PD have the sporadic, 'typical' variant without a familial inheritance pattern. Sporadic PD is assumed to be a complex condition in which genes and environment interact. The traditional focus of causal research in PD lies upon either environmental or genetic risk factors. Yet risk of PD by environmental factors may depend on a genetic background, and a genetically determined risk of PD is often realistic only when adequate information on environmental exposure is available. To date, neither the environmental nor genetic approaches have provided conclusive evidence for any toxin or gene that may raise the risk of the 'typical', common form of PD. The failure to identify *the* cause of PD is better understandable when acknowledging that PD is a nosological, but not a biological entity. It is most likely the clinical end point of several overlapping syndromes with common features and resulting from multiple events and interactions, which may be variably important in certain genetically susceptible individuals.

4.3.1 Methodological aspects

In the general population (e.g., urbanised areas with a history of inward migration), the genetic make-up is assumed to be heterogeneous. As was addressed in *Chapter 3.1*, a search for shared segments in the genome, as is suitable in genetically homogeneous populations, lacks statistical power in this setting, and the sample sizes required to detect any effect are astronomically large. An approach joining family-based and population-based methods is the assessment of siblings with PD, derived from the

general population. Series of sibling pairs with PD (in whom a stronger genetic contribution is expected) together with their unaffected relatives can be studied in a genomic screen for susceptibility factors. Practically, only when combining efforts of various research groups, sufficiently large numbers of sib pairs can be collected to obtain enough power to detect susceptibility regions for PD.[36,57-59]

Diagnostic pitfalls

The common, sporadic variant of PD as found in the general population has several characteristics hampering ascertainment. First of all, unlike the early-onset parkinsonism associated with DJ-1 deletions (*Chapter 2.2*), sporadic PD is generally of late onset. Younger and healthy relatives cannot be defined as definitively unaffected as they may still develop PD in the course of their lives. Hence their phenotype must be regarded as unknown, which reduces power in linkage studies. Secondly, a crucial diagnostic criterion for PD is progression of symptoms. If disease (in particular early-stage disease) is assessed at one point in time only, the feature of progression cannot be judged accurately. Therefore, (long-term) follow-up of PD patients to assess progression is necessary to obtain a valid impression of the rate of decline. Thirdly, the penetrance of PD (the extent to which a certain genotype is clinically observable) is reduced. This assumption, one of the features defining complex disease, can be illustrated by certain mendelian forms of PD (*PARK3*[8], *PARK4*[9], *PARK8*[13]). Reduced penetrance can thus give rise to false-negative results (exclusion of cases with the risk genotype who lack the according phenotype). Lastly, another feature of complex disease is the presence of phenocopies (phenotypes mimicking the trait under study without the underlying genetic mutation), which can give rise to false-positive results. In common PD, examples of phenocopies are parkinsonism due to neuroleptic medication, drug abuse or vascular disease (although these secondary parkinsonisms usually do not respond as well to levodopa treatment). In families with monogenic parkinsonism, phenocopies may be PD patients in which PD has a completely different cause and who lack the mutation(s) segregating with PD in other relatives. Due to a high baseline risk of PD,[60] the latter remains a possibility, even in families segregating other PD-associated mutations.

Genetic association studies

An approach for genetic research in the general population that is less sensitive to sample size is the study of genetic association in candidate genes. Based on their function in a certain (hypothetical) pathway, polymorphisms or mutations in candidate genes can be assessed in PD patients and compared to controls. By means of the case-control design, various groups of genes have been studied as suitable candidates for a role in the pathogenesis of PD. In Table 3, examples of commonly studied candidate genes are grouped by candidate pathway. Candidate genes marked by an asterisk maintained a significant association with PD in a large meta-analysis.[61]

Clinical and neuropathological similarities between sporadic and mendelian forms of PD form a plausible link between the PD genes and the common, sporadic form of PD. Polymorphisms in the first three PD genes (alpha-synuclein, parkin and UCH-L1) and markers in the *PARK3* locus have been studied extensively, but results are inconclusive.[62-70] Other groups of candidate genes for the common form of PD are classified by the hypothetical disease pathway (Table 3), and may overlap one another in terms of function or possible pathways. The main groups are dopaminergic metabolism (led by the predominantly dopaminergic deficiency which gives the clinical signs of parkinsonism); protein degradation (Lewy bodies, the proteinaceous inclusion bodies seen in PD brains, suggest a cascade of events giving rise to abnormal protein aggregates, which are lethal to the normal neuronal architecture); xenobiotic metabolism (slow-breakdown variants of detoxification enzymes could impair the ability of PD patients to handle neurotoxic substances); mitochondrial function (metabolites of MPTP, a neurotoxin extensively studied as the model for an exogenous cause of PD, cause mitochondrial damage in the substantia nigra) and, as studied in *Chapter 3.4*, iron metabolism and oxidative stress (considering the identification of the DJ-1 gene in parkinsonism and its putative role in the response to oxidative stress).

Table 3. Most frequently studied candidate genes in Parkinson's disease

Dopaminergic metabolism

Monoamine oxidase A, B (MAO-A, MAO-B)
Catechol-O-methyltransferase (COMT)
Tyrosine hydroxylase (TH)
Dopamine transporter (DAT)
Dopamine receptor 2-4 (DRD2-4)

Protein aggregation

α-synuclein (PARK1)
Parkin (PARK2)
UCH-L1 (PARK5)
MAPT (Tau)
Apolipoprotein E (APOE)

Xenobiotic metabolism

Debrisoquine-4-hydroxylase (CYP2D6)
N-Acetyltransferase 2 (NAT2)*
Glutathione transferases (GSTT1*;GSTM1;GSTP1;GSTZ1)

Mitochondrial metabolism

$tRNA^{Glu}$ *
ND2

* *Significant association in a meta-analysis*[61]

Validity and future of polymorphism studies

In recent years, the contribution of genetic association studies within genetic research has surged. PD represents disorders in which polymorphisms in biologically plausible genes have yielded few, if any, conclusive results. Inconsistencies in diagnostic criteria for PD, ethnic origin of the study population and composition of the control group all contribute to this.[61,69] Pooling samples and applying meta-analysis could substantially increase the statistical power to detect an effect of a particular polymorphism. The possibility of publication bias (a tendency of researchers to report, and publishers to publish, mostly the positive associations, or significant associations) distorting meta-analyses cannot be excluded. In a recent overview of genetic association studies, Hirschhorn et al.[71] collected over 600 polymorphism studies (various polymorphisms and various disorders) with positive results. They found that only six of the 166 associations studied repeatedly (about four percent) could be replicated in 75 percent of subsequent studies. Of these six robust and consistent associations, none was implicated in PD.[71] After *Nature Genetics*, *The Lancet* recently issued an editorial stating it no longer considered genetic association studies for publication, unless a causal gene had definitively been identified.[72] Their policy can be justified in terms of the limited space to place articles, but in the future. the precedent created by *The Lancet* may be adopted by more prominent scientific journals. Consequently, genetic association studies will be more difficult to publish, and a new form of publication bias against genetic association studies emerges. If other journals react on this development by encouraging submission and publication of negative *and* positive genetic association studies, this could result in a more balanced impression of all performed studies on a particular association and a minimisation of the skewedness towards positive associations, which is likely to be present in the currently available literature. Furthermore, the Internet, not hampered by the space restrictions often referred to by journals when rejecting manuscripts, creates ways to accommodate positive as well as negative studies. A centrally managed Internet database for genetic association studies, with results cast in a simple format rather than in article format would be a readily accessible and possibly more reliable source for meta-analysis. The requirement of compulsory yet brief descriptions of objectives and the characteristics of patient and control populations (age; ethnicity; co-morbidity; possible confounding factors; family history for disease) and tests for e.g., deviations from the Hardy-Weinberg equilibrium might lower the threshold to publish (negative) results and improve the validity and uniformity of the data. Such a database, however, would become successful only when there is continuous and critical quality monitoring of the data input and output. Paradoxically, the present ban on polymorphism studies by some journals can thus improve the validity and success of genetic association studies in the future.

4.3.2 Main findings and limitations

From the genetically isolated population (the GRIP population), we ascertained a series of 41 PD patients who could be connected to a common ancestor within 14 generations (*Chapter 3.2*). Mean age at onset in the patients with late-onset PD was significantly lower, and the proportion of early-onset patients was higher than in a group of PD patients from the Rotterdam Study, a population-based cohort. The age-at-onset distribution in the patients from the isolate was significantly bimodal with peaks in the early-onset and late-onset range. Although these characteristics suggest a genetic origin in at least the early-onset patients, a role of the known PD genes and loci could be excluded. Most importantly, although four other parkinsonism patients from the same community were homozygous for a DJ-1 deletion and the carrier frequency was almost one percent, no DJ-1 mutations were observed amongst these patients with 'typical PD'. The question whether heterozygosity for the DJ-1 deletion would predispose to parkinsonism at later age can, based on the absence of carriers amongst the typical-PD patients, thus provisionally be answered negatively. In conclusion, even though all patients originate from the same genetic isolate and a limited number of founders, yet another gene may be involved and thus genetic heterogeneity in PD is present in the genetic isolate.

In this study, 41 PD patients could be connected to a common ancestor. As mentioned in *Paragraph 4.2.1*, familial aggregation must be viewed relative to a reference group. The true degree of familial aggregation of the PD patients in the absence of a valid reference group is difficult to estimate. At the time of this study, a control group of healthy individuals was lacking. An important criterion is the sampling frame of a control group, which ought to be identical to that of the patients. Therefore, the patients' spouses would have constituted a most suitable control group.

In a genomic screen (*Chapter 3.3*) performed in 21 of the 41 PD patients from the genetically isolated population described in *Chapter 3.2*, significant linkage and association was found for a locus on chromosome 10q. After saturating the flanking region with 12 additional markers, confirmation was sought in a sample including all aforementioned PD patients and 88 control individuals from the GRIP population. Due to an unreliable marker order, haplotype construction proved to be impossible. Since not one but several alleles seemed to be associated, the CLUMP programme was used. Upon this analysis, two alleles of the original marker (amongst which the originally associated allele) and a single allele of another marker showed significant association, but combining those into haplotypes was not feasible.

This newly identified association on chromosome 10 is promising but requires further analysis. Since the CLUMP programme searches a maximal chi-square statistic in alleles or groups of alleles, as soon as alleles are clumped together, it is not possible to pinpoint the allele(s) truly associated with disease. When clumped alleles differ slightly in size, there are several explanations. Founders of the population may have introduced different alleles of the same gene, or many generations ago mutations may

have occurred. Furthermore, since the used markers have a mutation rate of approximately 10^{-3}, there is a possibility that marker alleles have changed in length, which could cause more than one allele to be associated. However, when the associated alleles vary strongly in length, as was the case for the associated alleles identified by CLUMP in our study, the question arises what is the finding's biological plausibility. The region of interest was saturated with 12 interpolating markers, but the order of the markers in the, recombination-prone, telomeric area remains uncertain. Apart from further study of the marker order, still larger case series are needed for confirmation of this association.

In *Chapter 3.4* we assessed the role of mutations in the HFE gene, a pivotal gene in iron metabolism and the commonly mutated gene in hereditary haemochromatosis, in patients with PD and patients with parkinsonism overall. Two independent patient series were studied, one from the Rotterdam Study and one from the Southwest of The Netherlands. In the PD patients from the Rotterdam Study, a significant excess of C282Y homozygotes was observed, whereas in the series from the Southwest, an excess was observed in parkinsonism patients overall. Although hampered by the rarity of the C282Y mutation, these findings suggest that C282Y homozygosity occurs more frequently among PD patients and possibly parkinsonism patients overall. If true, PD and/or parkinsonism may be a rare expression within the clinical spectrum of haemochromatosis. Alternatively, the abnormal iron metabolism associated with C282Y homozygosity may be considered to predispose to 'typical' PD. Hereditary haemochromatosis is a treatable disorder, and lessening the iron load of the body by regular phlebotomy lowers the risk of long-term complications such as diabetes and cardiovascular disease.[74] If PD were part of a C282Y-related disease spectrum, phlebotomy could lower an excess risk of PD amongst those individuals. In the current sample, the rarity of C282Y homozygosity severely limits the power to study and statistically test other variables, such as the phenotypic expression of the mutations (clinical presentation; transferrin, ferritin and total iron values; magnetic resonance imaging to assess brain-iron contents) in homozygotes and heterozygotes. Therefore, our observation requires confirmation in still larger samples.

Conclusion

This thesis aimed to dissect the genetic basis underlying PD. We were successful at identifying a new gene, DJ-1, involved in early-onset PD. Putative properties of DJ-1 yield clues for a novel aetiological mechanism in PD. At present, half a year after its identification, seven different mutations in DJ-1 have been identified worldwide and more may be added to this list in the future. Hopefully, it will soon be feasible to determine whether the recently identified polymorphisms in the DJ-1 gene play a role in common PD. Patients from the Rotterdam Study[60] would constitute a suitable population to assess this.

The results of our studies conducted in patients with 'sporadic' PD were less unequivocal. Problems we encountered were very similar to those in studies on other complex diseases. Future study would therefore benefit from improvement of statistical methods and study design to successfully identify genes involved in the common, late-onset form of PD.

Looking back, the setting for most studies in this thesis, the GRIP community, harboured an entire clinical spectrum, ranging from genetically complex late-onset PD to monogenic early-onset parkinsonism: PD genetics in a nutshell.

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CHAPTER 5

SUMMARY
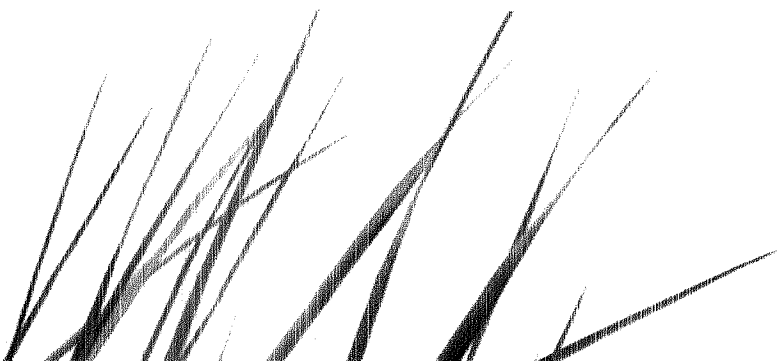
Parkinson's disease (PD) is a common movement disorder, estimated to affect 1/1,000 of the world's population aged 65 years and over. Although evidence for rare genetic subtypes is mounting, the vast majority of PD is of unknown cause. In Chapter **1.1**, advances in the genetics of PD are reviewed focusing on monogenic forms of PD, their clinical presentation and the extent to which they attribute to the risk of PD in the general population.

Individuals from genetically isolated populations are expected to be more genetically homogeneous than individuals from the general population. **Chapter 2** describes studies on familial parkinsonism, conducted in a genetically isolated population in the Southwest of The Netherlands. A consanguineous kindred segregating autosomal recessive early-onset PD was ascertained (**Chapter 2.1**). In a genomic screen, homozygosity mapping yielded significant evidence for linkage to a haplotype of 16 cM on chromosome 1p36. This haplotype could be clearly separated from the more centromeric PARK6 locus on chromosome 1p35-36 by 25 cM. This newly discovered PD locus was named PARK7. We identified the responsible gene contained in the PARK7 locus: DJ-1 (**Chapter 2.2**). In the kindred from the isolated population a deletion of the gene was found. Another parkinsonism family from Italy segregated a point mutation in the gene. DJ-1 codes for a small, highly conserved protein. The function of the DJ-1 protein is unknown, but there is evidence that the gene is involved in the response to oxidative stress. Our findings suggest that loss of DJ-1 function leads to neurodegeneration. The Dutch patients with DJ-1 mutations exhibited typical early-onset parkinsonism. In addition, however, other psychiatric and neurological traits were observed, as outlined in **Chapter 2.3.** The severity spectrum ranged from mild hypokinesia and rigidity, to early-onset parkinsonism with levodopa-induced dyskinesias and motor fluctuation. Three of four patients in this PARK7-linked kindred exhibited psychiatric disturbances. Structural neuroimaging was unremarkable, but functional imaging of the brain as performed in three patients showed significant dopaminergic dysfunction. Assessment of cerebral glucose metabolism performed in one patient showed possible involvement of the cerebellum. Furthermore, the phenotype observed in this parkinsonism kindred also comprised some remarkable physical traits (**Chapter 2.4**). At clinical examination of the parkinsonism patients, we also observed short stature and brachydactyly. This prompted us to further investigate body height and hand structure in patients and their relatives, as well as in a control group from the same genetically isolated population. Using the DJ-1 deletion as a marker for the homozygosity region on chromosome 1p36 (PARK7), we found a significant association of the deletion with body height, adjusting for age and gender (p = 0.005). This association suggests that either DJ-1 itself or a gene or regulatory element elsewhere in linkage disequilibrium with DJ-1, is implicated in short stature. Analysis of hand-bone length showed incomplete segregation of the deletion with brachydactyly, rendering it unlikely that the brachydactyly is fully explained by a gene in the DJ-1 region. The, homozygous, patients had a more pronounced overall bone-

length reduction than their heterozygous unaffected relatives. Given these differences within the kindred, a modifier gene for brachydactyly may be present in the region containing the DJ-1 gene. Finally, we assessed whether (neurologically unaffected) heterozygotes for a DJ-1 mutation may have signs of preclinical disease at functional neuroimaging. Subclinical dopaminergic dysfunction in heterozygotes has been observed two other recessive forms of parkinsonism associated with mutations in parkin and with linkage to the *PARK6* locus. In order to assess cerebral dopamine and glucose metabolism, PET neuroimaging was performed in homozygotes (patients) and heterozygotes (clinically unaffected) for the DJ-1 deletion, as well as in a non-carrier relative (Chapter 2.5). The homozygous patients had, regardless of the degree of clinical severity, significantly reduced F-DOPA uptake concordant with typical PD, and putaminal FDG hypermetabolism. In the heterozygotes for the DJ-1 mutation and a relative not carrying a deletion, F-DOPA metabolism was unremarkable: an effect of the DJ-1 gene deletion was not observed. Due to the small number of participants in this study, however, dopaminergic function must be assessed in more individuals with DJ-1 mutations when they become available.

Chapter 3 describes genetic studies of sporadic PD (in which the mode of inheritance is difficult to pinpoint). How to assess the genetic basis of PD and other neurological diseases is reviewed in Chapter 3.1. The genetically isolated community described in Chapter 2 was the setting for a study on PD patients who were at first sight unrelated. Clinical characteristics of a series of PD patients, the extent of relationship and results of a candidate-gene study are discussed in Chapter 3.2. We studied 109 patients with parkinsonism from an area comprising the genetically isolated population. Of these 109 patients, 41 patients with typical PD could be linked to a common founder 14 generations ago. The distribution of ages at onset of these PD patients was significantly bimodal (one peak with a mean at age 67 years and another peak with a mean at 44 years), the latter being significantly larger than that in a population-based study, the Rotterdam Study. In other clinical features, the only statistically significant difference between early-onset and late-onset PD was a decreased motor and cognitive function in patients with late-onset PD. Association to other PD genes and loci, most importantly DJ-1, could be excluded in these other studied PD patients from the same community. The finding of a common ancestor in the 41 PD patients along with the exclusion of known PD genes and loci suggests the presence of at least one other susceptibility gene involved in PD in this population. In Chapter 3.3, the results of a genomic screen performed in 21 of these 41 patients are outlined. Upon analysis for linkage and association, two peaks were identified, on chromosomes 19 and 10. The association for chromosome 19 appeared to be false positive. The association for chromosome 10 was further examined by saturating the region with a denser set of markers. Unfortunately, haplotype construction was not possible due to an ambiguous marker order on the physical map. More than one allele appeared to be associated (the 211- and 229-bp alleles of D10S1651 and the 177-bp

allele of D10S1711). **Chapter 3.4** presents findings of a genetic association study. Mutations in the HFE gene, which are the major cause of the iron-storage disorder haemochromatosis, may result in excessive oxidative stress, as possibly do mutations in DJ-1. Two series of patients with common, sporadic PD, and, since it possibly concerning atypical neurological features, parkinsonism overall were therefore studied for mutations in the HFE gene. In one series, PD patients were significantly more often homozygous for the C282Y mutation than controls ($p = 0.03$), and in the other, parkinsonism patients were more often homozygous for this mutation than controls. Although hampered by the small numbers of patients, our data are suggestive of an increased risk of PD, and possibly of parkinsonism overall, in homozygotes for the C282Y mutation. Given the rarity of this genotype, large series of patients are required to prove this hypothesis.

Finally, **Chapter 4** provides a discussion of the results, limitations and implications of the studies in this thesis.

SAMENVATTING
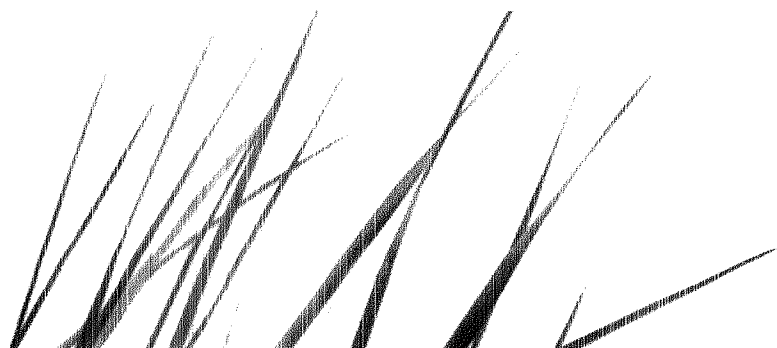
De ziekte van Parkinson (ZvP) is een bewegingsstoornis waar ongeveer één op de duizend wereldbewoners van 65 jaar en ouder aan lijdt. Hoewel het bewijs voor zeldzame erfelijke subvormen gestaag toeneemt heeft is de oorzaak in het overgrote deel van de ZvP onbekend. In **Hoofdstuk 1.1** passeren ontwikkelingen in de genetica van de ZvP de revue met een nadruk op monogene vormen, hun klinische presentatie en de bijdrage aan het risico van de ZvP in de algemene bevolking.

Mensen uit genetisch geïsoleerde bevolkingsgroepen zijn waarschijnlijk genetisch homogener dan mensen uit de algemene bevolking. **Hoofdstuk 2** beschrijft onderzoeken naar familiair parkinsonisme, uitgevoerd in een genetisch geïsoleerde gemeenschap in Zuid-West Nederland. Daar werd een consanguiene familie met autosomaal-recessief parkinsonisme onderzocht (**Hoofdstuk 2.1**) waarin een genomische zoektocht met homozygotie-mapping een significant bewijs voor linkage opleverde voor een haplotype van 16 cM op chromosoom 1p36. Dit haplotype lag op een afstand van 25 cM van het dichterbij de centromeer gelegen *PARK6* locus (chromosoom 1p35-36). Dit nieuw ontdekte ZvP-locus kreeg de naam *PARK7*. Vervolgens werd het verantwoordelijke gen in het *PARK7*-locus geïdentificeerd: DJ-1 (**Hoofdstuk 2.2**). De familie uit het genetisch isolaat had een deletie en een andere familie met parkinsonisme uit Italië een puntmutatie in het DJ-1 gen. DJ-1 codeert voor een klein en sterk geconserveerd eiwit. De functie van het DJ-1 eiwit is onbekend, maar er zijn aanwijzingen dat het gen betrokken is bij de reactie op oxidatieve stress. Onze bevindingen doen vermoeden dat verlies van DJ-1 functie tot neurodegeneratie leidt. De Nederlandse patiënten met DJ-1 mutaties hadden een typisch, jong beginnend parkinsonisme. Ook werden echter andere psychiatrische en neurologische kenmerken waargenomen, zoals wordt uiteengezet in **Hoofdstuk 2.3**. De ernst van de aandoening liep uiteen van milde hypokinesie en rigiditeit, tot jong beginnend parkinsonisme met levodopa-geïnduceerde dyskinesieën en schommelingen van motore activiteit. Drie van de vier patiënten in de familie met *PARK7*- parkinsonisme hadden psychiatrische symptomen. Structurele beeldvorming van de hersenen liet geen bijzonderheden zien, maar funktionele beeldvorming (verricht in drie patiënten) toonde een significante dopaminerge dysfunctie aan in het striatum. Beeldvorming van de glucosestofwisseling (verricht in één patiënt) wees op een mogelijke betrokkenheid van de kleine hersenen. Verder betrof het fenotype in deze familie met parkinsonisme betrof ook enkele opvallende lichamelijke kenmerken (**Hoofdstuk 2.4**). Tijdens het klinisch onderzoek van de patiënten met parkinsonisme bemerkten we ook een korte gestalte en brachydactylie. Dit vormde aanleiding tot nader onderzoek van de lichaamslengte en handstructuur in de patiënten, hun familieleden en een controlegroep uit dezelfde gemeenschap. Met de DJ-1 deletie als marker voor het homozygotiegebied op chromosoom 1p36 (*PARK7*) vonden we een significant verband van de deletie met lichaamslengte, na correctie voor leeftijd en geslacht (p = 0.005). Dit verband doet vermoeden dat ofwel DJ-1 zelf, of een gen/regelelement elders in linkage disequilibrium met DJ-1, betrokken is bij een beperkte

lichaamslengte. Daarentegen toonde analyse van de handbeenderlengte een onvolledige segregatie van de deletie met brachydactylie aan, die het onwaarschijnlijk maakt dat de brachydactylie volledig kan worden verklaard door een gen in het DJ-1 gebied. De verkorting van handbeenderen was meer uitgesproken bij de homozygote patiënten dan bij hun heterozygote gezonde familieleden. Gezien deze verschillen binnen de familie zou er een modificerend gen kunnen liggen in het gebied waar ook het DJ-1 gen ligt. Tenslotte hebben we onderzocht of (neurologisch niet-aangedane) dragers van een DJ-1 mutatie op hersenscans wel aanwijzingen hebben voor een preklinisch ziekteproces van de hersenen. Subklinische dopaminerge dysfunctie bij heterozygoten werd ook gevonden in twee andere recessieve vormen van parkinsonisme, geassocieerd met mutaties in het parkin-gen en met linkage met het *PARK6*-locus. Teneinde de dopamine- en glucosestofwisseling in de hersenen te bestuderen werden PET-scans gemaakt bij zowel homozygoten (patiënten) en heterozygoten (klinisch gezond) voor de deletie, alsook bij een familielid zonder deleties (normaal genotype) (**Hoofdstuk 2.5**). De homozygote patiënten hadden ongeacht de ernst van hun symptomen een significant verminderde F-DOPA opname zoals in de typische ZvP, en bovendien hypermetabolisme van glucose in het putamen. De heterozygoten en het familielid zonder de deletie hadden geen verminderde F-DOPA stofwisseling: een effect van de DJ-1 deletie werd niet waargenomen. Aangezien het aantal deelnemers aan dit onderzoek klein was is onderzoek van het dopaminerg systeem bij meer mensen met DJ-1 mutaties, zodra deze beschikbaar zijn, vereist.

Hoofdstuk 3 beschrijft genetische studies over de sporadische ZvP (met een onduidelijk overervingspatroon). Op welke manier de genetische achtergrond van de ZvP en andere neurologische aandoeningen onderzocht kan worden, kan gelezen worden in **Hoofdstuk 3.1**. In de genetisch geïsoleerde gemeenschap die werd beschreven in Hoofdstuk 2 hebben wij Parkinsonpatiënten bestudeerd die op het eerste gezicht geen familie van elkaar waren. De klinische kenmerken van een reeks van deze patiënten, hun onderlinge verwantschap en de uitkomsten van een kandidaatgenstudie worden uiteengezet in **Hoofdstuk 3.2**. We onderzochten 109 parkinsonismepatiënten die afkomstig waren uit een gebied dat het genetisch isolaat bevat. Van deze 109 patiënten konden 41 patiënten met de typische ZvP verbonden worden met een gemeenschappelijke voorouder 14 generaties terug. De verdeling van de beginleeftijden van deze Parkinsonpatiënten was significant bimodaal (één piek met een gemiddelde leeftijd van 67 jaar en één piek met een gemiddelde van 44 jaar). De laatstgenoemde piek was significant hoger dan die bij Parkinsonpatiënten uit het ERGO-onderzoek, een onderzoek in de algemene bevolking. In klinisch opzicht was het enig statistisch significante verschil tussen een vroeg en laat beginnende ZvP het verminderd motorisch en cognitief functioneren bij de patiënten met een laat beginnende ZvP. Bij de patiënten afkomstig uit hetzelfde isolaat in deze studie kon een verband met andere Parkinson-genen, met name DJ-1, kon worden uitgesloten. De

bevinding van een gemeenschappelijke voorouder in de 41 Parkinsonpatiënten, samen met de exclusie van bekende Parkinson-genen en –loci wijst op de aanwezigheid van tenminste één ander gevoeligheidsgen voor de ZvP in deze gemeenschap. **Hoofdstuk 3.3** bevat de uitkomsten van een genomische zoektocht in 21 van deze 41 patiënten. Met een analyse op linkage en associatie werden twee pieken gevonden, op chromosoom 10 en 19. De associatie op chromosoom 19 scheen vals-positief te zijn. De associatie op chromosoom 10 is nader onderzocht door het gebied te verzadigen met een dichtere markerset. Haplotypevorming bleek helaas niet mogelijk vanwege een onduidelijke markervolgorde op de fysieke genenkaart. Meer dan één allel bleek te zijn geassocieerd (de 211 en 229 baseparen lange allelen van marker D10S1651 en het 177 baseparen lange allel van marker D10S1711). **Hoofdstuk 3.4** bevat de bevindingen van een genetische associatiestudie. Mutaties in het HFE-gen, de voornaamste oorzaak van de ijzerstapelingsziekte hemochromatose, zouden overmatige oxidatieve stress kunnen veroorzaken zoals mogelijk ook mutaties in het DJ-1 gen dat doen. Twee reeksen patiënten met de 'gewone' sporadische ZvP, en - aangezien het ziektebeeld mogelijk atypische neurologische kenmerken behelst- parkinsonisme in het algemeen, werden daartoe onderzocht op mutaties in het HFE-gen. In één reeks waren de Parkinsonpatiënten significant vaker homozygoot voor de C282Y-mutatie dan de controlepersonen ($p=0.03$), en in de andere reeks waren parkinsonismepatiënten vaker homozygoot voor deze mutatie dan de controlepersonen. Hoewel belet door het kleine aantal patiënten, wijzen onze gegevens op een verhoogd risico van de ZvP, en mogelijk van parkinsonisme in het algemeen, bij homozygoten voor de C282Y-mutatie. De zeldzaamheid van dit genotype vergt echter grote groepen patiënten om deze hypothese te bewijzen.

In **Hoofdstuk 4**, tenslotte, worden de uitkomsten, beperkingen en gevolgen van de studies in dit proefschrift besproken.

Dankwoord

Hoewel alleen mijn eigen naam op de kaft staat kwam dit proefschrift tot stand dankzij veler inbreng. Twee mensen, Lodewijk Sandkuijl en dr. Piet Dijkstra, leverden een belangrijke bijdrage aan dit onderzoek maar hebben de uitkomst ervan helaas niet meer mee kunnen maken. Hun familie en collega's wens ik alle sterkte met het verwerken van het verlies.

Ik bedank

- alle patiënten en familieleden die aan het onderzoek hebben meegewerkt, hun huisartsen, verpleeghuisartsen en neurologen. Enkelen vermeld ik apart, namelijk de huisartsen van het GRIP-gebied PJLM Snijders (Pieter, gedacht ooit nog paranymf te zullen worden van Mevrouw Heintje Davids?!), ThWC Snieders, G Droge, P van Wouw, WCJ van Broekhoven, M. Kraanen, LJ Dronkers en hun praktijkassistentes, en neurologen dr. MWIM Horstink (UMC St Radboud Nijmegen) dr. MAM Bomhof (Amphiaziekenhuis Breda), A van Spreeken en diens secretaresse Karin, L van Hoof en H Wouters (Franciscusziekenhuis Roosendaal).

- mijn promotoren, Prof. dr. Van Duijn en Prof. dr. Oostra; Cock en Ben - voor alles wat ik in deze vijf jaar van jullie heb mogen leren. Het is een uiterst waardevolle ervaring en een goede basis geweest. Ik wens jullie een succesvol ERF-onderzoek toe en ben benieuwd naar de uitkomsten. Ook mijn copromotor dr. Peter Heutink bedank ik op deze plaats voor de samenwerking en wens ik van harte geluk met de benoeming tot hoogleraar aan de Vrije Universiteit in Amsterdam.

- dr. John van Swieten - voor de verificatie van de diagnoses, een reeks huisbezoeken ter verheldering van complexe neurologische ziektebeelden en uiteraard alle inspanningen als Secretaris voor de lees- en promotiecommissie. Ook Prof. dr. Peter de Jonghe van de Universiteit van Antwerpen dank ik van harte voor het doorlezen van het manuscript en het zittingnemen in de promotiecommissie.

- Vincenzo Bonifati - for the studies on 'our' family, and the verification of the Parkinson's diagnoses. To work with (and learn from) you has been an honour and a joy. I will miss the inspiring discussions about the phenotypic work-up, parkinsonism genetics and about altogether unrelated issues.

- Robert-Jan Galjaard - voor je klinisch-genetische expertise en counseling, en de vele tochten naar de onderzoeksfamilies, en naar het Jan van Bremen Instituut in Amsterdam voor de MCPP's. Daniëlle Majoor - voor de counseling van de Parkinsonfamilies in GRIP; radioloog Hugo Wüstefeld - voor het vervaardigen van extremiteitsfoto's in de onderzoeksfamilies; dr. Julia Zguricas - voor het gebruik van gegevens uit de TPT-families en Ruud Koppenol - voor de klinische fotografie

- dr. Monique Breteler, Prof. dr. Bert Hofman, dr. Maarten de Rijk, dr. Sanjay Harhangi, Patricia Giesbergen en de andere medewerkers en deelnemers aan het ERGO-onderzoek - voor het kunnen gebruiken van de gegevens uit ERGO

- Prof. dr. Leenders van het Academisch Ziekenhuis Groningen - voor de PET studies in de onderzoeksfamilies en, uiteraard, het kritisch lezen van mijn manuscript. Beste Nico, van het PSI ('98) tot in het AZG heb ik je achtervolgd met PET-cliënten, maar om dit jaar binnen 3 weken nog 2 bijna voltallige families te scannen leek ondoenlijk. Dankzij de vereende krachten van ook dr. Jan Pruim,

Sylvia Eshuis, Paul Maguire, Lammy Veenma en Renée Staal is het desondanks gelukt - in ongekend tempo en met goede herinneringen aan Groningen!

- dr. Booij van de afdeling Nucleaire Geneeskunde van het Academisch Medisch Centrum in Amsterdam - voor het verrichten van SPECT scans in één van de onderzoeksfamilies

- dr. Ed Boeren, Petra Veraart en Hilda Kornman - voor de schat aan genealogische gegevens die aan GRIP ten grondslag ligt. Petra, jouw hulp liep zelfs uiteen van de huisbezoeken in het kader van de Parkinson- en ADHD-projecten tot aan de schuddekopkes in je achtertuin. En Hilda, hoeveel stambomen voor mijn onderzoek heb je inmiddels niet met eindeloos geduld en geprigel uitgetekend?

- de ontwerper van PEDKIN - voor de prachtige open source software die kinship- en inbreeding-coëfficiënten uit HAZA-DATA gegevens berekent (URL: http://www.zwets.com/pedkin)

- Marjolijn, Petra, Marti (EPIB) en Jeannette (KGEN) - voor de secretariële ondersteuning; Nano en Rene - voor eerste hulp bij digitale calamiteiten; Koos, Yolanda, Jan, Mw. Schop (Studiezaken FGG) en Mw. Davids (College van Bestuur EUR) - voor het maatwerk van de combinatie AIO-aanstelling / coschappen

- de, letterlijk naaste, collega's van het genetisch-epidemiologisch lab: Aida, Jeannette, Hilda, Wilma, Bernadette, Els, Florencia, Ruud, Anand, Erik, Leon & alle medewerksters van het ERF centrum, en eerder ook nog Erwin, Bianca, Astrid en Angela - voor twee complete genomic screens en de vele andere typeringen, maar natuurlijk ook voor de thee, karnemelk en het muzikale behang van de labradio ♪

- the *Latin Quartet*: Aida, Florencia, Alejandro, Carolina - for sunny company, occasional Ida-babysits and OMA's cafeine boosts (causing me *and* my little passenger to bounce down the final stretch towards completion of this thesis)

- de collega-onderzoekers van de Genetische Epidemiologie: Anna, Behrooz, Fakhredin, Fernando, Ingrid, Kristel, MarieJosee, Mark, Tonnie, Yurii, de MSc-studenten en uit het verleden Margriet, Marleen, Norbert, Gerwin, Arjen. My room mates, Aida and Tessa - for countless cups of tea *and* a terrifc job on the PLO files. Aida, good luck in Clinical Genetics and on completing your gene chase! Your Highness Prince Omer - for all your wise tutorials on HFE, and on better not to run through corridors nowadays! Jeanine - voor je statistisch inzicht, en, door een kier van de vluchtdeur, de gesprekken over 10q-analyses en ieniemienie-meisjes. Veel geluk in Leiden. Esther - voor een significant verbeterde vijfjaarsoverleving. En bij *Hizkia Van Kralingen* kunnen we nog altijd aan de slag.

- David Holmes Morton, Valentijn Drexhage, Cees Louwerse, Marie Lepoutre, Atie Lepoutre Ravn - voor veelzijdig 'mentor'schap. Annelise - voor vriendschap in de vorm van een Noors wafeltje; Josine - voor het fijt dat we allebij Dom-blondje zein; WJ 'Herbie' - voor de hovenierskunsten op de kaft; Jocelyn - for *ever* sitting on a tincan! Tenslotte papa, mama, Floor, Broer, Gerdie, Edwin, Ingrid. En Zwets.

Biography

Marieke Dekker was born on May 20th 1975 in Sluis. In 1993, she graduated from the United World College of the Atlantic (Wales, UK) to study medicine at the Erasmus University in Rotterdam. In 1996 and 1997, she did elective research on type-1 glutaric aciduria and maple syrup disease in the Clinic for Special Children (a clinic for metabolic diseases in the Amish and Mennonites, Strasburg PA, USA), and genotype-phenotype correlations in Crigler Najjar disease (Sophia Children's Hospital Rotterdam). In 1997, she enrolled in a Master of Science programme for Clinical Epidemiology (n i h e s, Rotterdam) and received the MSc degree in 1998. In the same year, she started the project described in this thesis at the Department of Epidemiology & Biostatistics and the Department of Clinical Genetics of the Erasmus MC, Rotterdam. She obtained her medical degree cum laude in 2001. Married to Marco van Zwetselaar, they have a daughter Ida (June '02) and are expecting a second child in August. She is planning to become a paediatrician.

Stellingen

Mutaties in het DJ-1 gen (PARK7) veroorzaken een jong beginnend parkinsonisme met autosomaal-recessieve overerving. (*dit proefschrift*)

Parkinsonisme als gevolg van een deletie in het DJ-1 gen gaat gepaard met een verhoogd risico van psychiatrische symptomen. (*dit proefschrift*)

Dragerschap van een deletie in het DJ-1 gen is geassocieerd met een korte gestalte, hetgeen het meest uitgesproken is bij personen die homozygoot zijn voor de deletie. (*dit proefschrift*)

De F-DOPA PET scans bij dragers van een enkele deletie in het DJ-1 gen tonen een normaal presynaptisch dopaminerg neurotransmittersysteem. (*dit proefschrift*)

De deletie in het DJ-1 gen speelt geen rol in patiënten met een laat beginnende ziekte van Parkinson afkomstig uit de onderzochte genetisch geïsoleerde gemeenschap. (*dit proefschrift*)

Door de huidige ontwikkelingen in de genetica dient de ziekte van Parkinson te worden hernoemd tot de ziektes van Parkinson.

Het vóórkomen van diabetes mellitus type 2 op de kinderleeftijd toont het belang van voorkómen.

Eenieder die genetisch onderzoek verricht in een godsdienstige gemeenschap dient zich te verdiepen in -en te leren van- de heersende geboden en verboden. (naar McKusick, Medical Genetic Studies of the Amish, 1978)

Wanneer het taboe op clinical trials bij jonge kinderen wijkt, wordt de kindergeneeskunde waarlijk 'evidence-based'

Hoewel de media bijdragen aan de verwarring omtrent uitkomsten van epidemiologisch onderzoek (Breslow, Biometrics 2003;59:1-8) ligt de echte verantwoordelijkheid bij onderzoekers die hun resultaten onvoldoende duiden.

Voordat loopbaan verwordt tot renbaan is het goed om even stil te staan.

Rotterdam, 11 juni 2003