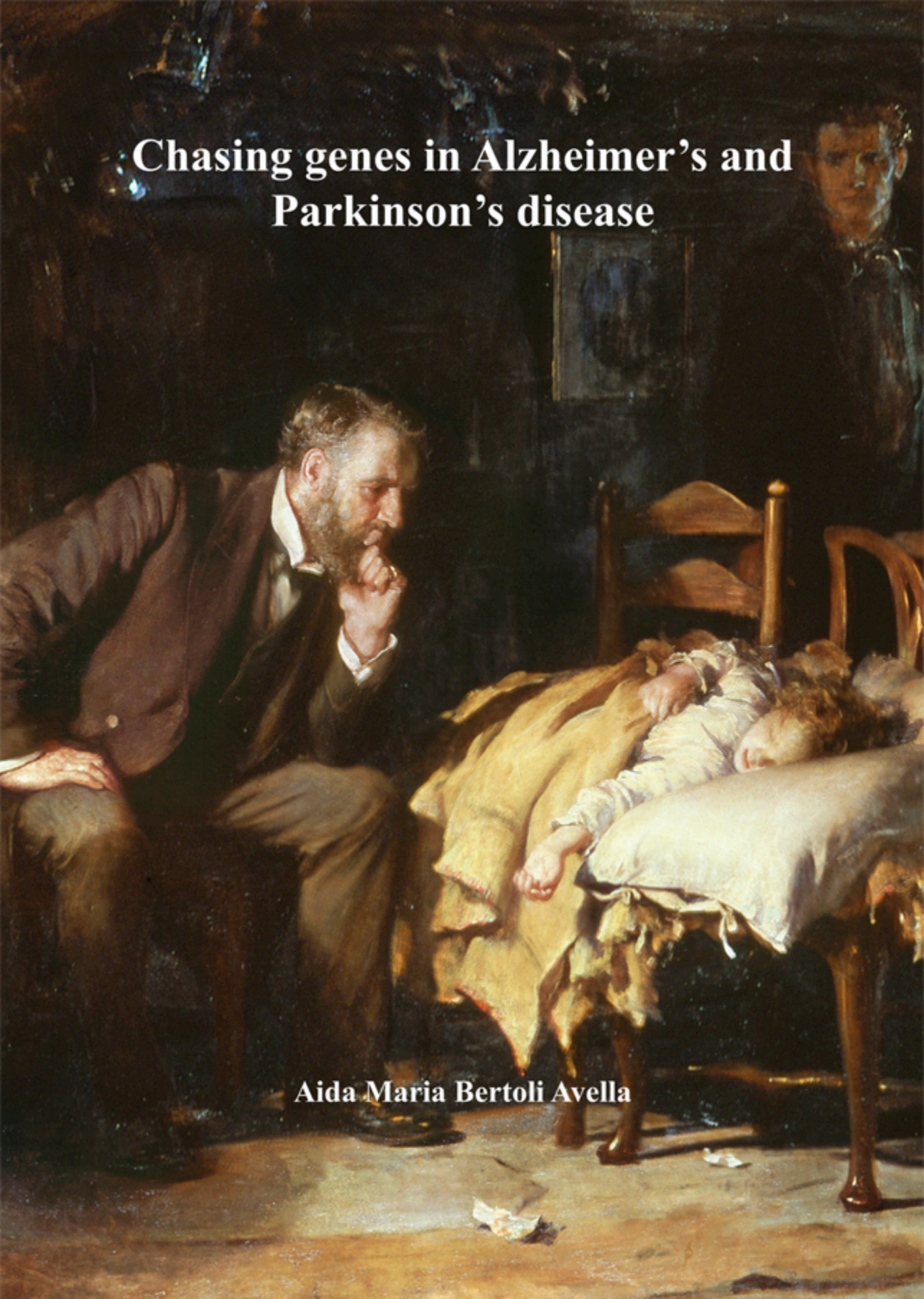
Chasing genes in Alzheimer's and Parkinson's disease



Aida Maria Bertoli Avella

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Chasing genes in Alzheimer's and Parkinson's disease

Zoektocht naar genen betrokken bij de ziekte van Alzheimer en de ziekte van Parkinson

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A mis padres, gracias por su amor...

A mis abuelos y a Javier, in memoriam

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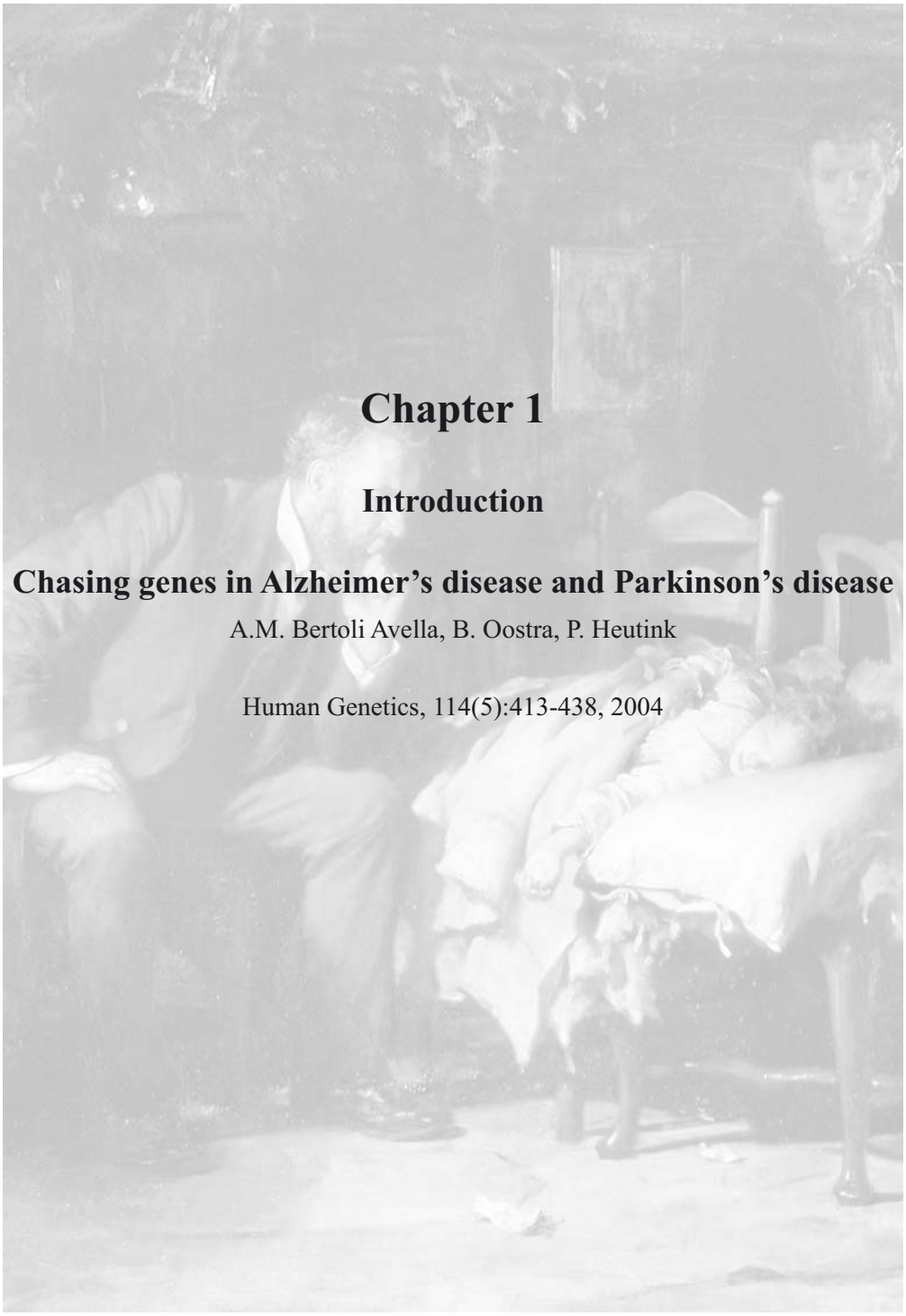
Chapter 7. General Discussion

El mundo era tan reciente, que muchas cosas carecían de nombre, y para mencionarlas había que señalarlas con el dedo. . .

The world was so recent, that things were nameless and to refer to them, people had to point with the finger. . .

Gabriel García Márquez, "Cien años de soledad", 1967



Chapter 1

Introduction

Chasing genes in Alzheimer's disease and Parkinson's disease

A.M. Bertoli Avella, B. Oostra, P. Heutink

Human Genetics, 114(5):413-438, 2004

Abstract

Alzheimer's disease (AD), the most common type of dementia and Parkinson's disease (PD), the most common movement disorder are both neurodegenerative adult-onset diseases characterized by progressive loss of specific neuronal populations and accumulation of intraneuronal inclusions.

The search for genetic and environmental factors that determine the fate of neurons during the ageing process has been a widespread approach in the battle against neurodegenerative disorders. Genetic studies of AD and PD have initially focused on the search for genes involved in the aetiological mechanisms of monogenic forms of these diseases. They later expanded to study hundreds of patients, affected relative-pairs and population-based studies sometimes performed on "special" isolated populations. A growing number of genes (and pathogenic mutations) is being identified that cause or increase the susceptibility to AD and PD. This review discussed the way in which strategies of "gene hunting" have evolved during the last years and the significance of finding genes such as the *presenilins,* α*-synuclein, parkin* and *DJ-1*.

In addition we discuss possible links between these two neurodegenerative disorders. The clinical, pathological and genetic presentation of AD and PD suggests the involvement of few overlapping, interrelated pathways. Their imbricate features point to a spectrum of neurodegeneration (tauopathies, synucleinopathies, amyloidopathies) that will need further intense investigation to find the missing links.

Introduction

In 1907, Alois Alzheimer, a German psychiatrist and pathologist, described the case of a 51-year-old woman with presenile dementia. In addition to diffuse cortical atrophy, he reported "peculiar changes of the neurofibrils...which are eventually seen clustering together in thick bundles" and a "deposit in the cerebral cortex of a peculiar substance which can be recognized without staining" [1]. Subsequently, these changes were recognized to be neurofibrillary tangles and amyloid plaques. This "new" disorder was named Alzheimer's disease by the German psychiatrist Emil Kraepelin in 1910.

In 1817, James Parkinson, a British physician, first described the clinical picture known as "shaking palsy" (paralysis agitans) based on six cases that he personally followed for several years. Armand Trousseau, a French internist, made two important additions to Parkinson's description; he recognized both the rigidity and the occurrence of mental impairment in some cases [2]: "The intellect...gets weakened at last; the patient loses his memory and his friends notice soon that his mind is not as clear" [3]. Forty years later, the term Parkinson's disease was first used by the French neurologist Jean-Martin Charcot. Since then, investigators have tried to elucidate the causes of Parkinson's and Alzheimer's disease in order to understand the pathophysiology of these disorders and to design effective therapies.

These late-onset neurodegenerative disorders display remarkable clinical and pathological overlaps. They are characterized by progressive dysfunction and, at a later stage, by the loss of specific neuronal populations, in addition to the formation of intra and extra cellular protein aggregates. Here, we describe the efforts and progress made in the mapping of genes involved in Alzheimer's and Parkinson's disease. The study of the identified genes,

proteins and pathways should help to dissect these complex disorders. In spite of the progress made, the chase has however just begun.

Nomenclature

Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee.

Alzheimer's disease

Alzheimer's disease (AD, OMIM 104300) is the most common type of dementia, with a prevalence that varies from about 3% in individuals around 65 years of age to nearly 50% in people aged 85 years [4]. AD is characterized by progressive memory loss and deterioration of cognitive functions that ends with the death of the patient in a period of approximately 10 years. However, the consequences of the disorder extend to family members and caregivers whose lives are also affected and to society in general. Since the prevalence of AD increases exponentially with advancing age [5], countries with high life expectancies are particularly confronted with this health problem.

Neuropathologically, the disease is characterized by extracellular amyloid plaque formation, the deposition of amyloid in the wall of blood vessels, the presence of intracellular neurofibrillary tangles and neuronal loss. Although the diagnosis of possible and probable AD can be made on the basis of clinical signs and symptoms, neuropathological confirmation is necessary to establish a definite diagnosis [6].

The most common form of AD (senile dementia of the Alzheimer type) usually begins in the seventh to ninth decade of life. A less frequent form of the disease typically presents before age 65 years and segregates in families as an autosomal dominant disorder. The later is known as presenile dementia or early-onset AD and corresponds to Alois Alzheimer's first description. It should be noted that the classification of AD in presenile (early-onset) and senile (late-onset, LOAD) dementia is not equivalent to familial (inherited) or non-familial AD.

Familial aggregation

The clustering of AD in families was recognized long ago. In 1932, presenile dementia was described in a four-generation family with pathological confirmation in one case [7]. Recent historical and genealogical research into one of Alzheimer's first cases (Johan F.) brought to light the occurrence of "mental illness" in several family members with transmission over several generations [8].

The classic "twin method" has often been applied in order to evaluate the contribution of genetic and environmental factors to the disease aetiology, based on the differences in concordance rates observed between monozygotic (MZ) and dizygotic (DZ) twins. Three Scandinavian twin studies, one Swedish [9, 10], one Finish [11, 12] and one Norwegian [13], found higher concordance rates for AD in MZ than DZ twins and concluded that AD is highly heritable and that genetic factors are of considerable importance for developing AD, even late in life. They also confirmed the need to identify environmental triggers. In younger twin patients (from North America, 62–73 years old), the observed concordance rates were lower but still twice as high in MZ than in DZ twins [14]. Furthermore, multiple-threshold models

have been used on AD twin studies to evaluate the genetic influences on age at onset (AAO). One study (average AAO=63.5 years) reported that additive genetic effects and a shared environment accounted for similar contributions to individual variation in AAO [15], whereas in a larger and older sample (average AAO=75 years), a considerable part of the variance was attributable to genetic factors [16].

Clinical and population-based studies have estimated the life-time risk of AD in first-degree relatives as being between 17% and 67% [17-20]. Different methodologies and sample characteristics might be responsible for the wide variation in risk estimates, but they all indicate a role for genetic factors in the aetiology of AD.

For familial aggregation studies, population-based samples are preferred over clinic-based samples, since the later may not represent the larger AD population (AAO, family history, severity of the disease) and might introduce bias in the ascertainment. Indeed, when comparing AD patients from tertiary centres and AD patients from geriatric nursing homes, the AAO in the clinical group was significantly earlier; this was inversely associated with familial aggregation [21]. The AAO of dementia among first-degree relatives of early-onset AD patients is lower than the AAO in first-degree relatives of late-onset cases. This observation had raised the question about the genetic control of the AAO in AD [18], even before that any gene involved in AD susceptibility had been identified. This issue has only recently been systematically investigated for both AD and PD (see below).

Population-based studies have consistently shown an increased relative risk for first-degree relatives in both early- and late-onset forms of AD [22, 23]. Recently, in a multi-ethnic population-based study comprising African-Americans, Caribbean-Hispanics and whites, familial aggregation of AD was found to be increased among families of patients compared with those of controls. Although differences between ethnic groups exist, a 50% increased risk for AD was found among first-degree relatives of patients when compared with first-degree relatives of cognitively normal controls [24] suggesting a genetic component for AD across different ethnicities.

Early segregation analysis on population- and clinic-based samples indicated that patterns of familial clustering observed in AD are best explained by a major autosomal dominant locus with reduced penetrance and a multifactorial component (in both early AD and LOAD), without ruling out the role of other genetic and non-genetic mechanisms [25, 26].

The boundaries between genetic and environmental factors are difficult to envisage. Despite uncertainties about the extent of the contribution of genetic factors in the aetiology of the common "sporadic" LOAD forms, it seems that they play an important role. Today, the genetic bases of the rare monogenic forms are better understood; they give insights into AD aetiopathology and help the mapping not only of genes, but also of biochemical pathways.

Known genes involved in familial AD aetiology

APP (AD1)

Based on the observation that middle-aged patients with Down's syndrome (trisomy 21)

commonly suffer from AD, researchers suspected that a gene on chromosome 21 could be involved in AD aetiology. In 1987, linkage analysis in families with early-onset AD identified a locus on chromosome 21q (AD1) close to the a*myloid precursor protein* gene (*APP* [27]). The APP protein is the precursor for β-amyloid, which is the major component of the plaques observed in AD and which also accumulates in small blood vessels in the brain. Therefore, *APP* was considered an obvious candidate gene for AD. In 1991, missense mutations in the *APP* gene were identified [28, 29] and the first gene involved in the aetiology of AD was recognized (Table 1).

Table 1. **Loci and genes related to Alzheimer's disease aetiology (AD autosomal dominant, AR autosomal recessive AAO age at onset, FBATs family-based association tests)**

Alzheimer's disease locus	Chromosomal location	Genes and tested "candidates"	Mapping method	Molecular defects	References
AD1	21q21	*APP*	Linkage analysis (AD)	Single base substitutions (missense mutations, exon 16 and 17)	27-29
AD2	19cen-13.2	*APOE*	Linkage analysis (AD)	Susceptibility factor (APOE-ε4)	30
AD3	14q24.3	*PSEN1*	Linkage analysis (AD)	Single base substitutions (missense, splicing) deletions, insertions	31-33
AD4	1q31-q42	*PSEN2*	Linkage analysis (AD)	Single base substitutions (missense mutations, exon 4, 5, 7 and 12)	34-36
AD5	12p11.23-q13.12	Unknown, *A2M*, *LRP1*, *LBP-1c/CP2/LSF*	Linkage analysis (parametric and non-parametric). FBATs	–	37-40
AD6	10q24	Unknown, *PLAU*, *IDE, CHAT*, *VACHT, TACR2*, *HK1*, *LIPA*, *NFKB2*, *VR22*	Linkage analysis (parametric, non-parametric) and association analysis	–	41-44
AD7	10p13	Unknown	Association and linkage disequilibrium	–	45, 46
AD8	20p	Unknown	Covariate-based linkage method	–	47

The function of APP is still largely unknown. Normal processing of APP involves cleavage by α-, β- and γ-secretases. The cleavage by α-secretase occurs within the Aβ domain, preventing the formation of β-amyloid (Aβ), whereas β- and γ-secretases are involved in the cleavage and production of Aβ40 and Aβ42 [48, 49]. Not surprisingly, all the identified pathogenic mutations (*n*=16) are located within exons 16 and 17 of the gene and are clustered around the β- and γ-secretase sites of the APP protein. Mutations such as Lys670Asn; Met671Leu [50], which is located upstream of the β-cleavage site, and Val717Ile [29] and Val717Phe [51], which are situated close to the γ-site, result in an increase in the formation of Aβ40 and/or Aβ42 [29, 52, 53].

The identification of *APP* as the first gene shown to be involved in AD led to the generation of transgenic animal models over-expressing normal or mutated human AD genes. As in human disease, Aβ over-expression and deposition greatly increases with age and occurs

selectively in those brain regions most heavily affected in AD (hippocampus and cortex). Interestingly, most of the mouse models successfully mimic important features of the human disease, such as the presence of amyloid plaques, gliosis and neurodegeneration with age-related cognitive impairment, but neurofibrillary tangles are absent [54].

PSEN1 (AD3)

It soon became evident that *APP* was not the only gene involved in AD aetiology and several groups continued their searches. Earlier evidence suggesting genetic linkage [55] and the genomic location on chromosome 14 of a putative candidate gene, the serine protease inhibitor α*1-antichymotrypsin* (*AACT*), turned the attention of researchers to this chromosome and, in 1992, a new AD locus was identified by linkage analysis in families with presenile AD (AD3) [31-33]. However, the physical localization of the *AACT* gene in a different chromosomal region (14q32.1) and the existence of several recombinants quickly ruled out the "candidature" of this gene. Interestingly, significant linkage to chromosome 14q was also observed in families previously reported to be linked to the AD1 locus (*APP*). None of these pedigrees had individually shown significant evidence of linkage to chromosome 21 [27] and no mutations in the *APP* gene had been identified [56]. After the exclusion of several candidate genes and the refinement of the chromosome 14 region, the *presenilin 1* (*PSEN1*) gene was identified as the responsible gene [57], only 3 years after the linkage findings.

Approximately 268 families have been reported with *PSEN1* mutations worldwide (AD mutation database, http://molgen-www.uia.ac.be/admutations/). Most of them display early-onset AD but some also show atypical features, such as parkinsonism, frontotemporal dementia, epilepsy, myoclonus [58-60], cerebral amyloid angiopathy [61, 62] and spastic paraparesesis. The last mentioned has been associated with the deletion of exon 9 (Delta9) and unusual large "cotton wool" plaques [63].

PSEN2 (AD4)

In 1995, evidence for a locus on chromosome 1 (AD4 [35]) was reported in several kindreds known as the Volga-German families, a group of related kindreds of German-Russian origin with multiple cases of early-onset AD. Subsequently, the *presenilin 2* gene (*PSEN2*) on chromosome 1 [34, 36] was quickly identified because of its high homology with *PSEN1* and its localization within the genomic region previously identified on chromosome 1 by linkage analysis.

Unlike *PSEN1* mutations that usually lead to an early disease onset, great disparity in AAO has been observed in carriers of *PSEN2* mutations [64-66]. Available neuropathological studies report moderate cortical atrophy and abundant neurofibrillary tangles with senile plaques throughout the cerebral cortex [65].

During the last decade, an increasing number of mutations in *APP*, *PSEN1* and *PSEN2* causing AD have been reported. Around 158 mutations have been identified in these three genes, most of them (*n*=133, 84.2%) in *PSEN1*, followed by the *APP* gene, which accounts for 10.1% of the mutations. Mutations in *PSEN2* represent only a small percentage (5.7%) of all the mutations; a mere nine mutations in 15 families have been described (AD mutation database, http://molgen-www.uia.ac.be/admutations/ and http://www.alzforum.org/).

In an effort to estimate the genetic contribution of the *PSEN1* and *PSEN2* genes in a Dutch population-based study of presenile AD, mutation frequencies of 6% and 1%, respectively, were found after mutational screening of 101 unrelated familial (48%) and sporadic patients. When family history was taken into account, mutation frequencies for *PSEN1* were 9% in familial cases and 18% in autosomal dominant cases [67].

Furthermore, 56% of the early-onset French families assessed for mutations in the *PSEN1*, *PSEN2* and *APP* genes were found to be positive for *PSEN1* mutations and 15% for *APP* mutations [68]. When four genes were investigated (*PSEN1*, *PSEN2*, *APP* and the *prion protein gene*, *PRNP*) in a heterogeneous group of European patients with presenile dementia, *PSEN1* accounted for 8% of the mutations, whereas frequencies of 5%, 8% and 11% were found for the other genes, respectively. A lower frequency of *PSEN1* mutations (5.2%) was found in a clinic-based sample of 76 unrelated Colombian patients (30% with familial AD) with early (34%) and LOAD. When considering the autosomal dominant cases only, *PSEN1* mutations were recognized in 27.2% of the patients. No *PSEN2* or *APP* mutations were identified [69].

True differences between populations and the different ascertainment and inclusion criteria applied across the studies might explain the wide differences observed. Similarly, final estimates of prevalence are difficult to make. However, the studies have found evidence for a significant contribution of these genes to the aetiology of presenile AD, especially when a positive family history is present. Today, in addition to pathological confirmation of the disease, the finding of pathogenic *PSEN1*, *PSEN2* and *APP* mutations is used to confirm the diagnosis of AD. Importantly, the majority of familial AD patients do not have mutations in the three known genes suggesting the involvement of other genes not yet identified.

Although the factors involved in the cause and progression of AD are not yet well known, the discovery of *PSEN1*, *PSEN2* and *APP* mutations involved in the monogenic forms of AD has made a huge contribution to the field of neuroscience.

Presenilin activity is essential for the normal processing of APP [70, 71] and is also required for the cleavage of Notch1 at the plasma membrane and the release of its intracellular domain [72, 73]. Transgenic mice over-expressing human wild-type or mutant *PSEN1* have consistently shown elevated amounts of Aβ, especially Aβ42, disturbing the ratio Aβ40/Aβ42 [70, 74] and double mutants (*APP*/*PSEN1*) produce more Aβ than either transgene alone [48, 74, 75]. Moreover, the double transgenic mouse, *PSEN2*/*APP*, develops age-related cognitive decline associated with severe amyloidosis and inflammation in discrete brain regions [76].

Mutations in the *presenilins* alter the γ-secretase cleavage of APP resulting in over-production of the amyloidogenic Aβ42 peptides [70, 74, 77]. Indeed, it has been suggested that the presenilin1 protein could itself be a γ-secretase [78].

Recent studies indicate that presenilin proteins interact with other proteins, such as niscatrin [79] (also implicated in Notch signalling), APH-1 and PEN-2 to form a complex responsible for γ-secretase activity [80-82]. Determination of the roles of each component of the complex may provide means of intercession to arrest or prevent the pathogenic processes leading to AD.

The "amyloid cascade hypothesis" emerged more than 10 years ago [83, 84] and placed APP and specially its Aβ40–42 residue as the central protein in the mechanisms leading to AD. Considered together, the available data is consistent with the notion that *presenilins* and *APP* mutations affect the normal processing of APP by the β- and γ-secretases favouring the

production of the Aβ residue, especially of the toxic Aβ42 fraction. The imbalance between Aβ production and clearance leads to its cerebral deposition and consequent plaque formation. The occurrence of neuritic dystrophy, microglial activation and astrocytosis within amyloid plaques supports the hypothesis that these mature fibrillar lesions can induce injury [85].

Furthermore, the tau pathology observed in AD is likely to be a consequence of the Aβ imbalance [85, 86]. Mutations in the *microtubule-associated protein tau* gene do not cause AD but rather a rare form of dementia known as frontotemporal dementia (FTD) that usually occurs with parkinsonism (linked to chromosome 17, FTDP-17). The identification of mutations in the *tau* gene in patients with familial FTD [87] has strengthened the link of the *tau* gene to neurodegeneration and has contributed to the recognition of a group of related disorders known as the tauopathies. Intriguingly, coexistent tau and amyloid pathology (diffused and cored plaques plus amyloid angiopathy) in FTD patients with *tau* mutations has been reported [88] suggesting an interaction between the two pathologies.

Loci implicated in the common form of AD

APOE (AD2)

Pathogenic mutations are generally causal for certain phenotypes or diseases, with some exceptions (i.e. reduced penetrance). In contrast, susceptibility factors are neither necessary nor sufficient to cause a disease but they increase the probability or risk of having the associated phenotype or disorder.

A major susceptibility factor for AD, the *apolipoprotein* gene (*APOE*), was initially discovered by linkage analysis. In 1991, evidence supporting linkage to this region on chromosome 19 (AD2) was found in a set of families with LOAD [30]. Subsequent association studies have confirmed an increased risk in carriers of the ε4 isoform (APOE allele ε4) for late and early-onset forms of AD [89-91]. Others have shown the protective effect of the ε2 allele [92] and have estimated that the APOE genotype (ε4/ε4 and ε2/ε3) can make a difference of around 17 years in the AAO of AD [93]. Furthermore, a contribution of 7%–9% of APOE to the total variance in AAO of familial AD has been reported [93]. The presence of the APOE-4 allele is also associated with an earlier expression of clinical symptoms in carriers of specific *APP* [94] or *PSEN1* mutations [95] suggesting epistasis between the genes.

The mechanisms by which APOE-4 increases the occurrence of AD and lowers the AAO are not clearly understood. However, studies on transgenic animals have shown that APOE influences Aβ metabolism early in the amyloidogenic process and that APOE facilitates, in an isoform-dependent way, fibril and plaque formation [96, 97]. Recently, APOE has been described to undergo proteolytic cleavage resulting in the accumulation of carboxyl-terminal-truncated fragments of APOE, which are neurotoxic. Moreover, the cleavage of APOE-4 occurs in a more efficient way than that of APOE-3. APOE-4 C-terminal-truncated fragments are present in AD brains and are sufficient to provoke AD-like neurodegeneration and behavioural deficits in transgenic mice [98, 99].

Not surprisingly, the association with APOE is one of the few that has been consistently replicated and, so far, is the risk factor with the highest effect on AD. At least four more genes are thought to contribute to AAO variation of AD, with an effect as large as or larger than that of *APOE* [93].

Regardless of the progress made in the mapping of genes for Mendelian forms of

AD, efforts aimed at identifying genes implicated in the common late-onset form (other than *APOE*) have not been extremely successful. The genetic basis of this form is largely unknown. To address this problem, two main types of genetic studies have been performed: (1) linkage-based studies that mostly involve a collection of small families with two or more patients, viz. affected sib-pairs (ASP) and affected relative pairs (ARP) or multiplex families; (2) association-based studies that attempt a candidate gene approach in samples of patients and controls.

The principle underlying association studies is straightforward involving the search for significant differences in alleles at a specific locus between a well-selected group of patients and controls. However, the performance of these studies and the interpretation of their findings remain a major challenge, as corroborated by the observation that the consistent replication of association studies has, in most instances, been difficult to achieve. A recent review has found that only six out of the 166 positive associations that has been studied three or more times have been consistently replicated [100]. Some of the possible reasons for inconsistent results are false-positive results attributable to Type I error, false-negative results attributable to lack or insufficient power to detect the association in potential replication studies, the presence of population stratification and true differences between studied populations [101].

A combination of strategies such as family-based association tests have been used by others [37, 102]. As a result, several groups have identified a number of loci across the genome (i.e. chromosomes 12, 10, 9, 20) and an association with approximately 40 genes has been reported [103], although none has as yet been definitely established as an AD-susceptibility factor locus.

Chromosome 12 (AD5)

In 1997, a genome-wide screen and multi-analytical procedure that employed both parametric and non-parametric analyses identified a region on chromosome 12 (D12S373-D12S390) in which a gene related to AD could be located [39]. Subsequently, the region (12p11.23-q13.12) was called AD5. Strikingly, no confirmation was reached during a second (fine mapping) study in the same sample set. Two peaks (~20 cM in between) were generated depending on the way in which data stratification was performed, i.e. by APOE genotype, diagnosis of Lewy-body dementia at autopsy or family size. The largest contribution to the peak at D12S1042 came from a group of families in which at least one patient had the neuropathological diagnosis of Lewy-body dementia and that were lacking the APOE-4 allele [104].

Following this initial report, several groups carried out replication studies on the chromosome 12 locus in different sample sets but with similar methodologies [40, 105, 106]. Evidence for linkage to a larger chromosomal interval that included AD5 (67 cM, D12S358–D12S96) in the presence of genetic heterogeneity was found in a group of 53 families. The level of significance was still reached after applying the Bonferroni correction for multiple testing [40]. However, in an independent and larger set of patients (ASP from 230 families with LOAD), the previous findings could not be replicated and a different region on chromosome 12p (12p13.3-p12.3) showed suggestive evidence of linkage, close to the *alpha-2macroglobulin* gene (*A2M*). Most of the evidence came from affected pairs where both members were APOE-4-negative [106].

Although several groups have attempted to confirm and refine the genetic location of the putative AD locus on chromosome 12, the results have been inconsistent. This inconsistency may arise because of several reasons: genetic heterogeneity, difficulties inherent in the diagnosis of dementia, lack of good “biological markers” for the disease

and sample admixture. Similarly, methodological problems such as multiple testing and improper corrections for this may obscure the actual significance of the findings. Furthermore, preliminary power calculations and the proper determination of significance levels are lacking in most of the studies.

The selection of the most appropriate method of analysis is also a complex issue and depends on several factors: the disease under study, the type and size of the available sample and the density and type of the genetic markers. It is alarming to observe the amount of positive linkage reports obtained after multiple analyses of the genetic data without the appropriate correction or definition of stringent significance levels having been made. Although it is valid to perform exploratory analyses to assess the best genetic model or a better method of study, the results emerging from such analyses should not be interpreted as true linkage findings.

In order to avoid some of these problems, several groups have chosen the "candidate gene approach" as an alternative strategy to identify the AD5 gene(s). The *alpha-2macroglobulin* (*A2M*), its receptor the *low density lipoprotein receptor-related protein1* (*LRP1*) and the transcription factor *LBP-1c*/*CP2*/*LSF* genes have been intensively studied as candidates influencing the susceptibility to AD because of their chromosomal location within the critical regions and their role in β-amyloid metabolism [39, 107]. Initial positive reports of association [37, 38, 108-110], have been followed by negative findings and the absence of confirmation by independent groups studying different populations [110-122]. Furthermore, little evidence of the functionality of the polymorphisms has been presented.

In conclusion, although the identified regions on chromosome 12 bear tentative candidate genes and the positional candidate gene approach seems to be a good strategy, the various studies have yielded inconsistent and controversial results.

Chromosome 10 (AD6)

A two-staged genome screening was performed in ASP with LOAD from the National Institute of Mental Health (NIMH) AD Genetics Initiative; the sample was stratified on the basis of whether both or neither of the ASP members possessed at least one APOE-4 allele. The first stage involved genotyping 292 ASP by using a 20-cM marker interval [123, 124]. During the second stage, 451 ASP were genotyped with an additional 91 markers, located within the 16 regions in which the multipoint LOD score was greater than 1 in stage I [124]. Significance levels were estimated by simulation calculations. The best results in both stages corresponded to a region on chromosome 10q for which the initially obtained maxLOD score of 2.3 increased to 3.9 in stage II. The region on chromosome 10 was refined to approximately 44 cM spanning from D10S1426 to D10S2327 [44, 124].

Subsequently, Myers et al. [124] attempted to track the region by examining linkage disequilibrium in the area but they could not find a positive association when independent groups of discordant sib-pairs and a case-control sample were assessed. The same genomic region has been found by another group [125] but, because of overlap in the sample set, these studies cannot be considered completely independent [124].

Evidence pointing to the same region on chromosome 10q was found in a sample of late-onset extended pedigrees that were selected based on probands with extremely high plasma values of Aβ42. The investigated region gave a maximum multipoint LOD score of 3.93 at 81 cM, when high plasma values of Aβ42 were considered as a surrogate trait in a quantitative trait analysis [42]. Abnormal levels of tau protein and Aβ42 in cerebrospinal fluid have been used

as potential biological and diagnostic markers of AD, although other neurological disorders may also cause anomalous protein levels [126]. Conversely, results of plasma levels of Aβ40 and Aβ42 in relation to AD have been contradictory. Whereas the plasma level of Aβ42 has been found to be elevated in familial forms of AD with *PSEN1*, *PSEN2* and *APP* mutations [127, 128] others have found no such relationship with "sporadic" AD [129, 130].

Several candidate genes located on chromosome 10q have been tested for association with LOAD in different sample sets by using single nucleotide polymorphisms (SNPs) within and around the candidate genes and by searching for association between the independent SNPs and/or SNPs haplotypes and the disease (Table 1). Most of these studies have produced negative results [131-135] however, some groups have reported a positive association for *PLAU* [133, 136] and *IDE* [137]. Moreover, hippocampal IDE protein levels are reduced by more than 50% in ε4-positive LOAD patients compared with ε4-negative patients and controls, suggesting that IDE may interact with APOE status to affect Aβ metabolism [138].

Recently, significant association has been found with two SNPs located within intron 10 of the *VR22* gene in the same set of families for which linkage to high plasma levels of Aβ42 was initially described (10q, 81 cM) [42, 43]. VR22 encodes a novel α-catenin (α-T catenin), which interacts with β-catenin in a functionally effective fashion [139]; β-catenin also interacts with presenilin 1 [140]. Biological evidence is now needed to explore the way in which *VR22* variants are associated with high plasma levels of Aβ42 and whether these variants modify the risk for LOAD.

On the other hand, there is a growing interest in the identification of not only "risk genes", but also "AAO genes" that may be involved in controlling the AAO of some genetic disorders. Using an interesting approach that merged a sample of 449 AD families and 174 PD families, a putative locus controlling AAO of these two neurodegenerative disorders was identified on chromosome 10q (133–135 cM) [141]. Recent data suggest that the *glutathion S-transferase omega-1* (*GSTO1*) gene, a positional candidate, is down-regulated in the hippocampus of AD cases and act as a modifier for the AAO in both AD and PD cases [142].

AD7

The AD7 locus has been assigned to chromosome 10 close to marker D10S1423 (10p13). After a genome screen, allelic association with AD was observed for six genetic markers (D1S518, D1S547, D10S1423, D12S1045, D19S178, DX1047) 46. Independent replication came from a German sample of 80 AD patients and approximately 300 controls 143 and a prospective longitudinal study (11.5 years follow-up) of 325 asymptomatic first-degree relatives who carried the 234-bp allele (D10S1423), the APOE-4 allele or both. Individuals carrying both risk alleles had the greatest age-specific risk of developing AD 144.

The same region (AD7, 10p13), 5 cM away from D10S1423, was found in a genome-wide linkage-disequilibrium mapping investigation in Finland. The study was performed in a sample of patients and controls chosen from a geographically restricted area in eastern Finland in which the population has descended from a small group of founders who migrated to the region in the late 16th and early 17th centuries. Another six chromosomal regions were identified that contained more than one marker associated with AD: 1p36.12, 2p22.2, 3q28, 4p13, 18q12.1 and 19p13.3 45.

AD8

In addition to the linkage signal repeatedly observed in the *APP* region (chromosome 21), a joint effect with chromosome 20p was reported in 272 ASP with AD by Olson et al. [47]. The authors revised the data that had previously been collected and genotyped as part of the NIMH AD Genetics Initiative [123, 124]. Using an interesting approach that incorporated covariates to the linkage analysis and that allowed for detection of genetic linkage in the presence of locus heterogeneity, this group was able to identify a region on chromosome 20p (AD8, 21 cM) when the current age of patients was taken into the analysis. The incorporation of the presence of the APOE-2 allele as a covariate yielded a higher LOD score (4.09). Moreover, a two-locus model provided evidence of strong epistasis between chromosome 20p and the *APP* region on chromosome 21, especially in those patients who were of an older age and lacked APOE-4. The authors suggested that the development and/or rate of progression of AD in such families is influenced by the presence of high risk alleles at both loci, which probably interact biologically to increase disease risk [47, 145].

During this last analysis, the regions previously reported as being positive in the same dataset were not detected, probably because of the different analysis methods applied. Incorporation of covariates and allowance for locus heterogeneity increases the probability of Type I errors attributable to multiple analyses of the same genetic data. The authors therefore recommended careful selection of covariates and cautious interpretation of the results [47].

A recent reanalysis of the genotypic data of 437 families from the NIMH Genetic Initiative, with a similar method that ordered subset of families by using covariates (in this case, AAO), detected regions on chromosome 2q34 (210 cM) in a subset of families with early-onset AD, 9p22 (42 cM) in families with LOAD and 15q22 (60–62 cM) in families with AD of very late onset (≥79 years) [146]. In spite of the overlapping of the sample sets, only the 9p region has been reported before. On the other hand, a region 12 cM away from AD8 was also identified when the specific set of families were reanalysed with the ordered subset method [146].

Other loci

Recently, a genetic isolate community (Israeli-Arab) with a high degree of inbreeding and unusual high prevalence of "dementia of the Alzheimer type" was described. The diagnosis of the patients was mainly clinical and only a few cases underwent neuroimaging studies; neuropathology was unavailable. Multiple loci were found after performing a genome scan and association analysis; all of the loci overlapped with loci reported previously. The chromosome 9 (32–45 cM) and chromosome 12 (~83 cM) regions were highly significant, whereas the chromosome 10 region (~105–115 cM) and the chromosome 2 region (~41 cM), which lies 6–10 cM away from a region previously detected by linkage-disequilibrium mapping in Finland [45], showed suggestive linkage [147]. Other studies also found several regions on chromosome 9: 9q22 [123, 124, 148] 9p22.1 and 9q34.2 [125].

Another recent genome scan carried out in the full NIMH Genetic Initiative sample (437 AD families, most of them with only one ARP) identified regions that overlapped with previous reports. For chromosome 1 (1q23 [123, 148]), a region close to the gene encoding niscatrin (*NCSTN*), which binds presenilin and is required for γ-secretase activity and Aβ generation was found [149]. The 6p21 [39, 123, 141, 148], 6q26 [39, 145], 6q27 [148] and 19q13 (close to *APOE*) [39, 123, 148] regions have been frequently reported. Patients sharing alleles at the 19q13 region had

significantly more APOE-4 alleles than those who did not share alleles at the chromosome 19 marker, suggesting that the linkage signal on this region corresponds to the *APOE* gene [124].

Because of the overlap of the sample sets used in the different studies, similar results are expected. Thus, the use of largely overlapping sample sets limits the ability of independently replication of the positive results. Moreover, the use of different statistics in the same sample sets has also resulted in the identification of diverse genomic regions.

We believe that the presence of genetic heterogeneity, the lack of comprehension of the actual genetic model in complex disorders and methodological problems are having an effect on the successful mapping of susceptibly genes involved in AD aetiology. Only our ability to overcome these difficulties will lead to the detection of predisposing genes that have modest effects and whose interaction ends in neurodegeneration and disease.

Parkinson's disease

Parkinson's disease (PD, OMIM 168600) is the second most common neurodegenerative disorder in humans. Similarly to AD, the prevalence of PD increases with age, age being one of the most important risk factors. PD prevalence has been estimated to be around 2% among people over the age of 65 years [150].

PD is a progressive disorder that is characterized by severe motor symptoms, including tremor, slowness of movement, muscular rigidity and postural imbalance. The main pathological hallmarks of this disorder are a pronounced loss of dopamine-producing neurons in the substantia nigra pars compacta [151] with Lewy bodies, cytoplasmic inclusions consisting of insoluble protein aggregates, in surviving group of neurons [152].

Clinically defined PD represents a heterogeneous disorder that encompasses a spectrum of a small proportion of individuals with disease inherited in a Mendelian fashion, families with more than one patient but unidentifiable pattern of inheritance and a larger population of patients with isolated (idiopathic) disease. Families with autosomal dominant and autosomal recessive inheritance can often be distinguished from idiopathic PD; they usually have an earlier AAO, especially in autosomal recessive juvenile parkinsonism (AAO<21 years) and early-onset Parkinson's disease (EOP, AAO<45 years).

The contribution of genetic factors to PD has been, for many years, the object of controversy. Early contentious findings in MZ and DZ twin studies, the role of environmental factors such as MTPT in the aetiology of PD-like phenotypes and that PD seems to be a syndrome rather than a single entity have challenged our understanding of the contribution of genetics to the aetiology of typical PD.

Familial aggregation

In the early 1980s, several twin studies reported low concordance rates for MZ and DZ twins with PD [153, 154], however, others subsequently noticed methodological pitfalls and recommended caution when interpreting those premature findings [155]. AAO [156] and the long latency (preclinical) period of the disease were also identified as potential pitfalls. In 1999, a large cross-sectional twin study (white males in United States) identified a high concordance rate for MZ over DZ twins that was only present when the disease started at or before the age of 50 years [156]. Concordance for subclinical striatal dopaminergic dysfunction was also found to be significantly higher in MZ than in DZ twins in a 7-year positron-emission-tomography

longitudinal study [157] suggesting that the low rates of concordance in previous studies could have arisen because of the inability to detect presymptomatic disease and the absence of follow-up.

In addition to the known monogenic forms, familial aggregation has been found for typical late-onset PD (>50 years of age) [158, 159]. First-degree relatives of PD cases are 2–7 times more likely to develop PD than the first-degree relatives of controls in community-based studies [159-161]. In a comparative clinic-based case-control study of early-onset and late-onset PD cases, the age-specific risk of PD increased 7.76-fold in the relatives of early-onset cases and 2.95-fold in relatives of those with late-onset disease [162]. A stronger association for younger than for older PD patients was also observed in an large community-based study in Europe, whereas the analysis showed a significant trend of increasing risk with increasing number of affected relatives [163].

Furthermore, segregation analysis by using information on families of PD patients ascertained randomly from a movement disorder clinic suggested that familial clustering in PD was best explained by a rare "familial factor" transmitted in a non-Mendelian way and influenced the AAO of the disease [164]. Complex segregation analysis performed in a Finnish population considered to be genetically homogeneous because it evolved from a small group of founders suggested that the role of a major gene in early-onset cases is greater than in late-onset PD. Moreover, in families with several affected members, the best fitting genetic hypothesis was a major locus influencing the susceptibility to PD, although the exact model (dominant or recessive) could not be defined [165]. Early- and late-onset patients were also ascertained in the Icelandic population and linked to the information from a population-based genealogical database. Calculation of kinship coefficients (a measure of genetic relationship between two persons) showed that PD patients were significantly more related than the control group, the clustering of PD extending beyond the nuclear family. Accordingly, all risk ratios for first- and second-degree relatives were high, for the complete sample (6.6–2.4, 772 patients) and for the late-onset group (6.7–2.7, n=560). The risk ratios for siblings were higher than those for offspring, suggesting a role for some environmental factors shared early in life, whereas the spouses of PD patients were not at an increased risk for the disease [159].

Recent segregation analysis performed on 948 nuclear families with 4351 persons being included in the study have found evidence for both a major dominant gene influencing the AAO and a major additive gene influencing the susceptibility to PD [166]. Finally, recent genome scans have presented evidence suggesting the localization of putative genes influencing the AAO [141, 167] and a locus influencing the susceptibility to late-onset PD [168].

Known genes involved in Mendelian forms of PD

Alpha-synuclein (PARK1)

The first gene for familial PD (α-*synuclein*, PARK1) was mapped in 1996 in a large kindred of Italian origin, known as the Contursi kindred, with an autosomal dominant form of the disease [169]. The Contursi kindred is a large family originating from a town with this name in Italy. Many of the family members immigrated to New Jersey where they were recognized as part of this multiplex kindred the founders of which can be traced back to the 18th century [170]. The illness

in this family is comparable to PD but the AAO is on average 15 years younger (46±13.6) and there is a rapid disease progression. Moreover, some degree of cognitive impairment occurs in some patients [170]. Autopsy of two affected individuals showed Lewy bodies and other changes typical of PD [171].

A genome scan performed in this large family lead to the identification of a chromosomal interval that yielded a significant LOD score (maxLOD=6.04) [169]. Soon after, a mutation in the α-*synuclein* gene, Ala53Thr, located in the former identified 6 cM candidate region was found in the large Italian family and three families of Greek origin [172]. Several Greek families with a similar clinical picture and the Ala53Thr mutation were reported; the presence of unaffected carriers older than the expected AAO suggested incomplete penetrance of the mutation [173]. Further genetic studies in these Greek families found evidence for a common founder haplotype in the α-*synuclein* region and the origin of these families was traced to an area in the northern Peloponnese in Greece. The same haplotype was found in the kindred from Contursi, a region geographically and historically linked to Greece, suggesting that patients from these families may all be descendants of a single founder [174]. Re-examination of one of the original brains from the Contursi kindred, with new immunohistochemical tools, showed a dense burden of α-synuclein pathology, "rare Lewy bodies" and tau inclusions. Therefore, the neurodegenerative process caused by the Ala53Thr mutation is not identical to that seen in idiopathic PD [175].

A second mutation resulting in a substitution of alanine with proline at position 30 (Ala30Pro) has been described in a German family [176]. However, mutations in the α-*synuclein* gene are a rare cause of PD; no mutations were found after screening large numbers of PD patients with familial, sporadic, late- or early-onset forms of the disease [177-180]. Genetic heterogeneity became evident for the familial forms of parkinsonism (Table 2).

Table 2. **Loci and genes related to Parkinson's disease aetiology (AD autosomal dominant, AR autosomal recessive, AAO age at onset, FBATs family-based association tests, QTL quantitative trait loci)**

PD locus	Chromosomal location	Genes	Mapping method	Molecular defects	References
PARK1	4q21	*Alpha-Synuclein*	Linkage analysis (AD)	Missense mutations: A53T (G209A, exon 4), A30P (G88C, exon 3)	169, 172
PARK2	6q25.2–27	*Parkin*	Linkage analysis (AR)	Single base substitutions (missense, nonsense, frameshift, splicing) deletions, duplications	181, 182
PARK3	2p13	Unknown	Linkage analysis (AD, reduced penetrance). For AAO: linkage and association (FBATs)	-	167, 183
PARK4 (actually PARK1)	4p15 (actually 4q21)	*Alpha-Synuclein*	Linkage and haplotype analysis (AD)	Gene triplication	184, 185
PARK5	4p14	*UCHL-1*	Mutation analysis (AD, reduced penetrance)	I93M (exon 4)	186
PARK6	1p35–36	Unknown	Linkage analysis (AR)	-	187
PARK7	1p36	*DJ-1*	Linkage, homozygosity mapping (AR)	Single base substitutions (missense, nonsense, splicing), deletions	188, 189
PARK8	12p11.2-q13.1	Unknown	Linkage analysis (AD)	-	190
PARK9	1p36	Unknown	Linkage analysis (AR)	-	191
PARK10	1p32	Unknown	Linkage analysis (population design, model-free). For AAO: QTL analysis	-	141, 168
PARK11	2q36–37	Unknown	Linkage analysis (model-free and AD model with reduced penetrance)	-	192, 193

Alpha-synuclein mutations provided one of the first clues for understanding the pathways leading to PD. The role of α-synuclein in PD was confirmed by the finding of this protein as the major component of the Lewy bodies [194]. This observation also revealed the role of abnormal protein folding and aggregation in PD and, indeed, early in vitro experiments showed accelerated mutation-induced (A53T) protein aggregation and fibril formation [195].

Animal models of neurodegenerative disorders have been crucial in the understanding of the pathogenesis of these diseases. Transgenic mice over-expressing the wild-type human α-*synuclein* gene [196] or with the A53T mutation develop adult-onset motoric dysfunction leading to death and α-synuclein pathology [197-199]. Results from experiments in knockout α-*synuclein* mice support the hypothesis that α-synuclein is an essential presynaptic activity-dependent negative regulator of dopamine neurotransmission [200]. A transgenic fly model (wild-type or mutant α-*synuclein*) of PD displays adult-onset loss of dopaminergic neurons, filamentous intraneuronal inclusions containing α-synuclein and locomotor alterations compatible with the clinical picture observed in humans [201].

Both α-*synuclein* mutations (see above) have subsequently been shown to accelerate the formation of prefibrillar oligomers (protofibrils) in vitro [202]. Biophysical studies and animal modelling aimed at elucidating the mechanism of fibril formation support the emerging notion that toxic protofibrils may be responsible for cell death and that the fibrillar formations are observed later [203, 204].

The over-expression of α-synuclein is believed to be a risk factor for PD. Some studies have shown associations with polymorphisms (e.g. Rep-1; [205, 206]) located within the promotor region of α-*synuclein* and related to variations in the gene expression [207-209].

Recently, in a large family with parkinsonism (originally PARK4, see below), Singleton et al. [185] have found evidence consistent with triplication of the α-*synuclein* gene. The authors suggest that the increased dosage of α-*synuclein* is the cause of PD in this family and that a "dosis effect" (complete gene triplication) can be considered as the third mutation in this gene.

Parkin (PARK2)

In 1997, the PARK2 locus was mapped to a 17-cM region in chromosome 6q by linkage analysis in Japanese patients with EOP and autosomal recessive inheritance [182]. Clinically, the classic signs of bradykinesia, rigidity, tremor and early dystonia characterize this form of juvenile parkinsonism, plus slow progression, sleep benefit and good response to levodopa treatment. Pathologically, neuronal loss, gliosis (substantia nigra and locus ceruleus) and absence of Lewy bodies have usually been reported [210-212].

The second gene involved in the pathogenesis of parkinsonism was identified, in 1998, by positional cloning; the gene was named *parkin* [181]. Since then, a wide variety of mutations have been described in this gene ranging from point mutations to complex rearrangements, including deletions and/or multiplications of complete exons in the homozygous or heterozygous state [213-217]. In a large European study, *parkin* mutations were found in almost 50% of the families with EOP [216]. Among sporadic patients with disease onset below 45 years, *parkin* mutations were detected in 15% of the cases showing that mutations in this gene are a major cause of EOP [216, 218].

The contribution of *parkin* mutations or polymorphisms to typical late-onset PD has been the subject of intense research. A recent study involved mutation analysis in a large number of ASP (early- and late-onset). Mutations were identified in 103 (18.4%) of these familial PD cases; among these, 60% had a single mutation. Thirty-five (35/103) had an AAO of more than 60 years [219] suggesting that *parkin* mutations should be considered when late-onset PD patients are evaluated, especially when they have a positive family history.

The clinical consequence of carrying only one *parkin* mutation has been an issue of debate. Several studies have hypothesized that haploinsufficiency attributable to the partial loss of parkin expression may be a risk factor for dopaminergic cell death [220] and may confer risk for late-onset PD [219, 221]. Others have suggested that this simply reflects the inability to find a second DNA change located in regulatory regions, promoters or introns. Whether one heterozygous *parkin* mutation is sufficient to confer susceptibly to PD or whether this happens through a combination with other changes within this or another genes remains unclear. Recently, a genome scan performed in families with only one *parkin* mutation also found suggestive evidence of linkage to chromosome 10q24 suggesting a potential epistatic or additive interaction between the *parkin* gene and a chromosome 10 susceptibility locus [222]. Interestingly, the same region has been linked to AD.

Although Lewy bodies are a remarkable pathological feature of PD, the underlying mechanisms leading to the formation and their significance are poorly understood. The absence of Lewy bodies in the majority of patients with *parkin* mutations suggests that parkin might be required for the formation of Lewy bodies [223]. The available reports on the pathological

aspects of "parkin disease" are based on cases with homozygous exon deletions [210, 211, 224] and compound heterozygous exon deletions and point mutations [212, 225]. Lewy bodies have been documented in one patient with EOP belonging to the last group (compound heterozygous: deletion of 40 bp in exon 3 plus Arg275Trp, R275W); positive ubiquitin and α-*synuclein* inclusions were also found in this patient.

Recent experiments in cellular models have shown that the R275W mutation, located within the RING finger 1 domain, leads to an unusual cellular distribution of the protein, with large cytoplasmic and nuclear inclusions [226]. Cookson et al. [226] suggest that some *parkin* mutations may confer a dominant gain of function depending on the protein domain affected. Simultaneously, a case-control study found a similar R275W frequency in cases and controls (unaffected at age ≥75 years) [227] suggesting that the R275W allele and possibly other point mutations that do not cause a complete loss of function may predispose to PD in compound heterozygotes.

Although the mechanisms leading to neurodegeneration, cell death and disease are not well understood, parkin is known to be involved in protein degradation as an ubiquitin ligase, collaborating with the ubiquitin-conjugating (E2) enzyme UbcH7 or UbcH8. Parkin itself is a RING-type ubiquitin (E3) ligase involved in the ubiquitination of targeted proteins. Loss of the parkin ubiquitin-protein ligase function leads to the accumulation of proteins such as the synaptic vesicle-associated protein, CDCrel1 [228], Paelr [229] and neural cell death [230, 231]. Parkin interacts also with synaptotagmin XI, an interaction resulting in its ubiquitination and degradation. The interaction with a member of the synaptotagmin family suggests an involvement of parkin in neurotransmitter trafficking, in the regulation of the synaptic vesicle pool and in vesicle release [232].

Several findings indicate that the relationship between "parkin disease" and PD extends beyond a simple clinical overlap. Loss of parkin function is expected to cause the pathological accumulation of a form of glycosilated α-synuclein (αSp22) because of its inability to polyubiquitinate αSp22 [233]. In addition, over-expression of parkin can mitigate α-synuclein-induced pathology and suppress its toxicity [234]. Parkin over-expression in cellular models with impairment of proteasomal activity also leads to the formation of aggresome-like inclusions; these are eosinophilic inclusions (resembling Lewy bodies) with a core and halo. Furthermore, wild-type parkin has a tendency to aggregate and form inclusions that may have implications for the pathogenesis of sporadic PD [235]. Parkin also interacts with and ubiquitinates the α-synuclein-interacting protein, synphilin-1 [236], which is enriched in Lewy bodies, specifically in their central core [237]. These findings potentially link two familial associated PD genes, viz. *parkin* and α-*synuclein*, through synphilin-1. Parkin ubiquitinates the Lewy-body-like inclusions that are formed when α-synuclein is co-expressed with synphilin-1; this ubiquitination is impaired by familial-associated parkin mutants [236]. These results aid our understanding of the molecular mechanisms underlying "parkin disease" and typical PD and the plausible interaction of two PD-related gene products in a common pathway: the ubiquitin-proteasome system.

Ubiquitin C-terminal hydrolase (PARK5)

A mutation (Ile93Met) in the *ubiquitin C-terminal hydrolase* gene (*UCHL-1*, PARK5) [186] was identified in a German family with autosomal dominant PD. The missense mutation was found in two siblings and seemed to have incomplete penetrance in the family, since the phenotype

was lacking in the father who was presumed to be a carrier. Partial loss of the catalytic activity in the mutant protein was found in vitro [186].

In the search for *UCHL-1* mutations, several polymorphisms have been described in this gene, S18Y (serine to tyrosine) situated in exon 3 has been associated with a low risk for PD [238, 239], suggesting a protective effect of this variant, but not all studies have found this association [240]. Recently, an interaction with AAO has also been suggested, since carriers of the Y allele were found to have a decreased risk of developing PD at a younger age [241].

The UCHL-1 protein is present in large amounts in brain [242]; it has been found in Lewy bodies of patients with PD [243] and its role in recycling free monomeric ubiquitin units is thought to stimulate protein degradation. An in-frame deletion including exon 7–8 of the *UCHL-1* gene in gracile axonal dystrophy mice (*gad* mice) leads to the altered function of the ubiquitin system by disturbing the re-utilization of free ubiquitin, which results in the accumulation of target proteins and neurodegeneration. Nevertheless, the clinical and pathological features of the *gad* mouse are not consistent with those of PD [244].

Although sporadic and familial PD cases have been assessed for mutations in the *UCHL-1* gene during the last few years, none has been found [238, 239, 245, 246] indicating that, if this is truly a PD gene, mutations in the coding region of *UCHL-1* are a rare cause of PD.

DJ-1 (PARK7)

The PARK7 locus was found by homozygosity mapping in a family with several consanguinity loops and autosomal recessive EOP; the family comes from an isolated community in the south-western part of the Netherlands [189]. The initial genomic region (1p36) spanned 16 cM and was separated from the nearby PARK6 locus by ~25 cM. Confirmation of this finding came from one additional family of Italian origin with early-onset autosomal recessive parkinsonism [247]. The identification of a homozygous point mutation, resulting in a leucine to proline substitution (Leu166Pro, Italian), and a 14-kb deletion (Dutch) in the *DJ-1* gene located in the PARK7 region provided the evidence for the fourth gene involved in PD [188].

To date, several mutations have been found in the gene including base changes, deletions and splicing mutations [248, 249]. Some of them are probably pathogenic: the homozygous (Met26Ile) mutation affecting a highly conserved amino acid has not been found in ethnically matched controls; a heterozygous single-base deletion and a base change in exon 2 (c.56delC, c.57G→A) are predicted to cause a truncated protein and, finally, the base change IVS6–1G→C is expected to lead to an aberrant *DJ-1* RNA because of defective splicing. Less clear is the case of one EOP patient with a heterozygous (Asp149Ala) mutation (not present in controls) but in whom a second mutation was not found. It is should be noted that the studies published to date only involve sequence analysis; gene dosage studies are still lacking. Furthermore, the finding of heterozygous sequence changes and other variants has raised the hypothesis (as for the *parkin* gene) as to whether these changes confer susceptibility to the disease, alone or in combination with other unknown factors. The contribution of *DJ-1* mutations to the aetiology of EOP seems to be rare; however, their prevalence needs to be established. Further studies involving mutational analysis, including exon dosage, and other studies to determine the functional implications of these mutations are required.

The *DJ-1* gene encodes a multifunctional protein that is ubiquitously expressed in body tissues and brain areas. Evidence has been obtained regarding its involvement in the oxidative stress response [250, 251].The finding of the *DJ-1* mutations and the putative role of the

DJ-1 protein in oxidative stress provide a link between this pathway and the pathogenesis of parkinsonism [188]. Recent studies on the crystal structure of the DJ-1 protein have shown that the protein may function only as dimers [252-254]; the Leu166Pro mutation abolishes the dimerization of DJ-1 [253, 255-257] possibly affecting its function. Moreover, a chaperone activity (which suppresses the aggregation of unfolding intermediates) has also been reported [256].The finding that DJ-1 is sumoylated, an ubiquitin-like protein modification, suggests that it might be a target for parkin, thus connecting it to the ubiquitin-proteasome pathway [258]. Indeed, mutant DJ-1^{L166P} is less stable than the wild-type protein [257] and its degradation might be promoted via the ubiquitin-proteasome pathway [259]. The identification of DJ-1 interacting proteins and *DJ-1* variants and their possible relationships with those from other PD-related genes would be an interesting approach to elucidate the mechanisms causing parkinsonism.

Nuclear receptor related-1

Two heterozygous mutations have been identified in exon 1 (–291delT and –245T to G) of the *nuclear receptor related-1* gene (*NR4A2*, formerly known as *Nurr1*) in 10 out of 107 cases with PD and a positive family history. The clinical picture is similar to those of individuals with typical PD, with a mean AAO of 54±7 years. The mutations have been found to co-segregate with the disease in all (n=10) investigated families, whereas none of the mutations has been observed in cases with "sporadic" PD. Haplotype analysis has also shown a common haplotype in three different families of German origin suggesting a founder effect of the mutations [260]. Therefore, the frequency of the mutations in the general population is likely to be rare. The mutations result in a marked decrease in NR4A2 mRNA levels in transfected cell lines and in lymphocytes of affected individuals.

NR4A2 is a transcription factor that has a role in the differentiation of midbrain precursors into dopamine neurons and their maintenance [261]. Mice lacking *NR4A2* fail to generate midbrain dopaminergic neurons, are hypoactive and die soon after birth. The brains of heterozygous animals, which are otherwise apparently healthy, contain reduced dopamine levels [262]. Despite the evidence that makes the *NR4A2* gene an attractive candidate gene for PD, the search for mutations has so far been fruitless [263-265] indicating that the contribution of *NR4A2* mutations to the aetiology of PD, if any, is very low.

Known loci implicated in PD aetiology

PARK3

This susceptibility locus for late-onset PD was mapped in German/Danish families to chromosome 2p13. The clinical characteristics of the ascertained families resembled those of typical PD. Significant evidence for linkage was found in four out of six families in the study by using parametric affected-only analysis [183].

Strikingly, the PARK3 locus has also been implicated as an AAO locus for PD. The positive association with allele 174 (bp) of marker D2S1394, which is also present in the common haplotype in two PARK3 families (German-Danish), suggests that this allele is in linkage disequilibrium with a susceptibility PD gene or a gene influencing the AAO of PD [167].

PARK4, PARK6, PARK8 and PARK9

A number of loci for familial parkinsonism have been found in various families around the world. The PARK4 locus was reported in a large family with early-onset levodopa-responsive parkinsonism (Iowa kindred, 4p15)[184]. The obtained LOD scores were not significant but were suggestive and the findings were not replicated. Four years later, re-evaluation of the genetic data (an unaffected individual who did not share the 4p15 haplotype became ill), showed a multipoint LOD score of 3.5 at the α-*synuclein* locus. No mutations were found by direct sequencing of the gene, but real-time polymerase chain reaction analysis yielded results consistent with a whole gene triplication co-segregating with parkinsonism. Carriers of the α-*synuclein* triplication are predicted to have four functional copies of the gene, suggesting that this increased dosage of α-*synuclein* is the cause of the disease in this family [185].

The PARK6 locus (1p35–36) was identified in an Italian family with autosomal recessive EOP, from Sicily [187]. Subsequently, suggestive evidence of linkage was found in other families from Europe [266, 267].

The PARK8 locus (12p11.2-q13.1) was found in a large Japanese family with a genome-wide significance level. Interestingly, neuropathological examination of two cases showed pure nigral degeneration without Lewy bodies [190]. Recent replication has come from two families (family A, German-Canadian; family D, western Nebraska) with genetic linkage to the same region. Remarkably, pathological data has shown a variable spectrum within one family with several individuals with and without Lewy-body pathology [268].

Kufor-Rakeb syndrome was described in the offspring of a consanguineous Jordanian couple. The disorder is an autosomal recessive nigro-striatal-pallidal-pyramidal neurodegeneration, with juvenile onset [269]. Linkage to chromosome 1p36 was reported (maxLOD=3.6)[191] and the PARK9 locus was assigned; however, this parkinsonian syndrome has apparently little in common with PD.

PARK10

This was the first susceptibility locus for PD mapped by using a "population-wide" design. The study included 51 extended families with 117 patients connected at or within six meiotic events and 168 of their unaffected relatives from Iceland [168]. Hick et al. [168] used a dense map of 781 markers and model-independent, affected-only and allele-sharing methods for the analysis. They specifically searched for genomic regions that were shared by affected relatives more often than expected under the assumption of no linkage. A maxLOD of 3.9 was obtained. After fine mapping, this value increased to 4.9 and the detected region spanned ~7.6 cM on 1p32 [168].

Significant evidence of linkage was found on chromosome 1p (D1S2134, 78 cM) [141] at the same location of PARK10, by using a different approach that considered AAO as a quantitative trait in a sample of 174 families with PD from the US and Australia. Remarkably, two loci (PARK3 and PARK10) were found to contribute to both disease susceptibility and the AAO of the disease. It will be interesting to decipher whether the same gene(s) are involved in mechanisms that increase disease susceptibility and lower the AAO.

PARK11

Recent non-parametric analysis of a sample consisting mainly of ASP (see below) found suggestive LOD scores for regions on chromosomes X and 2 [192]. Follow-up of the 2q36–37 region in a larger sample set provided additional evidence of linkage to this region. A LOD score of 2.47 was obtained in the expanded sample containing ASP and multiplex families (*n*=150 families) but augmented to 3.51 with the inclusion of 25 families with one or two *parkin* mutations. This suggests a potential epistatic or additive interaction between *parkin* and the PARK11 locus [193].

Other loci

Several genome searches aimed at the identification of new PD susceptibility genes have found other chromosomal regions that might bear genes conferring risk for PD. Two main strategies have been applied: (1) the family-based approach, which uses large pedigrees with an identifiable Mendelian pattern and parametric analysis, and (2) the sib-pair approach involving the use of large collections of ASP or ARP from smaller families and mainly non-parametric analyses that do not require specification of the genetic model.

As discussed above, application of the family-based approach has led to the identification of several genes/loci for AD and PD. Recently, we have also found suggestive evidence for a novel PD locus on chromosome 19p13.3-q12 in an extended Cuban family with PD (mean AAO of 61.2 years) and autosomal dominant inheritance [270].

Other studies have applied the sib-pair approach. The first study was performed in a sample of 113 ASPs. Parametric and non-parametric analyses yielded slightly positive LOD scores on chromosomes 1, 9, 10 and 16. A maxLOD of 1.3 was found for the region on chromosome 9 [271]. During a second analysis of the data by using AAO as the phenotypic outcome, suggestive evidence for linkage was obtained for the same region on chromosome 9 and for chromosome 2 (PARK3) [167], they both overlap with regions previously implicated in studies that had examined affection status.

The second study used a group of 174 multiplex families (with two or more PD patients, 260 ARP); the sample was stratified according to AAO and the response to levodopa. This investigation identified regions on chromosomes 5q, 6q, 8p, 9q and 17q [272]. The linkage observed on chromosome 17 (close to *tau*) was mainly supported for the group of families with levodopa non-responsive parkinsonism. It is tempting to speculate that this may represent a subgroup of patients for whom variations within the *tau* gene act as risk factors. The highest LOD score (MLOD=5.07) came from a sample of 18 families from whom at least one patient had an AAO≤40 years and was obtained for marker D6S306 located in intron 7 of the *parkin* gene. Nevertheless, mutations in the *parkin* gene were not identified in 10 of these families [272] suggesting the existence of unidentified *parkin* mutations or the involvement of other genes in EOP.

The third genome scan was performed in families with at least one pair of affected living siblings, the sample was stratified according to the diagnosis "stringency": model I comprised 96 sibling pairs with more stringent diagnosis criteria, whereas model II included all affected individuals (170 ASP). Linkage and sequence analyses allowed the identification of "*parkin* families" (*n*=22), reducing the genetic heterogeneity in the sample set. Pankratz et al. [192] found evidence for linkage on chromosomes 2 (236 cM) and X (105–122 cM), both under the more restrictive (model I) and broader model (model II) and by non-parametric analysis. The

linkage to chromosome X was supported mainly by the brother-brother pairs [192]. Follow-up of the chromosome 2q36–37 region resulted in the identification of the PARK11 locus [193].

The next study conducted by this group in a expanded sample set comprising 425 ASP from 362 multiplex families [222] confirmed the previous regions and identified others. A new interval on chromosome 14 was identified (besides PARK11) yielding a LOD of 2.4 in 85 families with strong family history of PD. The analysis of the remaining 277 families again revealed the chromosome X region and new regions on chromosomes 10q24, 1 and 18 [222]. The 10q24 region has been previously linked to both PD and AD [141] in an analysis that considered AAO as a quantitative trait.

Considerations of genome-wide scans in PD

In PD, as in other complex disorders, the success of gene mapping attempts based on genome-wide scans depends on several factors. They can be summarized as follows. First, a precise definition of the phenotype is essential for reliability of results. Even with an excellent clinical evaluation and phenotype delineation, the presence of clinical variability, phenocopies and age-dependent penetrance will complicate the selection of a homogeneous sample set. Consequently, without proper sample stratification, the effect of the potential loci is expected to be "diluted". The appropriate selection of both the choice of sample to collect (extended pedigrees, ASP, ARP) and the method of analysis is also a crucial step. In addition, the number of subjects included in the study and the origin of those individuals (population isolate, admixed population, ethnic group) are essential factors. Small or modest samples sizes tend to limit the power of the study [273] and increase the probability of Type I error [274]. The study of patients coming from special isolated populations or large extended families is a known strategy for reducing genetic heterogeneity in the sample set, with the inconvenience that they are not always available. Finally, the genotyping approach, type, heterozygocity and spacing of the markers used in the study are important issues to consider.

A recent review of 101 genome-wide scans on 31 complex disorders found that the majority (66.3%) of the them did not show significant linkage when the Lander and Kruglyak criteria [275] were used and the results of studies of the same disease were often inconsistent. Two factors were closely related to the success of studies: increased sample size and ethnic homogeneity of the samples [273].

If we look at data available from the genome searches performed on PD, limitations related to sample size and heterogeneity seem to be evident, limiting the power to detect linkage. Several regions have been reported with suggestive or significant LOD scores (PARK10, PARK11) but replication studies in independent datasets are lacking. Some regions have shown overlapping among the screenings, for both affected status and AAO (PARK3, PARK10, chromosomes 9 and 10). The distinction between a real but weak effect and a false-positive result remains problematic.

Searching for genes conferring risk to non-mendelian disorders have been more difficult that originally thought. However, they hold promise in the elucidation of the causes and pathophysiology of genetically complex disorders.

Final remarks of a gene-hunting story

Genetic studies of common neurodegenerative disorders such as AD and PD initially focused on the search for genes involved in the aetiological mechanisms of the monogenic forms. This search has been particularly difficult considering the complexity of such disorders and the number of factors involved: genetic heterogeneity, variable AAO, variable expression and reduced penetrance, among others.

During the last two decades of the 20th century, enormous progress was made in the field of molecular genetics and genetic epidemiology. Many tools and strategies were developed: dense marker maps, high-throughput genotyping and sequencing facilities, together with statistical methodologies and sophisticated programs for the analysis and interpretation of the genetic data. However, the elucidation of the genetic bases of oligogenic, polygenic and multifactorial disorders still remains a challenge.

What lessons can be learned from the existing data? First, we lack adequate tools (such as biological markers) that allow the unambiguous identification of the disease of interest and that permit the distinction of clinical sub-phenotypes. Second, the methods successfully applied to the analysis of monogenic Mendelian disorders have shown limited value in identifying the multiple genes with small effects that lie behind complex polygenic and oligogenic disorders. Third, "alternative routes" can be applied, by combining positional mapping and functional evidence obtained, for instance, from gene expression profiles (microarrays) or proteomics, although the latter may have limited value in neurodegenerative disorders.

The search for disease genes, which first focused on single family studies, has evolved to the analysis of larger samples with hundreds of patients, ASP and population-based studies that can sometimes be performed on "special" isolated populations. The fascinating gene-hunting story in AD and PD is an outstanding example of the way in which identified genes can contribute to clarifying the pathogenic mechanisms leading to these devastating disorders. As initial results, several genes have been successfully identified as contributing to the risk of both diseases. Further studies of the effects of abnormal gene products on cellular and animal models should help to elucidate the pathogenesis of AD and PD. Hopefully, they will lead to the discovery of effective therapies and eventual prevention.

Missing links in neurodegenerative disorders

AD and PD are neurodegenerative adult-onset disorders that characterized by the progressive loss of specific neuronal populations and the accumulation of extra- and intra-neuronal inclusions. They both display substantial clinical overlap: around one-third of AD patients show clinical evidence of parkinsonism and close to 33% of PD patients develop dementia during the course of the disease [276-278].

Increased genetic risk for AD and PD has been found for relatives of patients with either of the disorders [279, 280]. Recently, a locus that might act as a common modulator of the AAO in both disorders has been localized on chromosome 10q [141] and a positive association with the gene *GSTO1* has been found for both disorders [1412].

There are also overlaps between the pathologies of these disorders. Neurofibrillary tangles and amyloid plaques, cardinal features of AD, are also found in PD brains with a frequency six times higher than in age-matched controls. About half of the patients with pathologically confirmed PD have clinical features of dementia and an AD pathology at post-mortem examination [281]. Lewy bodies, the pathological hallmark of PD, also occur in up to 61% of the patients with "sporadic" AD [282, 283]. Indeed, the density of Lewy bodies is thought to correlate positively with the severity of dementia [284, 285]. Furthermore, Lewy bodies also occur in familial AD caused by mutations in the *APP*, *PSEN1* and *PSEN2* genes and their presence is not associated with any particular mutation, suggesting that the effects of these mutations converge into a final common pathway [286-288]. In addition, neurodegenerative changes in the substantia nigra consistent with the diagnosis of PD are found in autopsy-confirmed AD cases [289].

The genes and subsequent abnormal products found so far for the monogenic forms of AD and PD point to pathological protein processing and aggregate formation. Cerebral accumulation of β-amyloid, α-synuclein and tau has been found in patients with the genetic and "sporadic" forms of the disorders and in transgenic mouse models. Double-transgenic mice (β-amyloid/α-synuclein) display an intermediate phenotype that resembles human Lewy-body dementia, a disorder displaying both dementia and parkinsonism. Further, β-amyloid peptides may promote the aggregation of α-synuclein, exacerbate its intraneuronal accumulation in vivo and accelerate the development of motor deficits in transgenic mice; specifically, the overproduction of Aβ1–42 induces intracellular accumulation of α-synuclein in vitro [290].

Tauopathies are a group of heterogeneous disorders usually displaying primary or secondary tau pathology, such as FTD, corticobasal degeneration (CBD; a rare and rapid progressive movement disorder) and progressive supranuclear palsy (PSP; a disorder characterized by parkinsonism, early postural instability and vertical supranuclear palsy). The positive association found with a tau haplotype (H1) for PSP, CBD and PD [291-294] and the occurrence of tau pathology in AD and PD patients suggest a role for tau as a common denominator in neurodegenerative diseases. Although *tau* mutations are mainly found in patients with FTD, the finding of *tau* mutations in some patients with PSP and CBD [295-297] strengthens this view.

A pathogenic interaction between α-synuclein and tau is suggested by the co-occurrence α-synuclein and tau filamentous inclusions in humans, by transgenic mice expressing mutant α-synuclein, and by mice that express wild-type human α-synuclein plus mutant tau. Co-incubation of tau and α-synuclein synergistically promotes fibrillization of both proteins (induced by α-synuclein). Furthermore, tau inclusions have been found in PD patients carrying the Ala53Thr α-*synuclein* mutation [175]. This suggests that interactions between α-synuclein and tau can promote their fibrillization and drive the formation of pathological inclusions in human neurodegenerative diseases [298].

Double-mutant transgenic mice (tau/APP) exhibit enhanced neurofibrillary tangle pathology indicating a possible APP or Aβ1–42 influence in the formation of neurofibrillary tangles in vivo [299]. The finding that amyloid pathology precedes tangle formation in AD predicts that Aβ could trigger tau pathology [86, 300].

Unexpectedly, DJ-1 antibodies do not label Lewy bodies in brains of PD or Lewy-body dementia patients. However, co-localization of tau and DJ-1 in Pick bodies (characteristic inclusions containing tau and ubiquitin often observed in FTD) and DJ-1 immunoreactivity observed in neurofibrillary tangles and neuropil threads in patients with AD and Lewy-body dementia constitute further evidence towards common mechanistic pathways in neurodegenerative disorders [301].

The clinical, pathological and genetic presentation of AD and PD suggests the involvement of a few overlapping interrelated pathways. It is still uncertain whether the affected proteins converge in a common underlying pathway or whether they represent the end stage of parallel neurodegenerative processes. Their imbricate features point to a spectrum of neurodegeneration (tauopathies, synucleinopathies, amyloidopathies) that needs further intense investigation to find the missing links.

Acknowledegments

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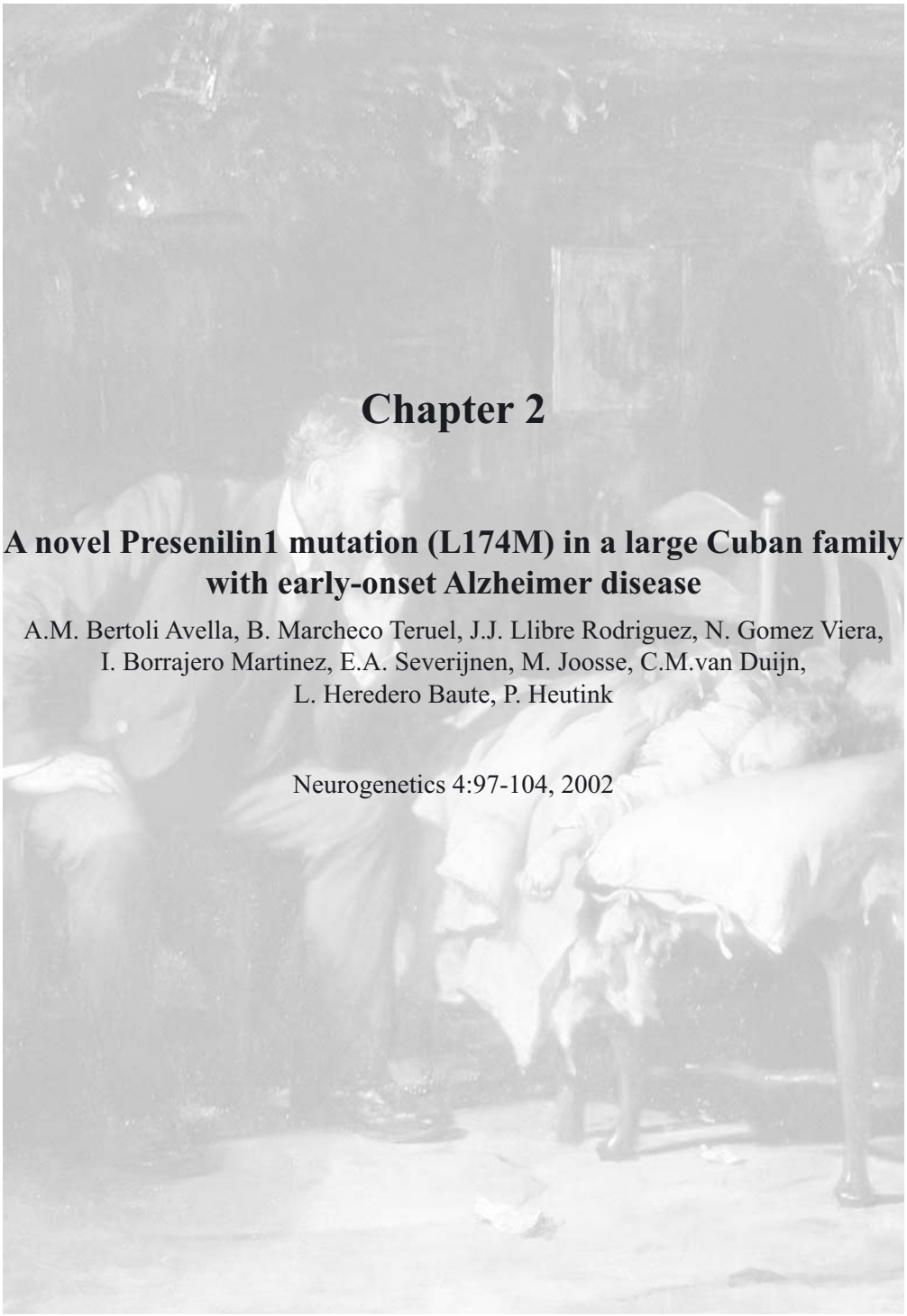

Chapter 2

A novel Presenilin1 mutation (L174M) in a large Cuban family with early-onset Alzheimer disease

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Abstract

We studied a Cuban family with presenile dementia (autosomal dominant) consisting of 281 members within six generations, the proband descended from a Spanish founder. Mean age at onset was 59 years of age. Memory impairment was the main symptom in all patients, additionally; ischemic episodes were described in 4 (n=18) patients. Neuropathological examination of brain material (1 patient) revealed neuronal loss, amyloid plaques and neurofibrillary tangles.

Thirty DNA samples were genotyped (regions on chromosome 1, 3, 10, 12, 14, 17, 19, 20 and 21). A maximum Lod score of 3.79 at $\theta = 0$ was obtained for marker D14S43, located in a 9 cM interval in which all patients shared the same haplotype. Sequencing of the *PSEN1* gene revealed a heterozygous base substitution, C520A (exon 6), which is predicted to cause an aminoacid change from leucine to methionine in the TMIII of the presenilin 1 protein. The mutation was found to co-segregate with the disease phenotype and the associated disease haplotype. The C→A change was not observed in 80 control chromosomes from the Cuban population. Leucine at position 174 is highly conserved among species and is identical in presenilin 1 and presenilin 2 proteins. We propose the *L174M* mutation might lead to an abnormal N-terminal and probably C-terminal fragments and malfunction of the protein complex.

In conclusion, we found a novel *PSEN1* mutation in a large family with clinical and pathological diagnosis of early-onset familial Alzheimer's disease, which may be relevant for other Hispanic populations.

Introduction

Alzheimer disease (AD, MIM 104300) is the most common type of dementia, characterised by progressive memory loss and deterioration of cognitive functions resulting in a state of complete dependency, with severe implications for patients, relatives and caregivers.

The complex aetiology of AD comprises both genetic and environmental factors; sporadic and familial (FAD) forms of the disease have been described. The high similarity in clinical and neuropathological features between the early-onset familial and "sporadic" late-onset AD suggests that similar pathophysiological factors are involved. Studies focusing on large families with the rare, early-onset, autosomal dominant form of the disease led to the discovery that mutations in the amyloid precursor protein (*APP*)[1], Presenilin 1 (*PSEN1*)[2], and Presenilin 2 (*PSEN2*)[3, 4] genes cause AD. Although these autosomal dominant families represent only a small percentage of the total AD cases, functional studies of the effects of the identified mutations have become an important way to dissect the causes and underlying disease mechanisms leading to AD.

Mutations in *PSEN1* are responsible for 6%-18% of the AD cases with early-onset [5]. To date the majority of the described *PSEN1* mutations are missense mutations giving rise to the substitution of a single aminoacid (www.alzforum.org/members/resources/pres_mutations/ and molgen-www.uia.ac.be/admutations/). It has been speculated that most AD related mutations result in a gain of function. Mutations in *PSEN1* alter the processing of βAPP by favouring the production of potentially toxic long tailed β-amyloid (Aβ) peptides ending at residue 42 or 43 [6]. Presenilin 1 activity is also required for the cleavage of Notch1 at the plasma membrane and the release of the Notch1 intracellular domain [7]. Mutations in genes involved in

Notch signalling also lead to late-onset neurological diseases such as CADASIL [8].

Although the provisional diagnosis of AD may be made on the basis of clinical symptoms, neuropathological confirmation is necessary to establish a definite diagnosis. AD neuropathology includes the presence in the extracellular space of amyloid plaques composed mainly of Aβ, deposition of amyloid in the wall of blood vessels and the presence of intraneuronal neurofibrillary tangles (NFT), consisting of hyperphosphorylated microtubule-associated protein tau.

Only a few *PSEN1* mutations have been described in Latin-American [9-11] and Caribbean populations [12]. In Cuba, a prevalence for AD of 5.13% has been reported for the population over 60 years [13] but to date genetic studies for AD had not been performed. Here we describe a large Cuban family with clinical and neuropathological diagnosis of AD carrying a novel *PSEN1* mutation, the first to be reported in the Cuban population.

Patients and methods

Family description

A large Cuban family with presenile dementia was ascertained through Patient IV-26 (Fig. 1A) descending from a founder (I-1) of Spanish origin (Canary Islands) who settled in Cuba in the early nineteenth century. We interviewed 50 people to complete family history, genealogy, and clinical data. The pedigree consists of 281 family members within six generations, 22 patients were reported, and a disease description was obtained for 18 patients of whom 6 were alive and 4 were available for clinical examination. No pathological studies had been performed. Medical records were available from patient IV-26 and III-26. Segregation of the disease in the family was consistent with an autosomal dominant mode of inheritance with high penetrance (Fig. 1A). Informed consent was obtained for both clinical examination and venous puncture for blood collection. The research project was approved by the Ethics Committee at the Higher Institute of Medical Sciences in Havana, Cuba.

Clinical studies

Two independent neurologists examined 4 patients and 6 at risk family members, some of them with memory complaints, 9 were admitted to hospital for a complete examination. Blood and cerebrospinal fluid tests, electroencephalography (EEG), brain computed tomographic (CT) scan and neurophysiological tests were performed. Neuropsychological studies, which consisted of a battery of several tests: Mini-Mental State examination [14], Wechsler Memory Scale, Word List Memory test and Wechsler Adult Intelligence Scale (WAIS) [15] were applied. Follow-up of some patients was necessary and a second evaluation was performed after 1 year. The NINCDS-ADRDA criteria [16] were used to establish the clinical diagnosis.

Pathology

Patient (III-26) died at age of 73 years during the course of this research. Neuropathological studies such as classical hematoxylin/eosin staining, Bielschowsky silver impregnation, Luxol fast Blue, Congo red, and trychromic Masson techniques were carried out on brain tissue (temporo-occipital region fixed in paraformaldehyde). In addition immunohistochemical studies using antibodies against Presenilin 1 N-terminal (dilution 1:50, from Chemicon International), presenilin 1 C-terminal (1:100, from Santa Cruz Biotechnology), Aβ (1:100) and Ubiquitin (1:500, DAKO), AT8 (1:40, Innogenetics, SA), PHF (a gift from P. Davies), α-

synuclein (1:1000) and Prion protein (1:100, Chemicon) were performed. Working conditions were implemented as recommended by the manufacturers. Diagnosis based on pathology findings was done according to CERAD criteria [17].

DNA studies

Blood samples were collected from 76 family members. DNA was isolated following standard procedures [18]. Thirty samples were tested with fluorescently labelled markers (short tandem repeat polymorphisms) from the CHLC Human screening set/Weber version 6, covering AD candidate regions on chromosomes 1, 10, 12, 14, 19 and 21 [1, 3, 19-22]. Additional regions where other dementia-related loci had been localised on chromosome 3 [23], 17 [24] and 20 [25] were also tested. Genomic DNA (20 ng) was amplified in 7.5 µl PCR reaction, using 1x GeneAmp PCR Gold buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer (forward primer labelled with FAM, TET or HEX), 250 µM dNTPs and 0.4 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR products were pooled and loaded on an ABI 377 automatic DNA sequencer (6.25% denaturing FMC Long Ranger acrylamide gel, filterset C and GS-500XL Tamra as size standard). Data were analysed using Genescan 3.1 and Genotyper 2.5 (updated) software from Applied Biosystems.

Haplotypes for every region were constructed based on the minimal number of recombinations. Additional markers from the Genethon linkage map [26] were tested for the chromosome 14q24.3 region.

Genotypes for *APOE* were also determined following the one-stage PCR method described elsewhere [27]. PCR products were digested with *HhaI* (10U/µl, Life Technologies), fragments were separated on Excell Gels (Pharmacia) using the Multiphor electrophoresis system (Pharmacia) and visualised by silver staining. Gels were scored by two independent observers.

Linkage analysis

Simulation calculations using the SLINK and MSIM programs [28] showed an average Lod score of 2.17 and a maximum of 5.55 at $\theta = 0$ demonstrating the family had enough statistical power to detect genetic linkage. In our linkage analysis, AD was assumed to be an autosomal dominant disease with a gene frequency of 0.001. Mutation rate was set equal to 0 and equal recombination rates were assumed for males and females. Age-dependent penetrance was defined by five liability classes based on the ages at onset observed in this family: unaffected at risk (< 40 years old): 0, 0, 0; 40-55 years old: 0.001, 0.5, 0.5; 56-70 years old: 0.01, 0.7, 0.7; 71-85 years old: 0.01, 0.8, 0.8; married in and affected: 0, 0.9, 0.9. Equal allele frequencies for markers were assumed since there were no data available from the Cuban population. Two-point linkage analysis was performed using the MLINK program from the LINKAGE package version 5.1. Marker order and genetic distances were used according to the Marshfield integrated linkage maps (www.marshfieldclinic.org/research/genetics/).

Sequence analysis.

All 12 exons from the *PSEN1* gene were sequenced by the use of intronic flanking primers as described previously [5]. For exon 2 (forward primer: 5'-TCGTGACAAATTAATACATTCC-3') and exon 6 (forward 5'-CAGTCTGGGCGACAAAGTG-3' and reverse 5'-TTAAATGATAGCTACACAGCA-3') we designed new primers, further away from the

exon-intron boundary (Genbank Accession No. AH004968). We also sequenced part of the regulatory region, 2 kb upstream exon 1 (primer sequences published elsewhere [29]). Genomic DNA (50 ng) from two patients and two healthy Cuban controls were used as template for PCR reactions. Specific conditions were 1x PCR buffer GibcoBRL, 1.5 mM $MgCl_2$, 200 µM dNTPs, Taq DNA Polymerase 2 U/reaction and 25 pmol of each forward and reverse primer in 50 µl of reaction volume. PCR product (5 µl) was checked on 1.5% agarose gel, the rest was purified (Qiagen kit) and used for a second reaction using the BigDye terminator kit (Applied Biosystems). Products were loaded on an ABI 377 Automatic DNA sequencer and analysed with Factura and Sequence Navigator program (Applied Biosystems). Once a heterozygous base change or another variation was found in the index case, all patients and 4 healthy controls were sequenced.

Allele Specific Oligonucleotide hybridisation.
Sixty family members and 40 unrelated Cuban controls were tested for the C→A change detected in exon 6 of the *PSEN1* gene. Allele specific oligonucleotides [30] were designed for the normal 5'- ATTGTTG**C**TGTTCTT-3' and mutated allele 5'-ATTGTTG**A**TGTTCTT-3' and radioactive labelled with α-dATP32. PCR products from exon 6 were fixed on two nylon membranes and hybridised with the normal and mutated oligonucleotide respectively, under specific conditions (35°C 1 hour). Filters were washed until a final stringency of 0.3x SSC and 0.1% SDS (35°C) was reached. Membranes were exposed to X-ray films.

Results

Clinical studies
Clinical characteristics of 14 patients are shown in Table 1. Memory impairment was the main symptom shared by all patients. Mean age at onset was 59 years of age, with a mean disease duration of 9 years. Patient III-17 showed late-onset dementia (76-83), but with similar clinical characteristics to the other patients. No cases of presenile dementia have been reported among his descendants.

The propositus, IV-26, started having problems at age 48 years when she suffered a cerebral ischemic episode. After recovering from unconsciousness (a few minutes), she had right hemiparesia in the face and body. The presence of any symptom before this episode could not be confirmed. Thereafter, she complained of memory loss, lack of interest, she started making mistakes at work, and due to progressive worsening of symptoms, she was admitted to hospital. The neuropsychological studies were altered, the EEG showed slow base activity and bifrontal irritative areas and head CT scan displayed cerebral and cerebellar atrophy and a hypodense lesion in left parieto-temporal area. At age 53 years she was unable to recognise even her closer relatives, she was completely dependent on caregivers and was bedridden until she died 6 years later.

Table 1. **Clinical description of patients**

	Patients													
Clinical characteristics	II-2[a]	II-4[a]	III-1[a]	III-3[a]	III-8[a]	III-14[a]	III-17[a]	III-23[a]	III-25[a]	III-26	IV-20[a]	IV-24	IV-26	IV-28
Age at onset/death (current)	65–80	61–68	55–72	63–68	51–56	59–67	76–83	58–69	63–72	67–73	53–62	51–(56)	48–59	50–(54)
Signs and symptoms														
Memory loss	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Impairment of daily activities	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Altered behavior	–	–	+	+	+	–	–	+	–	–	+	+	+	+
Depression	–	–	+	–	+	+	–	–	+	–	+	–	+	–
Anxiety	–	–	+	–	–	–	–	–	–	–	–	–	–	+
Aggression	–	–	+	+	+	–	–	–	–	–	–	–	–	–
Aphasia	+	+	–	+	+	+	+	+	+	+	+	–	+	–
Apraxia	–	–	–	+	–	+	+	+	–	+	+	–	+	+
Agnosia	+	+	–	–	+	+	+	+	+	+	+	–	+	–
Dementia	+	+	+	+	+	+	+	+	+	+	+	DCF	+	DCF
Ischemic episodes	–	–	–	–	–	–	–	+	–	+	+	–	+	–
Gait disorder	+	+	–	+	+	–	+	–	+	–	–	–	–	+
Seizures	–	–	–	–	–	–	–	–	–	–	+	–	+	–

[a] Based on family history + Feature present - Feature absent DCF= Deterioration of cognitive function

In addition to the propositus, 5 relatives were reported as patients; 3 of them were available for clinical examination (III-26, IV-24 and IV-28). Patient III-26 had a well-established dementia with five years of disease evolution and was bedridden. Generalised cortical atrophy, ventricular enlargement, and hypodense regions suggestive of vascular lesions were observed on brain CT scan. The patient died one year later at age 73 years.

Patient IV-24 was seen for the first time after 1 year of disease evolution. Neuropsychological tests were clearly altered. Patient IV-28 had a subtle alteration in neuropsychological studies a few months after the onset of symptoms. Follow-up of patients IV-24 and IV-28 confirmed a progressive deterioration of cognitive functions with impairment of daily activities. The patients had concentration and calculation deficits, difficulties in immediate and delayed recall of recently presented material, decreased emotional response, impairment of spatial skills, delay of speech and anosoagnosia. CT scans from patients IV-24 and IV-28 were normal at first-time examination. A second CT scan performed two years later (patient IV-28) showed signs of diffuse cortical atrophy. Other causes of dementia were excluded and the diagnosis of presenile dementia, probable AD was established.

Neuropathological studies

Microscopical examination of brain material (patient III-26) revealed neuronal loss and both diffuse and core neuritic plaques. The presence of amyloid plaques was confirmed by immunostaining with a Aβ antibody (Fig. 2A and B). Cerebral amyloid angiopathy was also observed (Fig. 2B). The existence of abundant amyloid plaques was the most prominent feature. Tau immunohistochemistry (AT8 and PHF antibodies) revealed numerous neurofibrillary tangles (Fig. 2C and D, respectively). Immunohistochemistry using antibodies against prion protein did not show any difference between normal controls and our patient. No inclusions were detected with the α-synuclein antibody. Pathological findings provided sufficient evidence to confirm the diagnosis of AD.

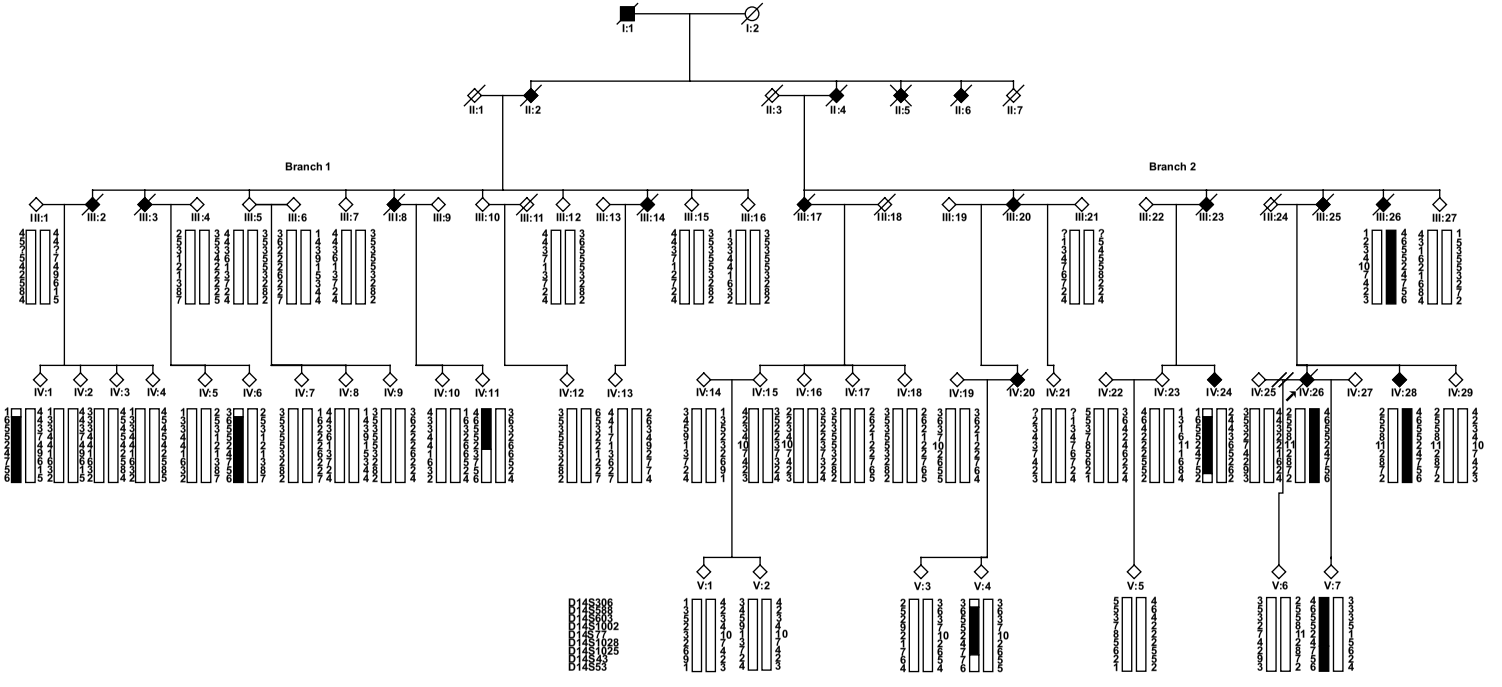

Figure 1A. *Pedigree of the family. Haplotypes corresponding to the chromosome 14q24.3 region are shown. Filled (black) symbols represent demented individuals. Gender and ages are not specified for confidential reasons. A diagonal line across the symbol means deceased individual; two diagonal lines indicate suicide. Filled (black) bars indicate the disease haplotype.*

DNA studies

APOE genotypes

Forty-seven DNA samples were tested, 32 were homozygous ε3, 13 had the ε3/ε4 genotype and 2 were homozygous ε4. Allele ε4 was only present in the second branch of the pedigree. Of 4 tested patients, 3 had the ε3/ε4 genotype.

Other genotypes

Genotypes for markers situated on chromosomal regions 1q31-42 (*PSEN2*), 10q24 (*AD6*), 12p11.23-q13.12 (*AD5*), 14q24.3 (*PSEN1*), 19cen-q13.2 (*AD2*), and 21q21 (*APP*) were determined for 30 family members. In addition, other dementia-related regions 3p11.1-q11.2 (*DMT1*), 17q21.1 (*MAPT*), and 20pter-p12 (*PRNP*) were tested. Haplotype and linkage analyses allowed us to exclude the presence of the gene responsible for most of them.

Evidence supporting genetic linkage was only found for the chromosome 14q24.3 region, two-point linkage analysis revealed positive LOD scores for several adjacent markers, and a maximum LOD score of 3.79 was obtained for marker D14S43 at $\theta = 0$.

We then saturated the region with additional markers from D14S52 to D14S617 covering 50 cM. Haplotypes from 45 family members are shown in Fig. 1A. The 4 patients shared a common region spanning 9 cM from marker D14S588 to D14S43. Patient IV-24 showed recombination events in both telomeric and centromeric parts of the haplotype, allowing us to determine the upper and lower limit of the region shared by all 4 patients.

Several demented patients were reported to have psychiatric symptoms (aggression, restlessness, anxiety, depression), but did have haplotypes identical to the other patients.

Sequencing of PSEN1

According to several genetic maps, the *PSEN1* gene is located close to markers D14S1002 and D14S77 in the middle of our critical region. Since mutations in the *presenilin* genes are the most common cause of the presenile dementias with autosomal dominant inheritance, we performed direct sequencing of 12 exons of *PSEN1* and part of its regulatory region for both sense and antisense strands.

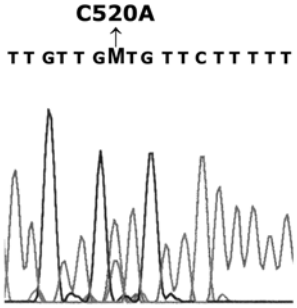

***Figure 1B**. Direct sequencing revealed an heterozygous base change C→A in exon 6 of the PSEN1 gene.*

A heterozygous base substitution at position +520 from the ATG starting site, resulting in a C to A transversion was found in exon 6 (Fig. 1B) which is predicted to cause an aminoacid change from leucine to methionine in the transmembrane domain III of the presenilin 1 protein. We then included additional samples; all available DNAs from the family (60 individuals) and 40 unrelated Cuban controls were tested with allele-specific oligonucleotides. The mutation was found to co-segregate with the disease phenotype and the associated disease haplotype and was present in 8 at risk subjects (5 are shown in Fig. 1A) but not in other healthy individuals

and spouses (96 chromosomes). The C→A change was not observed in any of the 80 control chromosomes from the general Cuban population.

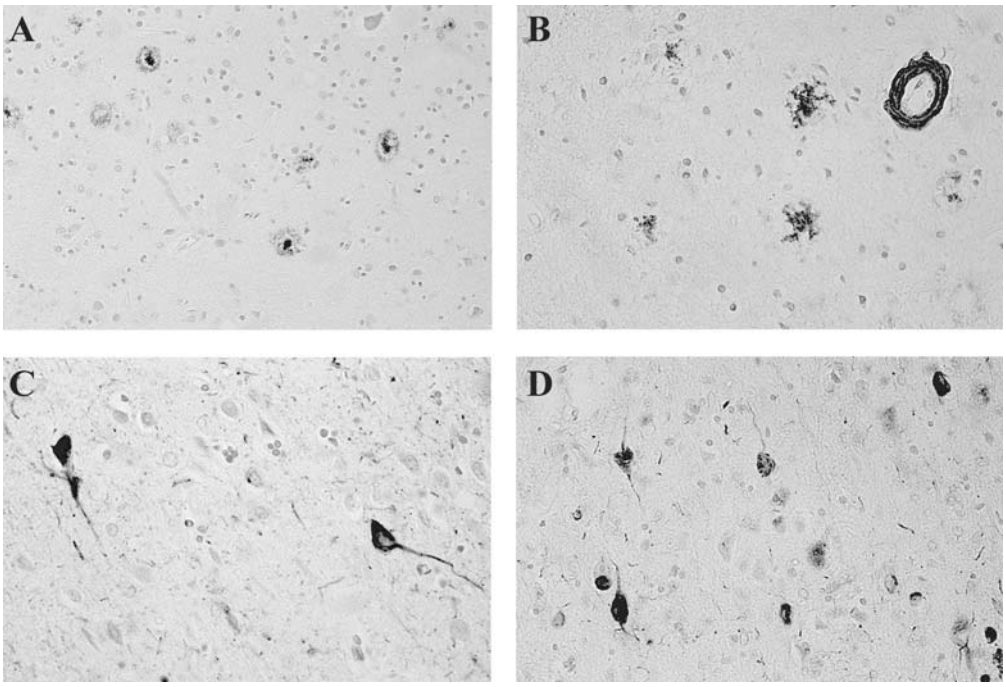

***Figure 2**. Sections of temporal cortex of patient III-26, stained with β-amyloid antibody (**A** and **B**), showing numerous extracellular plaques and amyloid deposition in the wall of a blood vessel (**B**), magnification 200x.*
***C** and **D**: immunostaining with AT8 and PHF antibodies shows several neurofibrillary tangles, magnification 400x.*

Discussion

The clinical picture was compatible with AD except for the fact that 4 out of 18 patients had vascular episodes such as transient ischemic crises.

Cerebral amyloid angiopathy (CAA) is best known as a leading cause of lobar haemorrhages in the elderly and as a common finding in AD. It has also been implicated in the production of other cerebrovascular lesions [31]. The presence of CAA might explain the vascular episodes described in patients III-23, III-26, IV-20, and IV-26. Hypodense zones suggestive of vascular lesions were observed on CT-scans from patients III-26 and IV-26; in addition, amyloid deposits were present in the wall of brain blood vessels (patient III-26), confirming the presence of CAA.

Factors such as the vasoactive character of β-amyloid which can produce vasospasm *in vitro* at low concentrations [32], the secretion of Aβ by activated human platelets [33] and the hyaline necrosis surrounding the amyloid deposit in the vessel wall [34] have been postulated as possible mechanisms by which amyloid in the vessels can lead to vascular lesions.

Furthermore, a relationship between *APOE* ε4 allele and the presence of CAA and cerebrovascular pathology in AD has been suggested, since the *APOE* ε4 allele is a risk factor

for the development of CAA in AD [35]. In this family, available patients with ischemic episodes carry also the *APOE* ε4 allele (patients III-26 and IV-26).

We found significant evidence for genetic linkage to chromosome 14q24.3. We were able to reconstruct haplotypes for 11 deceased patients using first degree relatives; all of them had the same "disease haplotype", except one patient (III-17) in which the reconstructed haplotype was different. This patient showed a much later disease onset (76 years old) while the observed range in the other patients was 48-67 years old. Unfortunately, only family history but no medical records were available. It might be that this patient had dementia due to other causes and therefore could be a phenocopy. Coexistence of both early-onset (with an associated *PSEN1* mutation) and late-onset forms of AD in the same family has been reported before [36].

Several heterozygous base changes were detected during *PSEN1* sequencing but none of the sequence alterations were found to co-segregate with the disease except the C520A change. Several facts indicate the pathogenicity of this novel *PSEN1* mutation. The C520A change was present in all examined patients and in all relatives carrying the disease haplotype, although they were still below the age at onset of AD in the family. The C→A change is unlikely to be a common polymorphism, we did not find it in 80 control chromosomes (from the same population) or in unaffected relatives (96 chromosomes) that did not carry the disease haplotype.

L174M is a conservative amino acid substitution seeing that leucine and methionine are both nonpolar amino acids. Nevertheless, close to half of the *PSEN1* and *PSEN2* reported mutations also lead to the same type of aminoacid change. The leucine at position 174 of the presenilin 1 protein is highly conserved among different species (Fig. 3) and between homologous proteins like presenilin 2 and sel-12, indicating that this position (TMIII) is important for protein function. Adjacent amino acid positions at 163, 165, 166, 169, 171, 173 and 184, where other mutations have been found, are also evolutionary conserved.

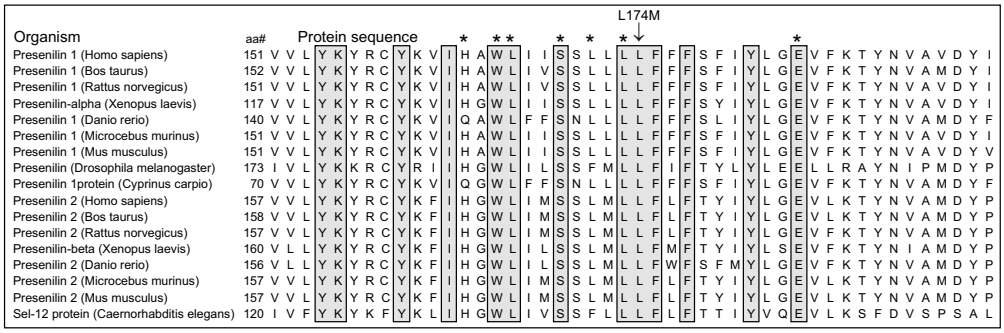

Figure 3. *Alignment of human presenilin 1, presenilin 2, and orthologous protein fragments. Highly conserved amino acids are shown in* boxes*. Leucine at position 174 is substantially conserved across several species.* Asterisks *indicate where other PSEN1 mutations are located.*

Under normal conditions Presenilin 1 holoprotein is rapidly converted into two fragments, an amino terminal fragment (NTF) and a carboxy terminal fragment (CTF) [37]. Presenilin 1 fragments can associate into tightly bound complexes that may represent the principal form in which presenilin functions in cells [38]. Formation of these high molecular weight complexes

composed of both presenilin 1 fragments may also explain why FAD-associated mutations within the N-terminal region of presenilin 1 (like *L174M*) result in the hyperaccumulation not only of the NTF but also of the CTF. It has been proposed that the observed elevation in the accumulated amounts of mutant presenilin 1 NTFs and CTFs must be the result of enhanced endoproteolitic processing of mutant presenilin 1 protein and/or greater stability of mutant presenilin 1-derived fragments [39], which appears to be associated with enhanced production of $A\beta_{42}$ and early-onset FAD.

Another possible mode of action of the *L174M* mutation might be that of an alternative translation start site. From the 82 mutations described in *PSEN1* only V94M in exon 4 [9] and the current mutation (exon 6) introduce a new methionine in the protein sequence.

Functional assays will be required to determine whether the *L174M* mutation affects presenilin 1 protein activity for the cleavage of Notch1 at the plasma membrane and the release of the Notch1 intracellular domain.

In conclusion, we found a novel *PSEN1* mutation in a large family with clinical and pathological diagnosis of early-onset FAD. This is the first genetic study on AD that identifies the involvement of the *PSEN1* gene in Cuba.

L174M is the seventh mutation described in Latin-American and Caribbean populations; for some of them (Colombia and Dominican Republic) a founder effect has been described. We are currently in the process of collecting new familial and sporadic cases with early-onset AD. It will be of interest to see the contribution of this mutation to other early-onset cases with AD of the Cuban population or in others of Hispanic origin.

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Disclosure: All experiments comply with the current laws in The Netherlands.

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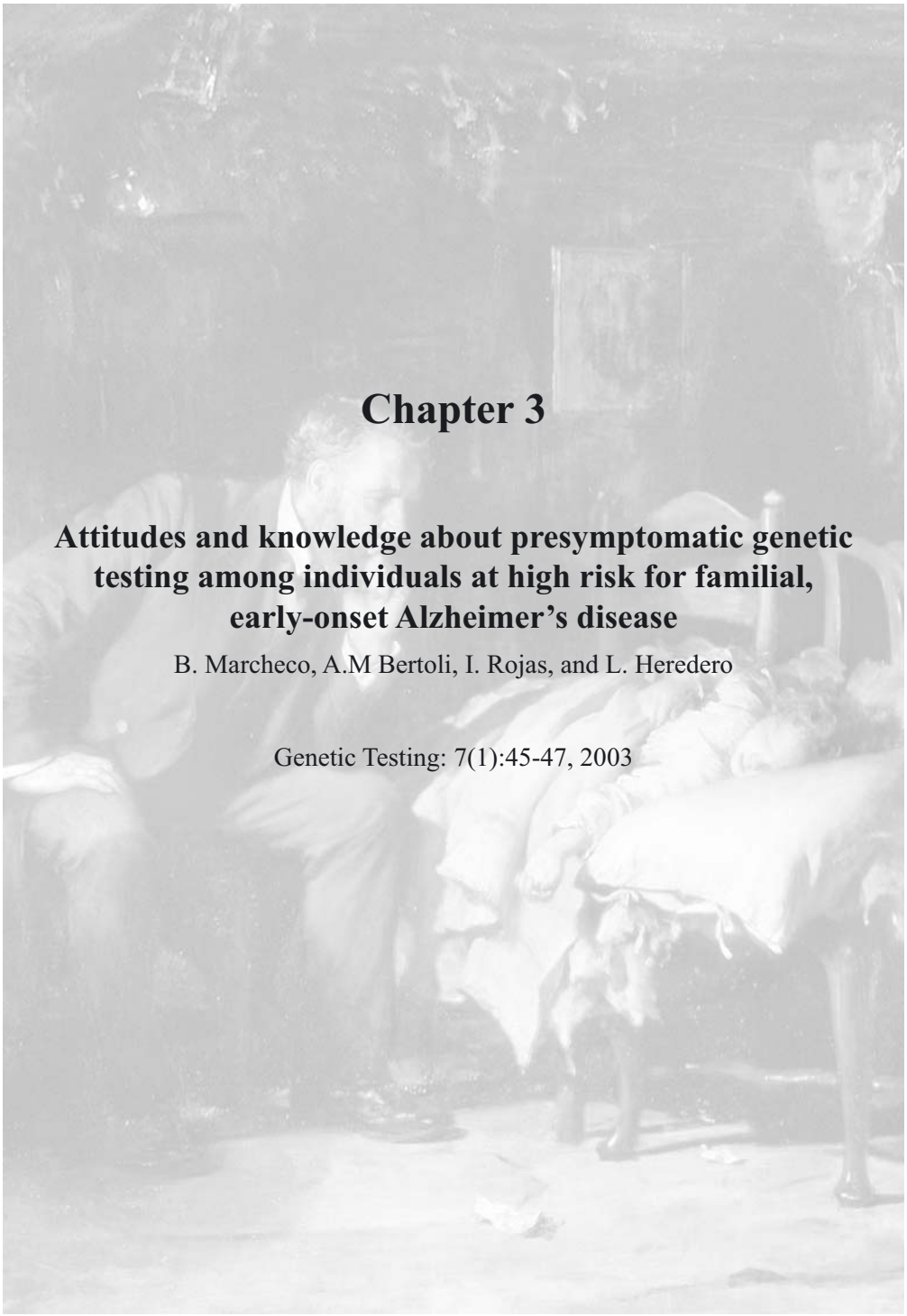

Chapter 3

Attitudes and knowledge about presymptomatic genetic testing among individuals at high risk for familial, early-onset Alzheimer's disease

B. Marcheco, A.M Bertoli, I. Rojas, and L. Heredero

Genetic Testing: 7(1):45-47, 2003

Abstract

The study was conducted in a large Cuban family with early-onset familial Alzheimer's disease (AD). Fifty-six first-degree relatives of familial cases with AD were interviewed concerning their clinical and genetic knowledge about AD and their attitudes toward the possible use of presymptomatic genetic testing of AD. The individuals had only limited knowledge about their personal risk of developing AD. All 56 family members would use presymptomatic testing to know their own risk of AD. Confronted with a hypothetical reproductive choice, 50% would choose not to have children if they themselves had the mutation. A positive prenatal test would lead 48.2% of the participants to have an abortion, and 19.7% would continue the pregnancy regardless of the positive test result.

Introduction

Advances in molecular genetics have led to the development of tests that can determine the specific individual risk of having inherited the disease genes for several adult-onset diseases. The implications of widespread testing for such diseases have to be considered at the societal level, but the decision to be tested is an individual one. It is therefore important to investigate the attitudes toward genetic testing and the knowledge about the possible consequences of testing among individuals at high risk.

Alzheimer's disease (AD) is a progressive and degenerative disorder that attacks the brain, first described by Alois Alzheimer [1]. The main symptoms are memory loss, cognitive impairment, deterioration of motor skills, and withdrawal from social contact. The clinical diagnosis can be difficult to establish and is only confirmed by post-mortem microscopic examination of the brain. The disease has severe emotional and financial consequences for individuals, families, and society.

AD can be familial or sporadic. Around 5% of all AD cases have an early onset, *i.e.* before age 65 [2, 3]. A few autosomal dominantly inherited genes causing a small percentage of all cases with AD have been identified. These include the *amyloid precursor protein* (*APP*) gene on chromosome 21, the *presenilin-1* gene (*PSEN1*) on chromosome 14, and the *presenilin-2* gene (*PSEN2*) on chromosome 1. Mutations in these genes usually lead to early-onset AD. It is possible to test for mutations in these genes among cases with familial AD and some commercial tests are now available [4]. The tests can be used presymptomatically or to confirm a clinical diagnosis.

In general, genetic testing for AD, diagnosis or disease prediction is a controversial point and is presently recommended in rare, early-onset familial cases with presumable autosomal dominant inheritance only [5]. Among genetic and environmental risk factors for AD the most established involve the *APOE* gene polymorphisms, but genetic testing of the *APOE* gene polymorphisms in AD is not recommended (Nuffield Council on Bioethics, http://www.nuffieldfoundation.org).

Many relatives whose family members suffer from the disease, wonder about their personal risk of developing AD, but as with Huntington's disease, no prevention or efficient cures are available for AD. Thus, predictive testing for AD can become a profound dilemma for the individuals at high risk for the disorder. The consequences of providing potentially devastating information to the individual and the family should be weighed against the principles of autonomy and self-determination of the family members.

Very few studies have examined the wishes of, and knowledge among, family

members at high risk of AD concerning genetic testing [6]. The present study was conducted in a large Cuban family that originated in the middle of the 19th century when immigrants coming from the Canary Islands settled in the western part of Cuba. Nineteen family members from this seven-generation family have suffered from an early-onset form of AD caused by a newly described mutation in the *presenilin-1* gene [7]. In this large family, first-degree relatives of patients with AD, for whom the questions of testing are particularly relevant because of their personal experiences [8, 9], were asked about their clinical and genetic knowledge and attitudes concerning the possibilities of presymptomatic testing for early-onset familial AD.

Method

A total of 56 first-degree relatives participated in the survey, of which 37 (66.1%) were female. The age range of the participants was from 20 to 69 years, most of them living in smaller cities with a level of education between middle school and high school; all 56 family members were of European Caucasian descent. Most of the subjects have been personally involved in care giving for relatives with AD; 64.3% were children of an affected parent and the female children of the Alzheimer patients in this family had a larger burden of care than the male children.

Further description of the sample appears in Table 1. The mean age of onset of AD in this family is 62 years and the mean duration of AD around 9 years. The family members were informed that they might be at risk for early-onset familial AD, but not about their carrier status. The aims of the survey were explained and following informed consent, questionnaires were distributed to 56 people. No family members refused to participate.

The survey was composed of questions concerning the level of information about their individual risk for AD, their attitude toward possible presymptomatic testing in the future, and their main reasons for this attitude. The impact of a hypothetical positive test result on reproductive decisions and attitudes toward possible prenatal diagnosis was also determined.

Table 1. **Description of the sample set**

Socio-demographic characteristics	n	%
Gender		
Male	19	33.9
Female	37	66.1
Age		
20-29	8	14.3
30-49	29	51.8
50-69	19	33.9
Marital status		
Married	33	58.9
Divorced	14	25
Single	9	16.1
Living with family	53	94.6
Living alone	3	5.4

Results

The survey was conducted from May 1996, to January 1997. Thirty-one individuals (55.3%) showed some knowledge concerning their individual risk. These family members had obtained their primary information from either another member of the family (51.6%), their general practitioners (38.7%), or from newspapers and journals (9.7%).

All 56 participants were interested in presymptomatic testing, with their main reasons being to know their own disease risk (Table 2). Only 9 of the 56 individuals were tested for the mutation and no one was informed about their true carrier status.

Concerning a hypothetical reproductive choice, 28 participants (50%; 9 males and 19 females) of the 56 persons would choose not to have children to avoid passing on the disease if they personally had a dominant disease mutation for presenile Alzheimer. The remaining 50% (10 males and 18 females) would choose to have children regardless of their mutation status.

Thirty-three individuals (58.9%; 7 males and 26 females) were interested in prenatal diagnosis if it becomes available, whereas 19 (33.9%; 9 males and 10 females) answered that they would like more information concerning the magnitude of risk before making a decision. Four participants (7.2%; 3 males and 1 female) would not use the test to obtain a prenatal diagnosis.

The hypothetical scenario of a positive prenatal diagnosis was presented to explore the possible behaviour of the participants in such a case. Twenty-seven participants (48.2%; 9 males and 18 females) would like to have an abortion; 18 participants (32.1%; 6 males and 12 females) would like more information about a possible future treatment or cure for the disease before making a decision; and 11 individuals (19.7%; 4 males and 7 females) would continue the pregnancy.

Table 2. **Reasons for choosing presymptomatic testing**

Reasons	n	%
To be prepared for the disease	37	66.1
Curiosity	34	60.7
To modify choices of health behavior	24	42.8
To plan career and retirement from work	24	42.8
To plan marital and reproductive choices	24	42.8
To diminish anxiety	22	39.2

Discussion

This is the first reported study concerning attitudes regarding genetic counselling and predictive testing in early-onset familial AD from a Latin American country. It offers information about the expectations and attitudes of family members at high risk for the disease.

It is an important finding of the study that there were no differences in attitudes between the two sexes to any of the questions. Around 55% of family members were aware that they were at risk for developing AD. All participating family members at high risk for

early-onset familial AD were interested in presymptomatic counselling and testing given that a dominant mutation could be identified.

Concerning reproductive choice, 50% would choose not to have children if they themselves had the dominant mutation. Concerning possible prenatal testing, around 60% were interested in this option. Only 7.2% were not interested at all in prenatal testing. This is in contrast with previous results from other studies of early-onset familial AD and Huntington's disease, where the uptake of prenatal testing was low among counselled couples and participation in reproductive testing even lower overall (Nuffield Council on Bioethics).

Many factors affect the uptake of prenatal testing for single gene conditions, such as age of onset and severity of the disease, the certainty of the test, and especially the possibility of treatment. Almost 50% of the relatives would choose abortions as a possibility, if the test result was positive for the mutation. In Cuba, abortion is a legally and socially accepted intervention and such a decision would be met without any prejudice.

The decision to undergo genetic testing for diseases such as early-onset familial AD where no cure or adequate treatment are available is very complex and personal. The present study suggests a clear need for education and genetic counselling of family members at high risk, especially for those individuals at risk who are about to make reproductive decisions.

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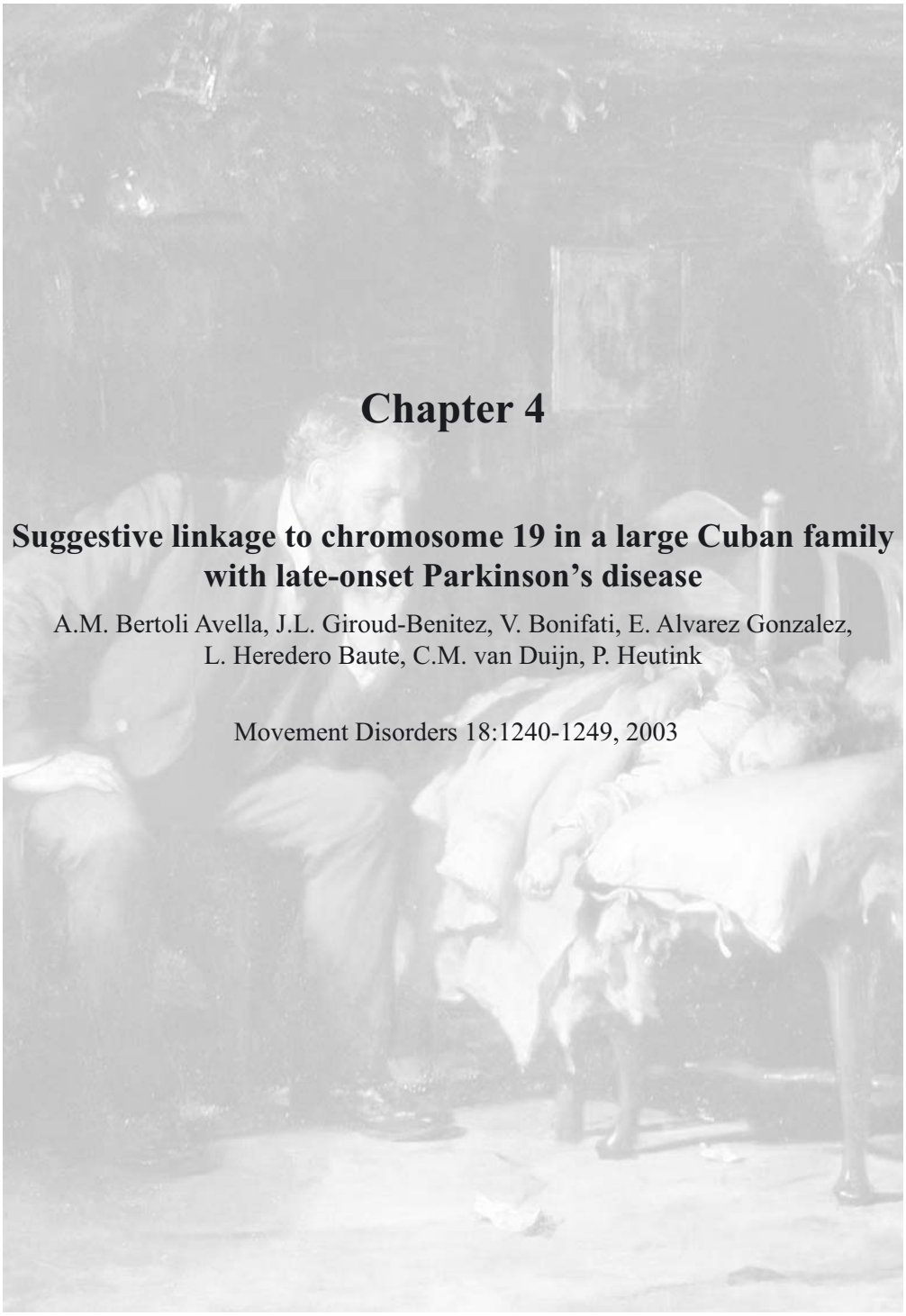

Chapter 4

Suggestive linkage to chromosome 19 in a large Cuban family with late-onset Parkinson's disease

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Abstract

The identification of disease genes using family-based approaches has provided important insights into the pathogenesis of Parkinson's disease (PD) demonstrating the importance of genetic studies on monogenic forms of the disease. We studied a large Cuban family with typical, late-onset PD and probable autosomal dominant inheritance. Mean age at onset was 61.2 years (±12.53, 45-76). Other phenotypes such as essential tremor and atypical parkinsonism were observed in this family.

We carried out a genome-wide scan and linkage analyses. The genetic data were analyzed using a conservative model in which only patients with clinically definite or likely PD were considered affected, other phenotypes were regarded as "unknown". Multipoint analyses yielded a maximum LOD of 2.26 between markers D19S221 and D19S840. Haplotype analysis showed a region on chromosome 19 shared by six of seven PD patients. The essential tremor phenotype and the atypical parkinsonism do not segregate with this haplotype, suggesting a different etiology.

Our findings suggest the presence of a novel locus for PD on chromosome 19p13.3-q12. We propose that an oligogenic model with moderate contribution of two or three genes rather than a "pure" monogenic model might explain better the wide range in age at onset, the reduced penetrance and the phenotypical variability observed in PD families.

Introduction

Parkinson's disease (PD, MIM168600) is the second most common neurodegenerative disorder after Alzheimer's disease, with a prevalence of around 2% among people over the age of 65 years.[1, 2] The cardinal clinical features of PD, bradykinesia, rigidity, resting tremor, and postural instability are associated with neuropathological findings such as loss of neurons in the substantia nigra and the presence of Lewy bodies.[3] The etiology of PD is largely unknown, but there is evidence that genetic factors play a role.[4, 5] Familial aggregation has been found for typical, late-onset PD (> 50 years of age)[6, 7]. First-degree relatives of PD cases are 1.5 to 9.5 times more likely to develop PD than first-degree relatives of controls[4, 8, 9] and segregation analysis supports a major gene influencing the susceptibility to late-onset PD[10, 11, 12]. Moreover, a susceptibility locus for late-onset idiopathic PD has been mapped to chromosome 1p32 (PARK10)[13] and mutations in the *nuclear receptor related-1* gene (*Nurr1* or *NR4A2*) have been found associated with familial PD.[14]

Until now, eight different forms of monogenic PD have been identified. Autosomal dominant forms are associated with mutations in the α-*synuclein* gene (PARK1 locus, chromosome 4q22)[15], *ubiquitin C-terminal hydrolase-L1* (PARK5, 4p14)[16], and three unidentified genes on chromosomes 2p13 (PARK3)[17], 4p14-16.3 (PARK4)[18] and 12p11.2-q13.1 (PARK8).[19] The *parkin* gene (PARK2, 6q25.2-q27)[20], the recently discovered *DJ-1* gene[21] (PARK7, 1p36)[22] and the PARK6 locus (1p35-36)[23] are associated with autosomal recessive forms of early-onset. In addition, several genome screens have been carried out recently on large groups of smaller PD families, revealing new regions on chromosomes 5q, 8p, 9q, 17q[24], 1, 9, 10, 16[25]; X and 2[26] (PARK11)[27] where further susceptibility genes for PD might be located.

The study of genes and their products identified using family-based approaches has provided substantial insights into PD pathogenesis and has implicated oxidative stress,

abnormal protein folding and protein degradation through the ubiquitin-proteasome system.[28-30]

The fact that several large families segregating PD have been found negative for the genes and loci known until now,[31-34] suggests the existence of other genes (perhaps pathways) involved in PD etiology. Large families suitable for linkage mapping, however, are rare and difficult to find. The study of an extended Cuban family with late-onset typical PD gave us the opportunity to localize the genetic defect for a late-onset form of Parkinson's disease. We carried out a genome-wide screen and linkage analyses and found suggestive evidence for genetic linkage and a haplotype segregating with typical PD on chromosome 19p13.3-q12.

Patients and methods

Family description

A large Cuban family with Parkinson's disease was studied (Fig. 1). The oldest known affected member, of Spanish descent was born in 1891 in the western part of Cuba and settled in Havana in 1898. Data was collected from 213 family members within five generations by review of baptism, marriage, death certificate and medical records when available, or by direct interview of family members. Informed consent was obtained for clinical examination, venous puncture for blood collection and genetic research. The Ethical Committee at the Higher Institute of Medical Sciences in Havana approved the research project.

Clinical studies

Two neurologists (J.L.G.B. and E.A.G.) independently examined 58 family members plus 11 affected individuals. Videotapes of most patients were reviewed by a third neurologist (V.B.). For all patients and 14 at risk relatives a second and third evaluation was done within an interval of 1-year follow-up.

The diagnosis of *clinically definite PD* was based on the presence of at least two of three cardinal signs (bradykinesia, resting tremor, rigidity), improvement with levodopa (L-dopa) treatment, asymmetric onset, no atypical features, and no other identifiable causes of parkinsonism. Together, these items reflect the widely accepted criteria for PD diagnosis[35], and they also include some supporting features that confer more than 90% accuracy in the identification of genuine Lewy body PD.[36, 37] In addition, the following diagnoses were made:
Clinically likely PD: when the clinical picture was otherwise typical, but information on L-dopa response was not available or unclear;
Clinically possible PD: only one cardinal sign and no atypical features were present;
Atypical parkinsonsism: two or more cardinal signs were present in the context of additional neurological signs (early cognitive deficit, ophthalmoplegia, pyramidal signs, cerebellar signs, peripheral neuropathy);
Essential tremor: when postural or action tremor were the only signs, and they had been present for at least five years without other neurological signs nor identifiable causes of tremor.[38]

The Unified PD Rating Scale (UPDRS)[39] and the Hoehn Yahr[40] scales were used for clinical evaluation in "on" (with medication) and "off" (without medication) states.
Blood biochemistry, EEG, neuroconduction studies, head CT scan or MRI (1.5 T) were carried out in all affected individuals. Mini-Mental state examination (MMSE)[41] was carried out in all patients and three at risk relatives. The Weschler Memory Scale and Weschler Adult Intelligence Scale (WAIS)[42] was carried out on patient IV-14.

Molecular analysis

Blood samples were collected from 46 family members. DNA was isolated after standard procedures.[43] A genome-wide screen was carried out with 382 fluorescently labeled markers from the ABI PRISM™ MD-10 Linkage mapping set v.2 (Applied Biosystems) covering all autosomes with an average spacing of 10 cM. Additional markers from the CHLC Human screening set v.6 and the Marshfield integrated genetic map (available online at http: www.marshfieldclinic.org/research/genetics/) were typed for fine mapping of some regions.

Genomic DNA (20 ng) was amplified in 7.5 µl PCR reactions, following the manufacturer's recommendations (Applied Biosystems). PCR products were pooled and loaded on an ABI 3100 automatic DNA sequencer, data were analyzed using GeneMapper v.2 (Applied Biosystems). Haplotypes were constructed based on the minimal number of recombinations and using the Cyrillic v2.1 program (available on line at http:cyrillicsoftware.com).

Statistical analysis

We used the SPSS v.10.0 program (SPSS, Chicago, IL) to tabulate some patient characteristics. Simulation studies (500 replicates) carried out using the SLINK and MSIM programs[44], yielded an average maximum LOD score (LOD_{maxav}) of 1.47 (SD ± 0.93) and a maximum LOD of 3.61. In addition, 31.6% of the replicates yielded a LOD above 2.0 and 3.2% above 3.0 respectively (θ=0). The family had sufficient statistical power to detect genetic linkage.

PD was assumed as an autosomal dominant disease with a gene frequency of 0.001. Age-dependent penetrance was defined by 4 liability classes based on the ages at onset observed in this family: unaffected at risk ≤45 years old: penetrance of 50%; 46 to 65 years old: 65%; ≥66 years old: 80%; married-in and affected: 95%. A phenocopy rate of 0.3% and 3% was defined for the group of 46 to 65 and ≥66 years, respectively.

Patients with definite and likely PD were considered as "affected". Individuals with: (1) normal neurological examination; or (2) those who could be retrospectively defined free of parkinsonian symptoms were considered "unaffected". Patients with other phenotypes including possible PD, atypical parkinsonism or essential tremor were assigned the "unknown" status.

One PD patient (IV-30) was referred to us later during the course of this research. For our linkage analyses (Model 1), the status of this patient was considered as "unknown" due to uncertainty of the diagnosis at the start of this study. After 1 year of follow-up, the diagnosis of definite PD was made and we carried out a second and exploratory analysis considering this patient as affected (Model 2).

Two-point linkage analyses were carried out using the MLINK program from the LINKAGE package (v5.1).[45] Equal allele frequencies were assumed, because no data on allelic frequency distribution from the Cuban population are available. For fine mapping, population specific allele frequencies were calculated based on 47 unrelated Cuban controls. Marker order and genetic distances were according to the Marshfield integrated linkage map. For some markers with unresolved order the Icelandic (deCODE) genetic map[46] was used. Parametric multipoint analyses (Model 1) were carried out using subsequent four point analyses for the complete chromosome 19 (Linkmap program v.5.1).

Results

Clinical features

Fourteen family members, nine of whom were living, were reported affected by PD (Fig. 1). Eight of these patients were examined, whereas one patient was not available for examination. Another three asymptomatic relatives (IV-1, IV-5 and V-4) showed mild parkinsonian signs during our examination.

After our clinical evaluation, the following diagnoses were established (Table 1 and Fig. 2): 5 cases of definite PD (III-4, III-6, IV-12, IV-25 and IV-30), 2 cases of atypical parkinsonism (IV-14, IV-19), 2 cases of possible PD (IV-1, IV-5) and 2 cases of essential tremor (IV-23, V-4). Another 5 cases (definite and likely PD) were ascertained among deceased or unavailable individuals based on their medical history. No pathological studies have been carried out.

Mean age at onset for PD (definite and likely PD, n=10) was 61.2 years (±12.53, 45-76). Asymmetrical onset, good response to l L-dopa therapy, no atypical features and a rather benign course were observed in five personally examined definite PD cases. CT scan or MRI from these patients showed mild, diffuse brain atrophy. In patient IV-25, CT evidence of a more pronounced fronto-temporal atrophy was not associated with overt cognitive or behavioral disorders.

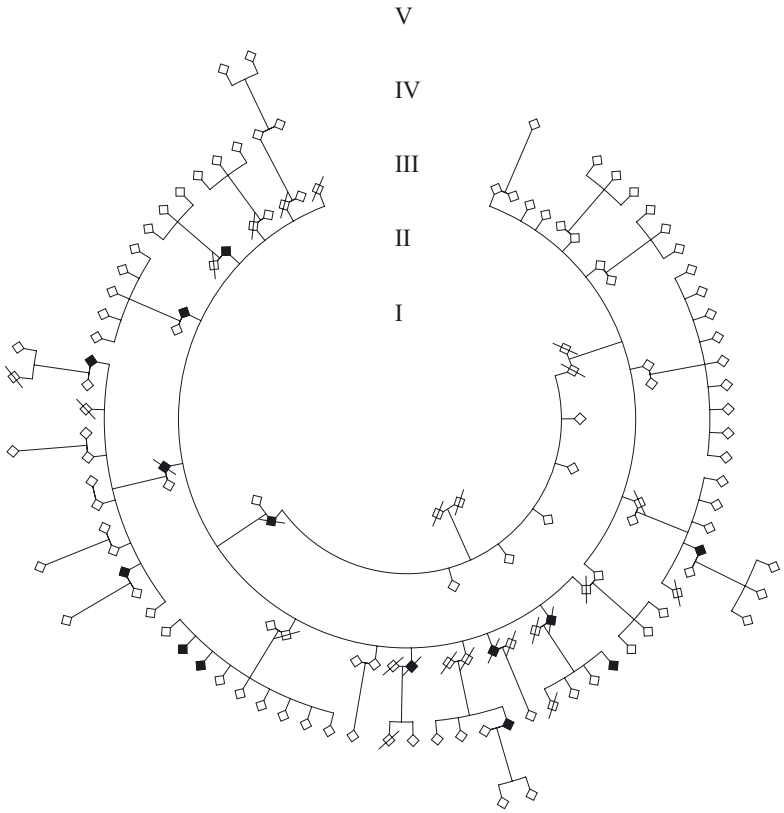


Figure 1. *Extended pedigree of the Cuban family. Filled symbols represent individuals that were described initially as affected by Parkinson's disease. Gender is not specified for confidentiality reasons.*

Table 1. **Clinical description of patients from the Cuban family**

Patient	Age at onset (y)	Disease duration (y)	Tremor (symmetry)	Brady-kinesia	Rigidity	Postural instability	Dyski-nesias	Other features	Hoehn Yahr Off	UPDRS Off/On	Response to L-dopa	Neuro-imaging	Source	Clinical Diagnosis
II-2	75	7 (dead)	RT (A)	+	+	+	-		III	-	Unclear	-	Anamnestic data	Likely PD
III-4	68	8	RT (A)	+	+	+	+		III	42/20	+	CT scan	Physical exam	Definite PD
III-6	73	8	RT (A)	+	+	+	+		III	46/17	+	CT scan	Physical exam	Definite PD
III-8	76	9 (dead)	-	+	+	+	-		III	-	Unclear	-	Clinical record	Likely PD
III-13	71	11 (dead)	RT	+	+	+	-		III	-	+	-	Clinical record	Definite PD
III-16	56	12 (dead)	RT	+	+	+	-		IV	-	+	-	Clinical record, photos	Definite PD
IV-1	66	4	-	+	-	-	-	Hypomimia	II	4/-	Not treated	-	Physical exam	Possible PD
IV-5	61	5	T (inferior lip)	+	+	-	-		II	6/-	Not treated	-	Physical exam	Possible PD
IV-7	45	18	RT	+	+	+	-		IV	-	+	-	Anamnestic data, photos	Definite PD
IV-12	48	9	RT (A)	+	+	+	+		III	56/12	+	MRI	Physical exam	Definite PD
IV-14	54	5	P/RT (A)	+	-	-	-	Cognitive decline, HBP	II	19/10	Unclear	CT scan	Physical exam	Atypical parkinsonism
IV-19	60	7	RT (S)	+	+	-	+	Hemiparesis (left side)	II	-/39	Unclear	-	Physical exam	Atypical parkinsonism
IV-23	45	14	P/AT (A)	-	-	-	-		N/A	12/-	Not treated	CT scan	Physical exam	Essential tremor
IV-25	48	15	RT (A)	+	+	+	+	Wearing-off	IV	69/30	+	MRI	Physical exam	Definite PD
IV-30	52	14	-	+	+	+	+	HBP, Severe dyskinesias	III	56/24	+	CT scan	Physical exam	Definite PD
V-4	34	5	P/AT	-	-	-	-		N/A	-	Not treated	-	Physical exam	Essential tremor

Y= Years, RT= Resting tremor, P= Postural, AT= Action tremor, A= Asymmetric, S= Symmetric HBP= High blood pressure N/A=Not applicable

Clinically possible PD cases: When first examined at age 59 years, patient IV-1 showed clear hypomimia with slight global bradykinesia. Signs have shown no progression, and no new signs have appeared during 3 years of follow-up. In 1997, patient IV-5 was treated with neuroleptics (trifluoperazine 3 mg/day) for a few months, due to severe agitation resulting from stress. At first examination in 1998, she had been free from neuroleptics for 2-3 weeks, and bradykinesia and rigidity in her right arm were found. At last examination in 2002, there was almost no progression of the described signs but tremor of the inferior lip was also observed.

Atypical parkinsonism: Patient IV-14 (aged 59) has been a heavy alcohol and tobacco consumer for 40 years. He first experienced behavioral changes and memory deficit at age 49. Five years later, he noticed tremor in the right hand, slowness, and gait difficulties. Our examination showed a coarse resting tremor, postural and action tremor in the right upper limb, hypokinesia, pyramidal and cerebellar signs, and sensory polyneuropathy. Neuropsychology studies revealed deficit in short-term memory and attention, slowness of thought, dyscalculia, bradylalia, with difficulty to initiate, execute and control complex motor programs, and severe depression. Brain CT scan showed multiple hypodense areas suggestive of vascular damage (subcortical, right putamen, left caudate nucleus) and cerebellar atrophy. We consider that this individual has parkinsonism in the context of a diffuse encephalo-neuropathy of likely alcoholic etiology.

His brother (IV-19) was 67 years old at first examination with the onset of neurological symptoms 7 years before, when he noticed bilateral hand tremors, difficulties in walking and postural imbalance. The clinical course was described as progressive, but at least three episodes of acute, transient neurological deficit have since then occurred. Our examination showed masked face, Meyerson's sign, dysarthria, dysphagia, mild left side hemiparesis, rigidity (mainly left-sided), mild but typical resting tremor, trunk dyskinesias and weak tendon reflexes in lower limbs. Posture, balance and gait were relatively preserved. Levodopa therapy has been irregular, and the response to this drug was unclear. Results of neuroimaging studies were unavailable. We consider this patient has parkinsonism in the context of a cerebral vasculopathy.

Molecular analysis

Linkage and haplotype analyses excluded all known loci for dominant PD (PARK1 D4S414 LOD= -3.25, PARK3 D2S2368= -2.36, PARK4 D4S391= -4.49, PARK5 D4S405= -4.16, and PARK8 D12S345= -3.62). Autosomal recessive PD loci (PARK2, PARK6 and PARK7) were excluded mainly by haplotype analysis. Other phenotypes associated with parkinsonism such as frontotemporal dementia-parkinsonism linked to chromosome 17 (FTDP-17), spinocerebellar ataxia type 2 (SCA2) on 12q and SCA3 on 14q were excluded by haplotype and linkage analyses (data not shown).

After performing two-point linkage analyses (Model 1), 60.3% of the markers from the genome screen gave a LOD score < -2.0, the accepted criterion for exclusion of linkage, 37.9% were between LOD=-2.0 and 0.99. We found positive (≥1.0) LOD scores for 1.8% of the markers (Table 2). The chromosomal regions surrounding these markers were subsequently excluded by haplotype analysis.

Table 2. **Regions with markers from the genome screen generating a LOD score ≥ 1**

	Two-Point LOD scores (Model 1)		
Chromosome	Marker	Location (cM)	LOD Score at θ = 0
Chr. 6	D6S264	179.1	0.37
	D6S446	189.0	1.12
	D6S281	190.1	0.88
Chr. 9	Telomere		
	D9S288	9.8	1.36
	D9S286	18.1	0.31
Chr. 11	D11S1320	141.9	0.66
	D11S968	147.8	1.34
	Telomere		
Chr. 13	D13S170	63.9	−4.02
	D13S265	68.7	1.51
	D13S159	79.5	$-\infty$
Chr. 15	D15S117	51.2	−0.54
	D15S153	62.4	1.09
	D15S131	71.3	−3.62
Chr. 19	D19S884	26.4	1.25
	D19S221	36.2	1.84
	D19S226	42.3	0.53
	D19S414	54.0	−0.08
	D19S220	62.0	1.94
	D19S420	66.3	−2.46

Only for chromosome 19, adjacent markers gave LOD scores higher than 1.0 and exceeded the average LOD score reached by our simulation studies (LOD_{maxav}=1.47). The CAG expansion of the *alpha 1A-voltage dependent calcium channel gene* (SCA6), which maps to chromosome 19 was assessed in three definite PD patients from the family, they all carried normal alleles of 10-12 CAG repeats (data not shown). Therefore, the chromosomal region was investigated more closely by typing additional markers between D19S209 to D19S210.

Results from the two-point linkage analyses are shown in Table 3. The maximum LOD score was 2.01 for marker D19S410 (45.48 cM). We used 24 markers from chromosome 19 for multipoint analyses, which provided better evidence for genetic linkage, with a maximum LOD score of 2.26 between markers D19S221 and D19S840 (fig.3).

Table 3. **Results from the two-point linkage analysis (Model 1) for chromosome 19 fine mapping**

Marker name	Location in cM[a]	θ						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
D19S209	10.97	-0.08	-0.06	0.01	0.04	0.05	0.03	0.01
D19S894	15.55	-4.66	-1.07	-0.44	-0.23	-0.11	-0.1	-0.07
D19S216	20.01	-0.19	-0.18	-0.15	-0.12	-0.1	-0.1	-0.07
D19S1034	20.75	0.91	0.89	0.81	0.69	0.45	0.22	0.06
D19S884	26.37	1.25	1.24	1.17	1.06	0.78	0.43	0.11
D19S865	32.39	1.03	1.02	0.98	0.90	0.67	0.38	0.11
D19S586	32.94	1.22	1.21	1.17	1.10	0.86	0.54	0.19
D19S221	36.22	1.84	1.82	1.72	1.58	1.22	0.79	0.32
D19S840	37.94	1.41	1.38	1.27	1.12	0.78	0.41	0.11
D19S714	42.28[b]	0.42	0.42	0.42	0.40	0.28	0.15	0.04
D19S226	42.28[b]	0.83	0.81	0.75	0.66	0.45	0.24	0.07
D19S410	45.48	2.01	1.98	1.85	1.67	1.24	0.72	0.20
D19S566	47.31	-0.25	-0.23	-0.18	-0.15	-0.12	-0.10	-0.05
D19S932	50.81	1.32	1.29	1.15	0.98	0.63	0.28	0.04
D19S433	51.88	-3.01	-0.21	0.38	0.53	0.48	0.29	0.08
D19S414	54.01	-0.08	-0.07	-0.06	-0.05	-0.03	-0.02	-0.01
D19S587	59.36[b]	1.70	1.67	1.56	1.41	1.04	0.60	0.18
D19S425	59.36[b]	-1.60	0.64	1.15	1.21	0.99	0.60	0.19
D19S220	62.03	1.96	1.91	1.73	1.49	0.98	0.48	0.12
D19S420	66.30	-2.48	-0.2	0.43	0.62	0.63	0.43	0.16
D19S902	72.72	0.16	0.2	0.28	0.32	0.28	0.17	0.06
D19S418	92.56	-4.30	-1.78	-1.02	-0.65	-0.28	-0.09	-0.01
D19S210	100.01	-0.47	-0.44	-0.35	-0.25	-0.10	-0.01	0.00

[a] Map location according the Marshfield genetic map.
[b] Marker orders according to the Icelandic genetic map.

Two-point linkage analyses under Model 2 yielded positive LODs higher than 1 for seven markers on different chromosomes (LODs between 1 and 1.51). Haplotype analyses showed in most of them the presence of common alleles shared for many individuals. In the case of chromosome 11q, marker D11S1320 and D11S968 gave positive LOD scores (1.15 and 1.34) with Model 2 and 1, respectively. We determined the actual allele frequencies using a group of 47 independent individuals (from the Cuban population) and recalculated the LOD scores that dropped to -0.61 and 0.97 (Model 1) and -0.15 and -1.5 (Model 2), indicating that the original results were likely false positive findings because of alleles inherited identical by state (IBS) rather than identical by descent (IBD). Linkage analysis with Model 2 resulted in a highest LOD score of 2.43 for marker D19S221 (θ=0). Therefore for both models chromosome 19 generated the highest LOD scores. Subsequent haplotype analysis showed a common chromosomal region shared by four definite PD patients. The same "disease haplotype" could be reconstructed in two deceased PD patients (III-8 and III-16). Patient IV-30 in Branch II (Fig. 2), however, has a different chromosome 19 haplotype.

For marker D19S221, all PD patients, including IV-30, shared allele 10. Estimation of true allele frequencies in 94 Cuban chromosomes showed that this is not a common allele (2.1%). Recalculation of LOD scores for D19S221 using the obtained allele frequencies resulted in LOD scores of 1.57 for Model 1 and 2.63 under Model 2. Haplotype analyses showed that for flanking markers no other allele was shared by this patient. This suggests that

allele 10 of D19S221 was probably shared IBS in patient IV-30 rather than IBD, although the possibility that this patient carried a very small "disease haplotype" due to a double recombination events cannot be ruled out completely.

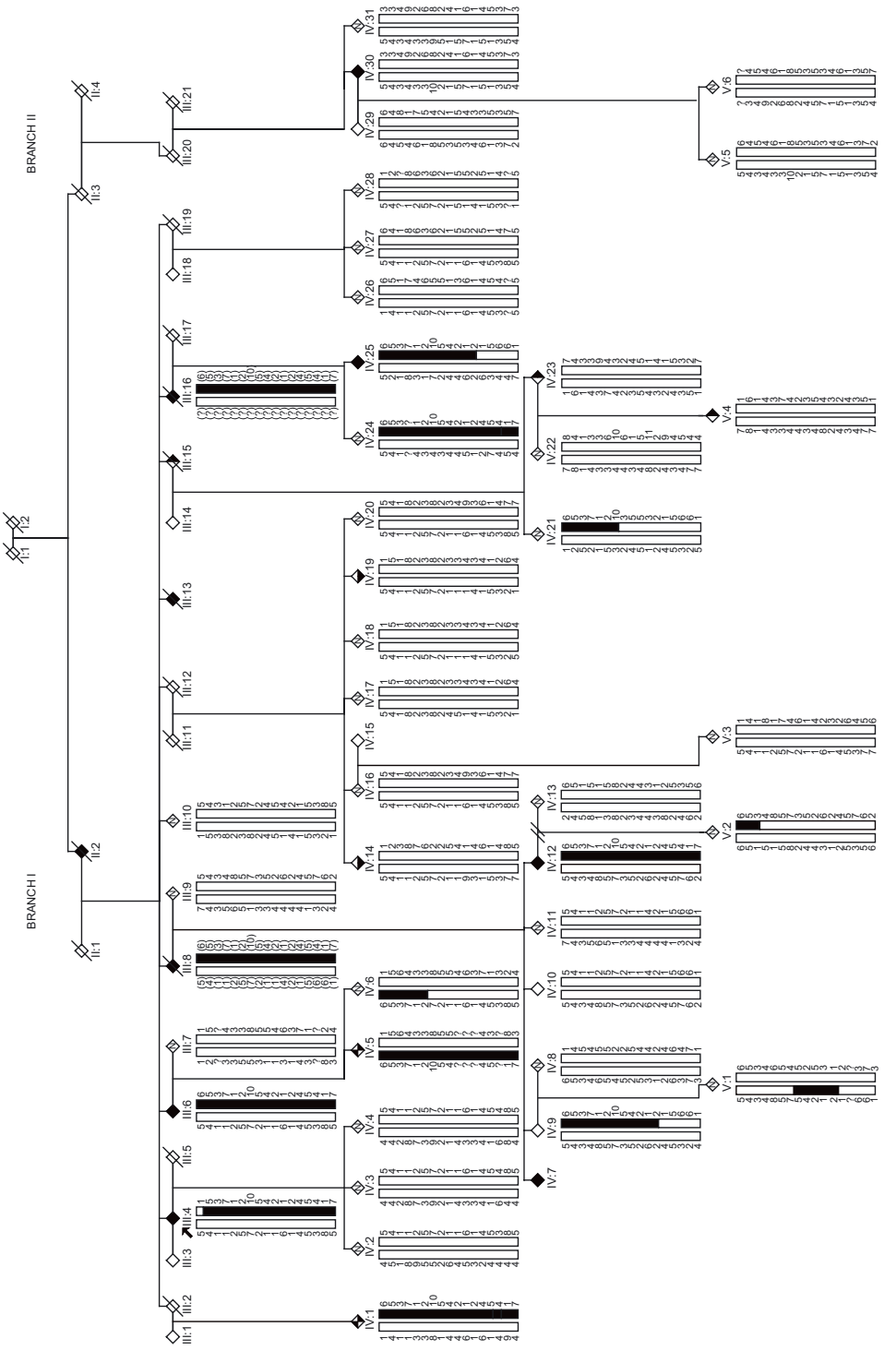
Although the age at onset is similar to patients of Branch I, this patient exhibited a slightly different clinical picture. Even though resting tremor is the more prominent feature in PD patients from Branch I, patient IV-30 has a severe akinetic form and almost no tremor. The parents of this patient (Branch II, Fig. 2) died free of parkinsonian symptoms at 79 and 80 years of age, in contrast to Branch I where every patient had an affected parent and unaffected parents have healthy offspring. If this patient has PD due to the same genetic cause of patients from Branch I, we have to consider the existence of non-penetrant individuals in two consecutive generations. Based on these findings it is conceivable that patient IV-30 is a phenocopy.

A recombination event between the telomeric marker D19S894 and D19S216 (patient III-4) delimited the upper border of the haplotype. In the centromeric side, we observed a recombination between markers D19S932 and D19S433 (patient IV-25). The minimal candidate region is spanning 35 cM from D19S216 to D19S932 (Fig. 2).

The whole disease-linked haplotype was present in 4 at risk individuals (ages between 59 and 65 years old); two of them (IV-1 and IV-5) have parkinsonian signs of hypomimia, bradykinesia, or rigidity that might suggest a prodromal clinical phase. Clinical examination of IV-6 and IV-21 who shared part of the haplotype was completely normal. Moreover, the disease-linked haplotype was not found in old, unaffected individuals (III-10, healthy, 76 years old), III-19 (unaffected, died at age 73 years of age), and III-12 (unaffected, died at age 85 years of age).

See next page:

Figure 2. *Condensed pedigree of the family showing individuals included in the genome screen and linkage analyses. In the figure, Generation IV has been divided into two levels in order to gain space. Filled symbols correspond to definite and likely PD patients, vertical and horizontal half-filled symbols show essential tremor and atypical parkinsonism phenotypes, respectively. Quarter-filled symbols indicate possible PD cases, 'N' inside a symbol means Normal neurological examination. Haplotypes of the chromosome 19 are shown; black bars correspond to the disease haplotype. Genotypes between brackets are inferred.*


BRANCH I
BRANCH II

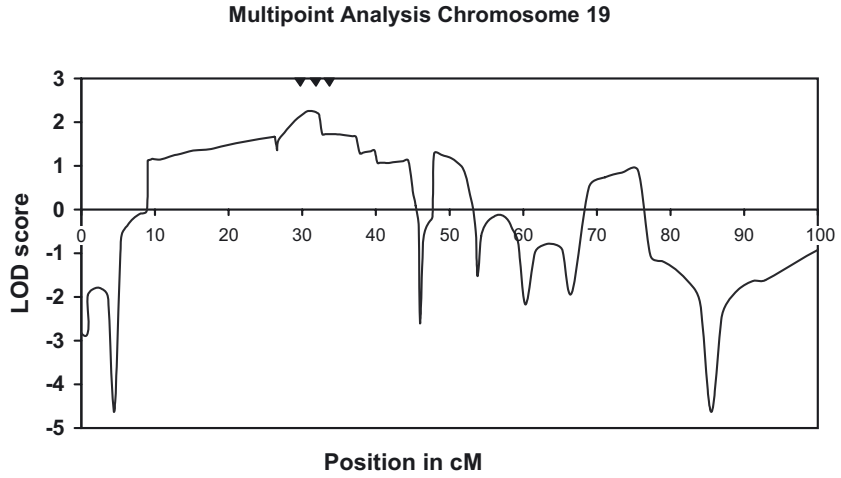


***Figure 3**. Multipoint LOD score graph for markers (fine mapping) from chromosome 19. Triangle-shaped labels correspond to markers D19S586, D19S221 and D19S840.*

Discussion

The presence of asymmetry of symptoms at onset and at examination in combination with typical resting tremor, bradykinesia, rigidity and good response to L-dopa, is consistent with the diagnosis of typical PD. None of these cases (definite and likely PD patients) presented any atypical features. Age at onset and disease duration are similar to those of typical, sporadic PD and to other PD families that had been reported[17, 32, 33]. They differ however, from several autosomal dominant families displaying a younger disease onset or more aggressive course (PARK1, PARK4, PARK8).

Segregation of PD in Branch I of the family (definite and likely cases) is consistent with an autosomal mode of inheritance with high penetrance (Fig. 2); a "vertical" segregation pattern, no generation gaps and male-to-male transmission were observed. In Branch II, only one patient was found, with no close relative being affected. Since PD in the rest of the family seems to be highly penetrant, this case might be a phenocopy.

Besides typical PD, we observed three cases with action, postural tremor compatible with essential tremor (ET) and apparently autosomal dominant transmission through three generations. Intrafamilial association of PD and an essential tremor-like phenotype has been reported [32, 33, 47, 48], supporting a relationship between PD and ET. In most cases, however, the nature of the relationship is unknown.

We also observed two cases with atypical parkinsonism. Personal history of cerebral vascular episodes or cognitive decline was reported and physical examination or neuroimaging revealed a vascular component in the clinical picture. It is remarkable that two siblings have the diagnosis of atypical parkinsonism. Considering that they have been living in different environments for more than 40 years, we think there could be predisposing genetic factors that may interact (or relate to vascular factors) resulting in a similar phenotype. In the case of patient IV-14, a toxic exposure such as heavy alcohol consumption could act as a modifier and is probably responsible for the "coarse" neurological picture.

Considering the presence of other phenotypes in our family and the uncertainties whether these phenotypes represent variations of the "PD gene", we have analyzed the genetic data using a conservative clinical model, in which only patients with clinically definite or likely PD were considered "affected" and atypical cases were regarded as diagnosis "unknown". Using this approach, the statistical power to detect linkage is less because the number of patients is lower, but it increases the probability of finding the true genomic location.

In general, the positive LOD scores obtained for the chromosome 19 region were close to the average value (1.47) but well below the maximum value (3.61) obtained with simulation analysis: low marker informativeness (marker heterozygozity) seems to be the most likely explanation. The multipoint analyses helped to overcome this problem partly, as more information was extracted when markers were analyzed together. Although no significant LOD score was obtained, the fact that 6 PD patients in two generations share a large haplotype for this region and not anywhere else in the genome indicates a true IBD allele sharing. Our results suggest that the region on chromosome 19 bears a gene responsible for this PD form.

Both the ET phenotype and the atypical parkinsonism in this family do not segregate with the chromosome 19 haplotype, suggesting a different etiology.

Several interesting genes are located in our candidate region (19p13.3-q12). The genes coding neurturin (*NTN*) and persephin (*PSPN*), two neurotrophic factors, are located on the 19p13.3 at the telomeric part of our region. *PSPN* is an attractive candidate gene for PD. The encoded protein is thought to function as a survival factor for midbrain dopaminergic and spinal neurons *in vivo*.[49] Other genes as *homer-3* and *telencephalin* could be considered as candidate genes. The homer protein family has been implicated in synaptogenesis, signal transduction, receptor trafficking and axon pathfinding.[50] Because we are considering a large chromosomal region, many other genes with known function can be considered as "candidates"; confirmation of our results in additional families are needed to reduce our candidate region and start the search for the responsible mutation.

In complex diseases such as PD, one of the advantages of studying large families is to avoid the heterogeneity problem. Many studies, however, have also encountered an intrafamiliar spectrum of parkinsonian phenotypes that required a precise phenotype refinement. For modeling the disease, we assumed an autosomal dominant inheritance. Although Mendelian models are useful for identifying the primary genetic cause of familiar disorders, they might be incomplete models for the true physiological and cellular nature of the defect.[51]

An oligogenic model with moderate contribution of two or three genes rather than a "pure" monogenic model might better explain the wide range in age at onset, the variable penetrance and the phenotypical variability observed in this and other PD families.

The study of additional extended families with the same ethnic origin will allow us to use different approaches such as a two-locus analysis. Using traditional linkage approaches will lead to the initial discovery of loci with major effects that follow Mendelian patterns of segregation, such as the chromosome 19 locus. Other methods, however, will then be necessary to detect "modifying" loci with a minor contribution or those who have a synergetic effect. A combination of methods can help us to understand the true genetic model for PD and can advance our efforts to dissect the genetic and molecular bases of the disease.

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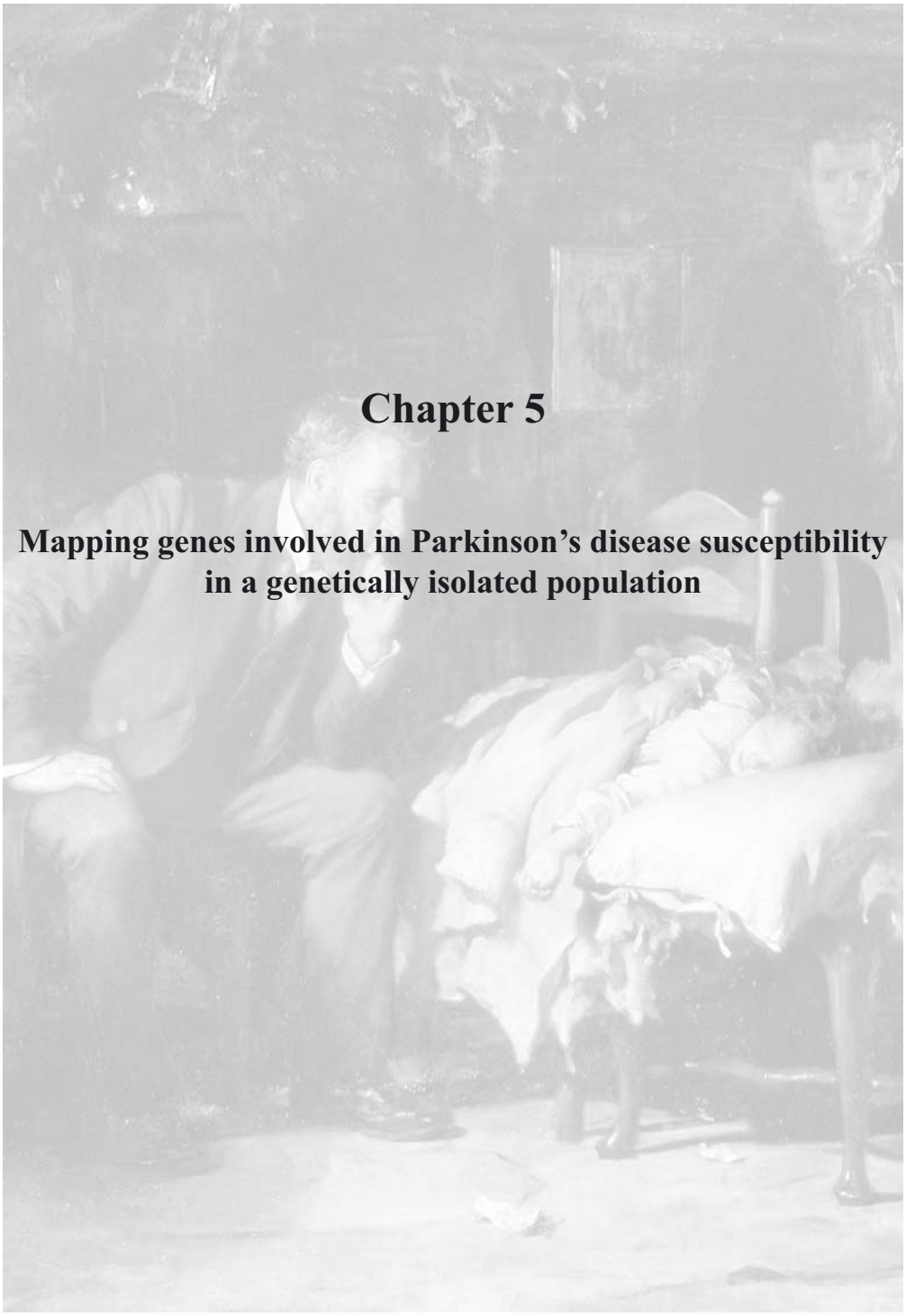

Chapter 5

Mapping genes involved in Parkinson's disease susceptibility in a genetically isolated population

Abstract

The study of genetically isolated populations, which are thought to be more genetically homogeneous than the general population, has proved to be a successful strategy for disease gene mapping, including the monogenic variants of complex disorders such as Parkinson's disease (PD).

In order to identify genomic regions containing genes that influence the susceptibility to PD, we studied 44 patients with idiopathic PD, 87 unaffected first-degree relatives and 25 spouses of patients who served as controls. The genealogic study showed that 40 of the patients could be connected to a common ancestor from an isolated community in the Netherlands. We performed a genome wide scan (STRPs, marker grid ~ 10 cM) and single-marker association analysis using the DISLAMB and CLUMP program.

With the DISLAMB analysis, four markers showed evidence of association at a nominal *p*-value of 0.01: D2S2333, D4S405, D9S158, D13S153. Furthermore for D9S158 (p=0.006) a neighbouring marker, D9S290, was also found to be associated (p=0.006) using the CLUMP analysis. Moreover, a common haplotype was observed in this 9q region for 10 patients (23%), and only one control individual (4%). This region overlaps with a previously identified interval (9q) that showed evidence of linkage with PD. In addition, marker D2S2333 (p=0.009) maps to the same location of a PD locus, PARK3, implicated in disease susceptibility and age at onset. The results of the study of this genetic isolate are consistent with previous studies suggesting the presence of genes contributing to Parkinson's disease susceptibility on chromosomes 2p and 9q. Further investigation of the identified regions will allow the confirmation and refinement of these candidate PD loci.

Introduction

Parkinson's disease (PD, OMIM 168600) is a late-onset neurodegenerative disorder characterized 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 nine recessive and dominant forms have been identified [1-9]. The genetic basis of the late-onset form of PD that has a complex inheritance pattern, however, is less evident. Although twin studies showed the genetic component of late-onset PD to be smaller than that of early-onset PD [10], the risk in first-degree relatives of PD patients to develop the disorder is still 2-14 times the risk in first-degree relatives of unaffected family members, arguing in favour of a genetic component [11]. Familial aggregation of the disorder was demonstrated by a genealogical search in Icelandic PD patients, leading to the mapping of a new locus for late-onset PD, *PARK10,* on chromosome 1p32 [12, 13].

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. Outbred populations are assumed to be genetically heterogeneous, therefore, genome-wide association studies using LD mapping would require very large case-control series and dense maps of polymorphic markers or Single Nucleotide Polymorphisms (SNPs).

In contrast, genetically isolated populations are more homogeneous due to the small number of founders and limited inward migration. Furthermore, LD might be maintained over sizeable regions around disease genes in recently isolated populations. Coarser marker maps should therefore be sufficient to successfully map disease genes in isolated populations [14]. The recent discovery of the *PARK10* locus [13] illustrates the potential of such approach.

We performed a genome scan in a group of 44 patients with typical PD from a genetically isolated population in The Netherlands. The aim of this study was to map gene(s) involved in PD susceptibility.

Methods

Patients

This study is part of a research program named Genetic Research in Isolated Populations (GRIP) [6, 15]. The scientific protocol of GRIP has been approved by 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. Since that time there has been a considerable population growth and until recently, a minimal inward migration [16]. 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 the 15th century has been computerised to a large extent, holding information on more than 60,000 individuals.

Patients with parkinsonism were traced through local general practitioners, neurologists and nursing-home physicians. 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 possible causes of parkinsonism and was verified by two independent neurologists according to the EUROPARKINSON criteria [17]. Data on the 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 the participants and from local municipal, church and computerised registers was extended up to 16 generations [18]. A total of 109 patients with parkinsonism were initially ascertained, of these, 57 could be connected to a common ancestor. A detailed clinical characterization of the patients is reported elsewhere [18].

Forty-four patients with the diagnosis of idiopathic PD were included in the genome wide scan. From these patients, 38 presented with late-onset PD (mean age at onset of 65.7 ± 10.8 years). Only 6 patients had a disease onset below 50 years (43.3 ± 3.2 years).

DNA analyses

The first genomic screen (stage I) comprised series of 23 PD patients, 48 first-degree relatives and 16 controls (spouses). We increased the sample size for further analysis, and the initial group was extended. In the second genome screen 21 PD patients and 39 first-degree relatives were included plus 9 controls (stage II). The inclusion of first-degree relatives in the study allowed for segregation checks and for the construction of haplotypes. The spouses of the patients served as controls in the analysis. They also originated from the same population. Our

total sample set comprised 156 individuals from whom 44 were PD patients, 87 first-degree relatives and 25 were controls. For fine typing of the regions identified during the genome screen, the group of controls was extended to 88 control individuals, all from the GRIP population.

Genomic DNA was isolated from peripheral blood as described elsewhere [19]. For the systematic genome scan we 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 maps. Information about marker order and distances were obtained from the NCBI physical map, Marshfield integrated genetic map and Celera human genome database.

Genomic DNA (20 ng) was amplified in 7.5 µl PCR reactions prepared using a Beckman Biomek 2000 robot system and performed in 384-well plates. PCR products were pooled and loaded on an ABI377 (filterset D; 6.25% denaturing FMC LongRanger acrylamide gel) for stage I, or ABI3100 automated sequencer for stage II. Data were analysed using ABI GeneScan 3.1 and ABI Genotyper 2.5 or Genemapper 2.1 software from Applied Biosystems.

All patients in this study tested negative for the deletion extending from exon 1 to 5 of the *DJ-1* gene, which was identified in a family with early-onset autosomal recessive parkinsonism from the same community [6, 18]. As previously reported, 3 early-onset, and 18 late-onset PD patients (from stage I) were studied for genetic markers flanking known PD genes and loci (4q22, 6q25.2-q27, 2p13, 4p14-16.3, 1p35-36, 1p36, 12p11.2-q13.1 and 1p32) but showed no association [18].

Statistical analysis

After completion of the genotyping phase, a single analysis of the complete data was performed. Because the genome scans had been performed with different equipments: gel electrophoresis for the first 87 samples (stage I) and capillary electrophoresis for the last 69 individuals (stage II), differences of 1 to 6 base-pairs were observed between alleles coming from the different stages. To merge the genetic data, a program was developed "in house", "pooling" the data based on the allele lengths and frequencies observed in each group. The program uses a maximum likelihood approach to obtain the best correspondence between alleles. Simulation studies showed that with sample sizes of about 100 individuals, 70% of the allele correspondences were achieved with an error rate less than 1% (manuscript in preparation, Y. S. Aulchenko et al.).

Genotypic data were analysed using a likelihood-based disequilibrium analysis program to investigate for single locus association (DISLAMB program version 2.1) [20], under the assumption that one marker allele will be over-represented on chromosomes that carry the disease mutation. The program estimates allele frequencies for cases and controls and performs a likelihood ratio test (LRT), the corresponding chi-square and *p-value* are obtained. The proportion of patients in whom the disease is associated to an ancestral allele is represented by the parameter lambda (λ).

To assess association, we also used the CLUMP software, which allows for allelic heterogeneity. The method clumps alleles together into a 2-by-2 table in such a way that the highest chi-square statistic is obtained [21]. Its corresponding *p-value* is obtained empirically by Monte-Carlo simulations. A total of 10, 000 simulations were performed each time. By using this program, it is

feasible to test the possibility that more than one ancestral allele is associated with PD.

A nominal value of $p \leq 0.01$ corresponding to a LOD score of 1.18 was used to decide whether a region was interesting for further study [20, 22]. For these regions, we determined haplotypes with the MERLIN program, using data from the first-degree relatives.

For the saturation-mapping analysis (chromosome 10), haplotype construction was not possible due to ambiguous order of markers on the physical map (NCBI, build 34, version 3). We performed single-point analysis of each marker separately using the CLUMP program.

Results

Forty of 44 patients with idiopathic PD could be connected to a common ancestor within 16 generations. The average kinship coefficient for patients was 7.2×10^{-4}. There were no affected first-degree relatives included in the analysis, 3 pairs of patients were related in 3-4 meiosis, and for the rest the genetic relationship extended beyond 5-6 meiosis. For the control group only 13 out of 25 individuals could be found in the genealogical database, their average kinship coefficient was lower than the patients (1.15×10^{-5}).

Table 1 shows all 14 markers with *p-value* ≤ 0.01 corresponding to a LOD ≥ 1.18, with either DISLAMB (n=4) or CLUMP (n=11). For the DISLAMB analysis only 4 markers had a *p-value* ≤ 0.01 with lambdas of 0.21 (2 markers), 0.29 and 0.37. Furthermore, three of these four markers (D4S405, D9S158, D13S153) also yielded low *p-values* (Table 1) with the CLUMP analysis.

In addition, the associated allele detected with the DISLAMB analysis was also identified with the CLUMP analysis; for D4S405 allele 6 and alleles 6 and 7 were detected with each analysis; for D9S158, allele 3 and alleles 3, 5, 6; for D13S153 allele 2 and alleles 2, 3, 5, showing consistency between results coming from both analyses.

Only one marker, D9S158, showed evidence for association with PD for both analyses (DISLAMB, p=0.006 and CLUMP, p=0.009). Furthermore, this was the only region where close markers showed association. D9S158 and D9S290 are both located at the 9q34 region, 21 cM apart according to the Marshfield sex-averaged genetic map. We constructed haplotypes in all individuals for the complete chromosome 9. A common haplotype was observed extending from D9S1776-D9S1682-D9S290-D9S1826-D9S158 (Fig. 1). The marker D9S164 was initially positioned between D9S290 and D9S1826 but its precise location is not certain yet; data from the draft sequences (NCBI physical map, build 34, version 3) positioned this marker on two different chromosomal regions on 9q34. Therefore D9S164 was excluded from the haplotype analysis. Ten out of 44 patients (23%) and only 1 out of 25 controls (4%) carried a common haplotype extending 3 or more markers. Patients carrying the common chromosome 9 haplotype were also more closely related (average kinship coefficient of 1.31×10^{-3}) than the complete group of 40 patients; most of them could be connected to a common ancestor within 10 generations (Fig. 2). For the remaining markers none of the haplotypes were associated with PD.

Table 1. **Results form the PD-GRIP genome screen**

		DISLAMB					CLUMP		MAPS		
Chromosome	**Marker**	**Associated allele**	**Frequency in cases**	**Frequency in controls**	***p-value***	**λ**	**Associated alleles**	***p-value***	**Cytogenetic Band**	**Marshfield cM**	**Physical Mb**
1	D1S230	-	-	-	0.192	0.16	1,3,5,6	***0.002***	1p31-32	95.3	?
1	D1S498	-	-	-	0.470	0.01	2,3,6	***0.004***	1q12	155.9	148.5
2	D2S2333	5	37%	24%	***0.009***	0.29	(1,5)	0.118	2p12	103.1	85.5
3	D3S1304	7	12%	3%	0.014	0.17	1,3,5,6,7	***0.004***	3p25-26	22.3	6.9
4	D4S405	6	15%	3%	***0.002***	0.21	6,7	0.014	4p14-15	56.9	40.2
5	D5S436	-	-	-	0.50	0	1,2,3,4,7,8	***0.005***	5q31-33	147.5	145.2
7	D7S684	-	-	-	0.50	0	1,2,4,5,7,10	***0.01***	7q34	147.2	137.5
9	D9S290	5	23%	12%	0.022	0.21	3,5	***0.006***	9q34.13	140.8	126
9	D9S158	3	49%	34%	***0.006***	0.37	3,5,6	***0.009***	9q34.3	161.7	134
10	D10S1651	-	-	-	0.22	0.15	1,2,3,4,5	***0.001***	10q26	168.8	132
11	D11S4175	-	-	-	0.49	0	1,2,3,4,5,6,7,10,12	***0.005***	11q14	91.5	89.9
13	D13S153	2	18%	1%	***0.009***	0.21	2,3,5	0.021	13q14	45.6	46.6
14	D14S280	2	47%	34%	0.015	0.32	2,3	***0.007***	14q24	105	90.1
17	D17S938	7	12%	3%	0.030	0.16	3,6,7	***0.002***	17p13	14.6	6.9

Marker D2S2333, which showed evidence of association during this study (DISLAMB p=0.009, λ=0.29), is at the same location as the *PARK3* locus. Markers on chromosome 4, D4S1534 at 136 cM (p=0.015, λ=0.31), and on chromosome 17, D17S949 at 102.3 cM (p=0.021, λ=0.17) were also interesting despite the modest statistical evidence for association, because of their location close to known genes related to PD aetiology (α-synuclein and *tau)*.

The analysis with CLUMP yielded *p-values* ≤ 0.01 for 11 markers. The strongest evidence for association came from marker D10S1651 (p=0.001). Therefore we tested a larger control group (n=88) originating form the GRIP population in order to obtain a better estimate of the allele frequencies. Twelve additional markers on chromosome 10 around D10S1651 (D10S217, D10S1676, D10S1439, D10S1655, D10S1248, D10S169, D10S1770, D10S555, D10S1675, D10S590, D10S1711 and D10S1700) were tested. Attempts were made to construct haplotypes by using the originally associated marker 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 region of chromosome 10q, especially for D10S1651 and the most telomeric markers, was unreliable (NCBI or Celera physical maps) as large parts of this region consist of draft and unfinished sequence.

Using CLUMP, one of the additional 13 markers (D10S1711, telomeric to D10S1651) showed evidence for association that was found with the 177-bp allele (p=0.006). For the original marker, D10S1651, two alleles (211 and 229-bp) were detected (p=0.049). All other markers had *p-values* > 0.30.

Discussion

We here report the results of our genome scan performed in a group of 44 patients with typical PD from a genetically isolated population in The Netherlands. After performing the association analysis with DISLAMB, 4 markers were of interest (p≤ 0.01): D2S2333, D4S405, D9S158, D13S153.

D9S158 (LOD=1.37) was the only marker for which both analyses showed association. In addition, a neighbouring marker, D9S290, also showed association. Both markers are located in the long arm of chromosome 9, with 8 Mb in between according to the NCBI physical map. Even with the coarse marker grid used for this screening (10 cM), a common haplotype extending up to five markers was observed for several patients. The complete region extends around 21 Mb based on the NCBI physical map. A common haplotype extending at least 3 markers was observed for 10 patients (Fig. 1). In the control group this was observed in only one individual.

Patient	D9S1776	D9S1682	D9S290	D9S1826	D9S158
1*	5	1	5	2	3
5*	5	1	5	2	3
16*	3	1	5	2	3
17*	6	1	5	2	3
2*	2	1	5	2	3
3*	5	2	5	2	3
7	5	1	5	2	3
8	6	1	5	2	3
4*	5	1	5	2	8
9*	5	1	5	4	3
12*	5	1	5	2	3
14*	5	1	5	2	3
6	5	2	5	2	3
10*	5	2	5	2	3
11	6	2	5	2	3
13*	7	1	5	2	3
15	5	1	5	6	6
18	3	1	5	2	4

Figure 1. Patients with PD showing a common haplotype in the chromosome 9 region. Dark grey boxes represent alleles which phase could be determined, light grey means phase unknown. The asterisks indicate all 12 patients that were related to a common ancestor.

This region overlaps with marker D9S1825 previously identified during a sib-pairs analysis that found suggestive evidence of linkage with this marker [23]. Marker D9S290, detected during this study is ~ 4 cM centromeric from D9S1825, suggesting that this interval might contain a gene involved in PD susceptibility. Moreover, evidence of linkage to PD age at onset was found at a region located ~ 20 cM (telomeric, marker D9S930, maxLOD=2) [24]. Similarly, the *PARK3* and *PARK10* loci have been implicated in PD susceptibility and age at onset of the disease [24, 25]. This line of evidence suggests that the same gene(s) could be involve in increasing susceptibility to PD and in modulating the age at onset of the disease.

Marker D2S2333 maps to the *PARK3* locus and also showed association. Several studies have related this locus to PD susceptibility and age at onset of the disease [3, 24], and recently a haplotype harbouring the *sepiapterin reductase* gene, implicated in dopamine synthesis, was found associated to a younger onset of the disease [26].

Markers D4S1534 at 136 cM and D17S949 at 102 cM, close to the *α-synuclein* and *tau* gene, respectively, showed modest evidence of association. Both genes have been implicated in PD. Three *α-synuclein* mutations are known to cause autosomal dominant PD. Moreover,

a polymorphism (Rep1) located within the gene promoter has been related to increased risk for late-onset PD in case-control studies of "sporadic" PD patients (mean onset age 64 and 66 years in each study) [27, 28]. Alpha-synuclein over-expression is believed to be a risk factor for PD and variation of the gene expression related to this polymorphism has been reported [29-31]. In addition, a specific *tau* haplotype (H1) has been related to PD as well, in case-control studies with "sporadic" PD patients (mean age at onset 64 years, range 31 to 91 years) [32]. We will next explore a possible association with *α-synuclein* and *tau* gene variants in this sample set and investigate potential interactions with other detected regions such as the chromosome 9q.

Two markers showing association with PD in the CLUMP analysis are of special interest because of their genomic location. Marker D1S230 (Table 1, p=0.002) is located at the *PARK10* region, which is the only locus mapped for typical late-onset PD. This region on chromosome 1p32 was found after a genome wide search in a group of 117 PD patients from Iceland [13]. Marker D5S436 (147 cM, p=0.005) is also interesting because of its location close to an interval (~ 139 cM) detected during the analysis of 174 small families comprising 378 PD patients [33]. Furthermore, excess of allele sharing between Icelandic PD patients was found for a marker in the same region (D5S666, 135 cM, LOD=1.6) [13]. However, for marker D5S436, a total of 6 alleles were found associated with PD during this analysis. Because most of the patients are related to a common ancestor, such allelic heterogeneity is not expected. Several associated alleles were detected for markers on chromosome 7 and 11 as well (D7S684, 6 alleles and D114175, 9 alleles) weakening the evidence of association.

The lowest *p-value* obtained during our analysis corresponded to marker D10S1651 (CLUMP, p=0.001). Upon saturating this region with a denser set of markers in an augmented sample, the control allele frequencies could be estimated more accurately. Besides marker D10S1651, marker D10S1711 yielded a *p-value* of 0.006. 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. Although the association was maintained in the follow-up analysis, a marker order could not be determined with sufficient certainty to construct haplotypes. This limits the interpretation of this last finding.

Further fine mapping of the positive regions with subsequent haplotype analysis will be required to confirm and eventually refine the detected regions. During this study we identified candidate regions showing overlap with others previously described. This suggests that genes located within those regions might be involved in PD susceptibility in this isolated population.

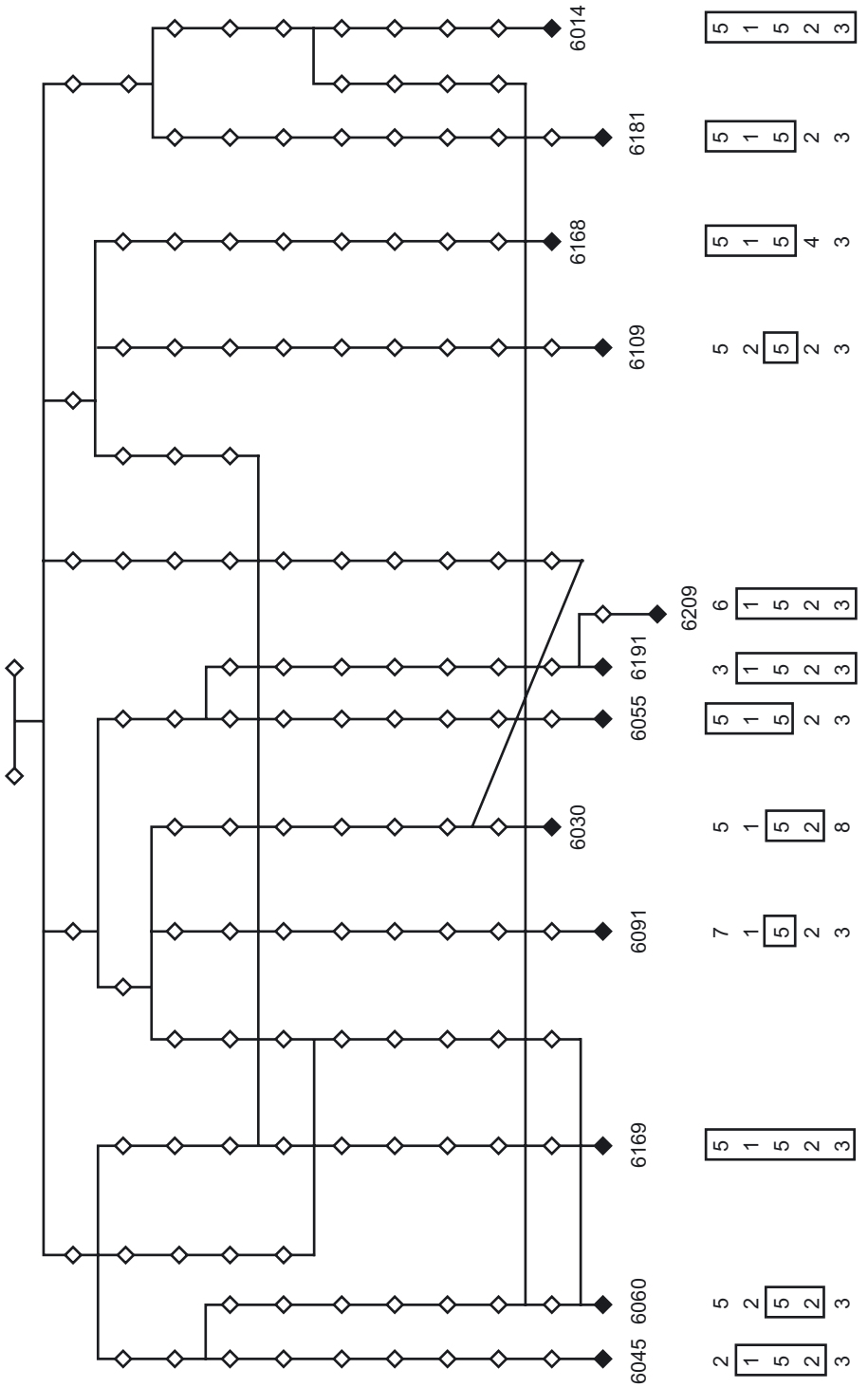

Figure 2. Pedigree showing the PD patients (filled symbols) with the common chromosome 9 haplotype, only the spouses connected to the pedigree are shown. The "boxes" in the haplotypes indicate known allele phase. Marker order is as follows: D9S1776, D9S1682, D9S290, D9S1826, and D9S158.

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Chapter 6

Novel parkin mutations detected in a large, multiethnic series of cases with early-onset Parkinson's disease

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Abstract

A large, multiethnic series of patients with early-onset Parkinson's disease (EOP) was studied in order to assess the frequency and nature of *parkin*/PARK2 gene mutations, and to investigate phenotype-genotype relationships. Forty-six EOP probands with onset age <45 years, and 14 affected relatives were ascertained from Italy, Brazil, Cuba, and Turkey. The genetic screening included direct sequencing and exon dosage using a new real-time PCR method. Mutations were found in 33% of the index patients overall, and in 53% of those with a family history compatible with autosomal recessive inheritance.

Fifteen *parkin* alterations were identified, including 10 exon deletions and five point mutations. Four of the mutations have not been described previously: Arg402Cys, Cys418Arg, IVS11-3C>G, and deletion of exon-8-9-10. Heterozygous genomic rearrangements represent 28% of the mutations found, confirming the importance of testing exon dosage when screening *parkin*. The patients with *parkin* mutations showed significantly earlier onset, longer disease duration, slower disease progression and more frequent symmetric onset than the patients without mutations, in agreement with previous studies. This study confirms the frequent involvement of *parkin* and indicates the importance of genetic testing in the diagnostic work up of EOP. The novel, cost-effective exon dosage method described here can also be applied to the study of other genes.

Introduction

Previous studies suggest that genetic factors are more important in the aetiology of early-onset Parkinson's disease (EOP) than in the common late-onset forms [1], and autosomal recessive forms are increasingly recognized among EOP cases [2]. Mutations in two genes, *parkin* [3] and *DJ-1* [4], have been identified in autosomal recessive EOP, and a third locus, PARK6 [5] bears a gene responsible for a further recessive form. So far, mutations in the *parkin* gene are the most frequent identifiable cause of EOP [6].

Parkin mutations were first identified in Japanese patients with autosomal recessive juvenile parkinsonism [3]. Since then, several mutations have been described in EOP cases, varying from point mutations to complex rearrangements including deletions and/or multiplications of complete exons [7-15]. Some studies emphasized the importance of gene dosage assays as part of the mutational analysis of *parkin*, but the estimated frequency of exon rearrangements varies greatly (33 to 67%) [10, 11, 16].

Most *parkin* mutations lead to the loss of the ubiquitin E3 ligase activity of the encoded protein, which normally tags specific substrates for degradation through the ubiquitin-proteasome pathway [17]. However, the mechanisms by which *parkin* mutations cause neurodegeneration remain to be elucidated. Studies on *parkin* knock-out mice failed to fully reproduce the picture of the human disease, but did suggest a role for the parkin protein in the regulation of nigrostriatal dopaminergic neurotransmission [18, 19].

Patients with *parkin* mutations are difficult to distinguish from other EOP patients on the basis of their clinical phenotypes [8, 20]. Moreover, due to the complexity of the *parkin* gene and the wide spectrum of mutations, the genotype-phenotype correlations have been difficult to establish. There is a wide variation (intra and inter-familial) in the clinical presentation and age at onset, even in patients with the same gene defects [20]. Atypical clinical and genetic presentations, including pseudo-dominant inheritance have also been described [21, 22]. Last, in a few patients, only one heterozygous mutation has been detected, suggesting that a second

mutation still escapes detection by current screening methods, or that some mutations in heterozygous form are sufficient to cause this disease [11, 12, 14]. It is clear that much work is still ahead to disentangle the complexity of the disease associated with *parkin* mutation (the "parkin disease"), and the analysis of large series of patients is therefore warranted.

Here we report on the nature and frequency of *parkin* mutations and on phenotype-genotype relationships in a newly ascertained, multiethnic group of EOP patients. Genetic screening included direct sequencing of the *parkin* coding region and systematic exon dosage analysis. Previously developed methods for exon dosage use expensive fluorescently labelled probes (i.e. LightCycler [10], TaqMan [21]). Here we describe a novel and cost effective quantitative PCR method employing a fluorescent intercalating agent and non-labelled oligonucleotides.

Patients, Materials and Methods

Patients

We included in the study all the patients referred during the period 2000-2002, who fulfilled the following criteria: clinical diagnosis of Parkinson's disease (PD), and either: (a) positive family history compatible with autosomal recessive inheritance and age at onset ≤45 years in the index case; (b) isolated presentation with age at onset ≤40 years. According to these criteria, we collected a multiethnic group of 46 EOP index patients from Italy (n=39), Brazil (n=4), Cuba (n=2), and Turkey (n=1), plus 14 affected first-degree relatives (total sample set n=60). Seventeen index cases were from families compatible with autosomal recessive inheritance, and 29 were isolated patients. Consanguinity was reported in eight families and two isolated cases.

The clinical diagnosis of Parkinson's disease was established according to the UK Parkinson's Disease Society Brain Bank criteria [23, 24], when at least two of the three cardinal signs (resting tremor, rigidity and bradykinesia) and a positive response to dopaminergic therapy were present, in absence of atypical features or other causes of parkinsonism. Neurological examination of each patient was performed by neurologists with experience in movement disorders and included the Unified Parkinson's Disease Rating Scale (UPDRS, motor part) [25] and Hoehn Yahr scale [26] in "on" and (if possible) in "off" status. Clinical data were collected using a standard form. Informed consent was obtained from all patients. Venous whole blood was taken and DNA isolated according to standard procedures [27].

Molecular studies

Haplotype analysis

In the families compatible with autosomal recessive inheritance of EOP, we typed Short Tandem Repeats (STR) markers from the PARK2 (*parkin*) [3], PARK6 [5] and PARK7 (*DJ-1*) [28] regions, by PCR using fluorescently labelled primers and an ABI 3100 automatic DNA analyser, as described previously [29]. Haplotypes were constructed based on the minimum number of recombinations.

Direct sequencing of parkin

Families showing no haplotype sharing for both alleles at the PARK2 locus were excluded from the mutational screening (n=3). For the remaining families and the isolated patients, direct sequencing of the *parkin* gene was performed using the BigDye terminator chemistry (Applied Biosystems). All 12 exons and exon-intron boundaries of the *parkin* gene were amplified using intronic primers, as described [30]. For exon 1, 6, and 10, we designed new

intronic primers (primers and PCR conditions available on request). PCR products were loaded on an ABI 3100 Automatic DNA sequencer and analysed with the SeqScape software version 1.1 (Applied Biosystems).

The frequency of the novel detected variants was assessed in panels of at least 96 and up to 500 chromosomes from ethnically matched control individuals by digestion with restriction enzymes or by the allele specific oligonucleotide hybridisation technique (ASO).

Splicing effect analysis

We used five computer programs to predict the possible consequences on splicing of sequence changes in the proximity of the exon/intron boundaries [31-35].

Exon dosages studies

All index patients without mutations or with a single heterozygous mutation detected by sequencing were investigated further for exon rearrangements. Exon dosages studies were performed through quantitative PCR using an iCycler iQ™ Real-time PCR machine (Bio-Rad) and SYBR™ Green I as intercalation dye.

Exonic and intronic primers for the 12 exons of the *parkin* gene were designed (available on request), allowing amplification of genomic fragments ranging from 81 to 139 bp. Fifty nanograms of genomic DNA was used per 25µl of single PCR reactions (qPCR™ Core kit, Eurogentec) for *parkin* and the "control gene" (*β-globin, HBB*). All samples were tested in triplicate and at least one positive and two negative controls were included in every plate (96 well plates). The thermal cycling parameters were: 95˚C - 10 minutes, 40 cycles of 95˚C - 20 sec., 60˚C - 45 sec., 75˚C - 15 sec., allowing for real-time data collection. A melting curve was generated for each sample, enabling the detection of non-specific products during the amplification.

The fluorescence of the SYBR™ Green increases significantly as it binds and intercalates into double-stranded DNA during the extension step of the amplification cycle. During the PCR the accumulation of product results in a measurable change in fluorescence of the reaction mixture, this point is called the Threshold cycle (CT). We used this value to perform our calculations, given that there is a linear relationship between the log of the starting amount of template and the corresponding CT during real-time PCR [36].

The iCycler software (version 3.0a) calculates automatically the CT for every reaction. Since three different measurements are obtained per sample, the average CT and standard deviation (SD) are calculated for both *parkin* and *β-globin*. The Average CT was used to calculate the ratio *parkin/β-globin* ($R_{P/\beta}$) using the following formula:

$$R_{P/\beta} = [(CCT_{Parkin} - CCT_{\beta globin}) - (PCT_{Parkin} - PCT_{\beta globin})]^{2}$$

where CCT is the average threshold cycle for the negative (normal) control sample and PCT is the average threshold cycle for the patient sample. On the basis of the observed variability of the values of the ratios in normal individuals and positive controls with *parkin* heterozygous rearrangements, we considered as normal the ratios between 0.8 and 1.2. Values lower than 0.7 or higher than 1.3 are interpreted as heterozygous deletion or duplication of the assessed exon, respectively. All positive results were confirmed at least twice and an average ratio was calculated. Furthermore, all cases with homozygous or heterozygous exon deletions affecting only one exon were confirmed with an independent set of primers to avoid false positive results due to primer mismatch caused by undetected polymorphisms. Segregation of detected rearrangements was investigated whenever DNA samples from relatives were available.

Evaluation of the consequences of the exon deletion on the protein (in-frame or

frameshift) was predicted based on the *parkin* cDNA sequence published with accession no. AB009973.

Statistical analysis

All calculations were done using the SPSS package version 11 (SPSS, Inc., Chicago, IL). We used the nonparametric Mann-Whitney U test or the Student's T test for comparison of means and the χ^2 or Fisher's exact test for comparison of proportions when appropriate. Differences of means (disease severity, UPDRS and Hoehn Yahr score in "off") were tested using ANCOVA. *P* values for trend were obtained from simple linear regression models where type of mutation was included as a continuous term (0=No *parkin* mutation, 1= two *parkin* exon deletions, 2=*parkin* heterozygous point mutation).

Results

Clinical studies

Patient characteristics are summarized in Table 1. The mean age at onset (AAO) was 33 ±11 years ranging from 14 to 65 years. Four of the 14 affected first-degree relatives had a disease onset later than 50 years of age, while in the remaining cases the AAO was before 50. Resting tremor and bradykinesia at onset were found in around half of the patients (53 and 54%). The onset of signs was asymmetric in most of them (79%). Bradykinesia was the most frequent sign found at clinical examination (96%) followed by rigidity, present in 91% of the examined patients. Other characteristics such as sleep benefit and slow disease progression were occasionally noticed.

Some patients presented other features: severe depression, anxiety, panic attacks, dysphoria, and emotional liability were noted. In addition to EOP, one case presented a chromosomal abnormality (Robertsonian 13q-14q translocation), and another patient had mild mental retardation and facial dysmorphism. Eighty-eight percent of the patients received treatment with levodopa; the majority of these also presented with levodopa-induced dyskinesias (79%) and motor fluctuations (73%).

Molecular studies

Haplotype analysis of the PARK2 region was performed in 8 families. In three of them the PARK2 locus was excluded since no haplotype sharing (on both chromosomes) was observed among the affected individuals. In the five remaining families, haplotype analyses supported a causal role of *parkin*, and they were included in the mutational screening (three of them were consanguineous). Haplotype analysis could not be performed in 9 families because DNA samples from additional family members were not available.

Parkin sequence analysis

Several intronic and exonic changes were identified. Two novel intronic changes, IVS11-3C>G and IVS2-18T>A, were found close to acceptor splice sites. The coding variants included two novel point mutations in exon 11: 1305C>T predicted to cause the aminoacid change Arg402Cys, and 1353T>C leading to Cys418Arg. The previously described missense mutations Arg42Pro in exon 2, and Thr415Asn in exon 11 were also detected. We also found a novel synonymous change in exon 4, 620G>A (Thr173Thr).

The known polymorphisms [7] 1239G>C (Val380Leu), 1281C>A (Asp394Asn), IVS2+25T>C,

IVS3-20C>T, IVS7-35A>G were also repeatedly found (data not shown).

The novel IVS11-3C>G change was identified in a consanguineous Cuban family with three EOP patients. Haplotype analysis of the PARK2 region previously suggested the possibility of heterozygous mutations in the *parkin* gene, since all patients shared the same parental haplotypes without evidence of homozygosity (fig. 1). The second mutation in this family was found by exon dosage analysis (see below).

The IVS11-3C>G change introduces a new cutting site for the restriction enzyme BseRI. We tested 96 chromosomes from unrelated Cuban controls and none was found to carry the base change, indicating this is not a common variant. All programs anticipated the abolition of the normal splicing acceptor site and the activation of the cryptic splice site AC<u>A</u>G/GAG to A<u>G</u>/AGGAG. Unfortunately, RNA from these individuals was unavailable. The PARK6 and PARK7 locus were both excluded by haplotype analysis (not shown).

The remaining intronic change IVS2-18T>A, found in an Italian patient, was located further away from the splicing site. The same computer programs predicted no affectation of the normal splicing, and therefore the pathogenicity of this sequence change remains doubtful. No other changes were found.

The novel sequence alterations 1305C>T (Arg402Cys) and 1353T>C (Cys418Arg) were found in heterozygous state (Table 2), they are located close to and within the second RING finger motif of the parkin protein and both affected highly conserved amino acids, suggesting they are pathogenic changes. However, in the patient carrying the 1305C>T (Arg402Cys) change, a second mutation was not found by the methods used in this study. This base change was found in 1 out of 500 control chromosomes (320 and 180 chromosomes of Italian and Dutch origin, respectively).

The Arg42Pro mutation, located within the ubiquitin-like domain of the protein, and the Thr415Asn mutation were both detected previously in homozygous state in families of Italian origin with EOP [7, 37]. Nucleotide changes were not found in the remaining patients by sequencing of the 12 *parkin* exons.

Exon dosages analysis

Homozygous deletions spanning up to 3 consecutive exons were found in eight probands (Table 2). One patient carried a novel deletion involving exons 8, 9 and 10.Using a quantitative, real-time PCR method, six index cases were found to carry heterozygous exon rearrangements. These include four out of the five probands carrying heterozygous point mutations, and two probands carrying two different heterozygous exon rearrangements (Table 2).

In three families, co-segregation and phase of the heterozygous deletions could be resolved. In an Italian family (Ver-01) the proband carried a heterozygous point mutation (C418R) and a heterozygous deletion of exon 3. We confirmed the presence of the C418R mutation in the mother and the deletion of exon 3 in the father, delineating the patient as a compound heterozygote (C418R/ex3del) of *parkin* mutations.

In the aforementioned Cuban family (Cu03), all patients carried the IVS11-3C>G change and a heterozygous deletion of exons 3-4 (fig. 1). The IVS11-3C>G change was then found in the unaffected mother, whereas the unaffected father carried the heterozygous deletion of exons 3-4.

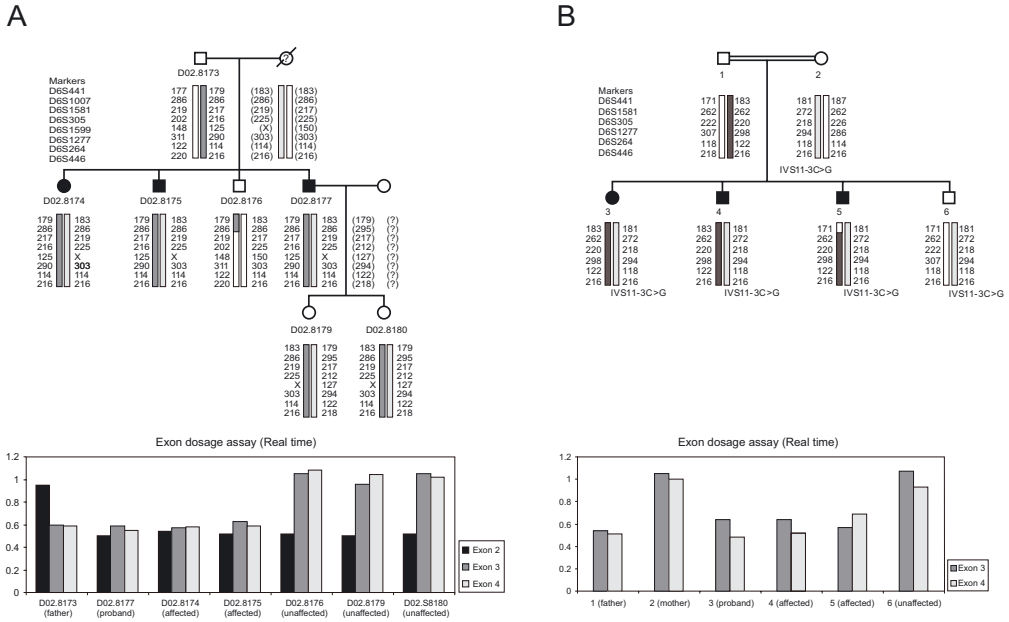

Figure 1. *The pedigrees from a Turkish, Ayd01 (A) and a Cuban family, Cu03 (B) are shown. Filled black symbols represent the patients with EOP. Haplotypes for the PARK2 region are displayed; alleles between brackets correspond to inferred genotypes. In pedigree A, for marker D6S1599, hemizygosity was observed; the missing allele is represented with "X". The bar graphs below the pedigrees are showing the results from the exon dosage assay for the corresponding individuals.*

Finally, in a Turkish family, haplotype analysis previously showed parental non-transmission of alleles suggesting hemizygosity for one intragenic marker (D6S1599), raising the possibility of a deletional event within the *parkin* gene. Subsequently, by means of real-time PCR, the three patients were characterized as compound heterozygous for two different exon deletions involving exon 2 and exons 3-4, respectively. The phase of the alleles was distinguished by exon dosages assessment in other family members and by haplotype analysis (fig.1). This revealed the possibility that the deceased mother (from whom no clinical data or DNA material was available) carried a homozygous exon 2 deletion.

Frequency of parkin mutations

We found *parkin* mutations in 15 out of 46 index cases (Table 2). In our series, *parkin* mutations are therefore responsible for 33% of EOP. When we look at the form of presentation (familial or isolated cases), mutations were detected in 53% (9 out of 17) of the families with EOP, and in 21% (6 out of 29) of the isolated cases.

Among the 15 patients with *parkin* mutations, eight carried homozygous exon deletions, two were compound heterozygote for two different exon deletions and four were heterozygote for an exon deletion plus a point mutation. Homozygous and heterozygous exon deletions represented 55% (16 out of 29) and 28% (8 out of 29) of the observed mutant alleles, respectively. Exon 3 deletions were found repeatedly in both homozygous and heterozygous states in Italian patients. Deletions encompassing exon 3 to 4 were found in heterozygous state in three families from Cuba, Turkey and Italy. Point mutations represented 17% (5 out of 29 alleles) of all *parkin* mutations; in each case the patient was heterozygote.

Table 1. **Phenotype description of the complete sample set, and according to *parkin* genotype**

Characteristics	Total sample set	n	Patients with *Parkin* mutations	n	Patients without *Parkin* mutations	n
Gender Male (%)	34 (57)	60	11 (48)	23	23 (61)	37
Age at onset, years (range)	33 ± 11 (14-65)	60	28 ± 9 (15-44) *	23	39 ±10 (14-65)	37
Disease duration, years (range)	15 ± 9 (1-36)	59	20 ±9 (6-36) **	22	13 ±8 (1-30)	37
Age at examination, years (range)	49 ± 10 (19-71)	59	49 ±10 (32-70)	22	49 ±10 (19-71)	37
Symptoms and signs at onset						
Bradykinesia (%)	31 (54)	57	10 (48)	21	21 (58)	36
Resting tremor (%)	30 (53)	57	10 (48)	21	20 (56)	36
Asymmetry (%)	46 (79)	58	13 (62) *	21	32 (89)	37
Dystonia (%)	7 (12)	57	3 (14)	21	4 (11)	36
Clinical signs at examination						
Bradykinesia (%)	55 (96)	57	21 (100)	21	34 (94)	36
Resting tremor (%)	37 (66)	56	14 (67)	21	23 (66)	35
Rigidity (%)	52 (91)	57	19 (90)	21	33 (92)	36
UPDRS off (range)	49 ± 21.2 (6-90)	24	41± 20.5 (6-70) ¥	8	53 ± 22 (18-90)	16
UPDRS on (range)	20 ± 11.0 (2-45)	42	20 ± 12.8 (2-43)	14	21 ± 10.1 (2-45)	28
Hoehn&Yahr off (range)	3.3 ± 0.9 (1-5)	30	2.9 ± 0.9 (1-4) ¥¥	10	3.4 ± 0.9 (2-5)	20
Hoehn&Yahr on (range)	1.8 ± 0.7 (0-4)	48	1.9 ± 0.9 (0-4)	17	1.7 ± 0.6 (1-2.5)	31
Treatment						
Treatment with L-dopa (%)	46 (88)	52	17 (81)	21	29 (93)	31
Daily dose of L-dopa in mg (range)	556 ± 304 (100-1250)	44	497 ±337 (150-1250)	15	587 ±288 (100-1200)	29
Duration of treatment in months (range)	123 ± 92 (3-336)	36	139 ±102 (8-290)	13	115 ± 87 (3-336)	23
Other features (%)						
L-dopa induced dyskinesias	34 (79)	45	12 (75)	16	21 (72)	29
L-dopa induced motor fluctuations	33 (73)	43	10 (63)	16	24 (89)	27

* p=0.02, ** p=0.005, ¥ p=0.06, ¥¥ p=0.006 (the last two after adjustment for disease duration)

Genotype-Phenotype correlations

Significant differences between the patients with and without *parkin* mutations were observed for AAO and disease duration (Table 1). The patients carrying *parkin* mutations have an earlier onset (28 ±9 vs. 39 ±10 years, p=0.02) and longer disease duration (20 ±9 vs. 13 ±8 years, p=0.005) than those where no mutations have been identified. When the groups were compared according to the type of mutation (point mutation or exon deletion) the difference came mainly from the group of patients with heterozygous point mutations. In the patients carrying a point mutation (missense or splicing) we observed a mean AAO of 23 ±8 years (n=7) vs. 31 ±9 years (n=16) in the group with two exon deletions and 39 ±10 years (n=37) in the patients without *parkin* mutations (p for trend=0.002). A similar effect was observed for the disease duration; patients with point mutations have the longest disease duration, 22 ±10

years, vs. 19 ±9 and 13 ±8 years for the group with exon deletions or no *parkin* mutations, respectively (*p* for trend=0.002). Although these results are statistically significant, they are based on small numbers and therefore should be interpreted with caution. However, the data suggest an influence of the nature of mutation on the AAO.

Regarding the clinical features, both groups of patients were comparable, except for the asymmetry of signs at onset, which was found in 89% (32 out of 37) of the patients without *parkin* mutations and in only 62% (13 out of 21) of the patients with *parkin* mutations (*p*=0.02).

After adjusting for disease duration, we observed a slower disease progression in the patients with *parkin* mutations according to the UPDRS motor scale (41±20.5 vs. 53±22, *p*=0.057) and Hoehn-Yahr scale measured in "off" status (2.9±0.9 vs. 3.4±0.9, *p*=0.006). Levodopa-induced motor fluctuations were more frequent in the group without *parkin* mutations that also have higher doses of levodopa (587 vs. 497 mg), but these differences were not significant.

Discussion

We have characterized clinically and genetically a series of 46 EOP index cases plus 14 affected relatives, identifying 15 different *parkin* mutations in 15 index cases, including the first Cuban family with EOP due to *parkin* mutations.

From the five identified point mutations, three are novel changes: Arg402Cys, Cys418Arg and the splicing mutation IVS11-3C>G. Recent functional studies suggest that the Cys418Arg mutation is pathogenic because it decreases parkin solubility in cells, and leads to the formation of cytoplasmic aggregates [38]. On the contrary, if the Arg402Cys variant is a rare polymorphism or a pathogenic mutation remains unclear, and further, functional studies are required to clarify this issue.

To our knowledge, only three splicing mutations [12, 14] have been reported in the *parkin* gene. It is known that the efficiency of splicing is dependent not only on the GT and AG dinucleotides present at the 5' and 3' exon/intron junctions respectively, but also by consensus sequences spanning both 5' and 3' sides. For the splicing mutation reported here, five different computer programs that predict splice sites in human genes, consistently showed a predicted abolition of the natural acceptor splicing site and the activation of a cryptic site that competes with the authentic one. As a consequence a 2 bp frameshift in the sequence of exon 12 (coding part of the second RING finger motif of the parkin protein) is introduced.

In the same family (Cuban origin) we also found a heterozygous exon 3-4 deletion in *trans* with the IVS11-3C>G change. The presence of compound heterozygous *parkin* mutations in a consanguineous family illustrate that the absence of homozygous haplotypes is not enough to rule out the involvement of the gene/locus under study, and the possibility of compound heterozygous mutations should be considered also in consanguineous pedigrees.

Up to now, semi-quantitative and quantitative methods have been used for determination of exon dosages in the *parkin* gene. The first is based on the peak heights corresponding to each of the exons amplified in a given reaction, compared to the peak heights of the control gene exon, obtained after assuming the log-linear phase of the multiplex PCR reactions [8].

Quantitative methods, i.e. LightCycler, TaqMan, offer a precise (real-time) measurement of the threshold cycle. All methods used until now employ expensive fluorescent primers or probes in multiplex reactions [8, 10, 15, 21].

Here we described a novel method to assess exon dosages in the *parkin* gene. The method uses an intercalating dye, in this case SYBR® Green I, which function as a fluorescent reporter, and non-labelled primers. The amplification reaction was performed independently for both sets of primers (*parkin* and β-*globin*) using the same master mix and the same starting amount of DNA. The β-*globin* gene has been used by several groups that currently perform exon dosages assessments, and has been proved to be a reliable "control gene" [8, 10].

Our method facilitates a rapid and accurate detection of exon rearrangements using a simple and cost effective technique. However, since this method employs only one fluorescent reporter, multiplex reaction cannot be performed. The advantage of the lower starting costs needs therefore to be balanced toward the throughput of a given study design, and this assay is predicted to be especially convenient for low- or moderate throughput screenings.

Positive controls (i.e. parents and offspring of patients with homozygous deletions) were used in the respective experiments to confirm the results and validate the method. Segregation analysis in available family members allowed the identification of the allele phases and at the same time served as "quality controls". We also confirmed all exon rearrangements compromising only one exon with an independent set of primers, to avoid false positive results due to primer mismatch.

Heterozygous exon rearrangements were detected in 28% of the *parkin* patients in our study. The detected exon rearrangements were all deletions, confirming that they are more frequent than duplications [8, 14]. Without the exon dosage assay, either both or one mutation would have been missed in 11% of the index cases.

In one family (Ge-01) where a homozygous exon 3 deletion was initially found, the gene dosage assay also detected a heterozygous exon 4 deletion, delineating the affected siblings as compound heterozygote (exon 3del/exon 3-4del, Table 2).

In this newly ascertained EOP sample set, we detected *parkin* mutations in 53% of the familial cases, and in 21% of the isolated patients. These frequencies are consistent with previous studies that applied similar inclusion criteria but a different method for exon dosage: 49% for familial and 15% for isolated EOP patients [8, 15]. Our results suggest a contribution of *parkin* to one-third of the EOP cases overall, although referral bias can still be an issue to consider. Other studies have detected a lower frequency of *parkin* mutations (18% of all EOP patients), however, exon dosage assays were not performed [12].

Table 2. **Mutational screening of the *parkin* gene**

Index case (no. of aff, no. of tested siblings)	**Presentation**		**Age at onset**	**Disease duration**	**Parkin Mutation 1**	**Parkin Mutation 2**
Hom exon deletions						
TOR-34 (3, 1)	F		41, 42, 43	12, 14, 18	Exon 2-3 del	Exon 2-3 del
PK-09-01 (2, 2)	F	C	20, 20	17, na	Exon 3 del	Exon 3 del
TOR-18 (3, 2)	F		38, 42, na	14, 28, na	Exon 5 del	Exon 5 del
IVR-1 (3, 1)	F	C	20, 22, 29	23, na, na	Exon 5-6 del	Exon 5-6 del
PG-001 (3, 1)	F		23, 25, 25	36, 50, na	Exon 6 del	Exon 6 del
PAL-1	S	C	18	16	Exon 6-7 del	Exon 6-7 del
ME-03 (2, 2)	F		29, 40	19, 10	Exon 8 del	Exon 8 del
PV-24	S		20	19	Exon 8-9-10 del	Exon 8-9-10 del
Het exon deletions						
Ayd01 (3, 3)	F		34, 40, 44	6, 10, 10	Exon 2 del	Exon 3-4 del
GE-01 (2, 2)	F		31, 30	33, 28	Exon 3 del	Exon 3-4 del
Het exon del / het point mut.						
RM-417	S		16	30	Exon 3 del	1345C>A (Thr415Asn)
VER-1	S		15	21	Exon 3 del	1353T>C (Cys418Arg)
Cu03 (3, 3)	F	C	17, 23, 30	30, 16, 6	Exon 3-4 del	IVS11-3C>G (Splicing)
MI-006-01	S		28	34	Exon 6 del	226G>C (Arg42Pro)
Het point mutation						
RV-3	S		35	18	-	1305C>T (Arg402Cys)

Aff=affected F=Familial form S=Sporadic C=Consanguinity del=deletion Het=heterozygous Hom=homozygous na=not available

Phenotype-genotype correlations

Patients with *parkin* disease showed a significantly earlier onset, longer disease duration, slower disease progression and more frequent symmetric onset than patients without *parkin* mutations, confirming previous findings [8, 20, 39]. Among the isolated patients with EOP, *parkin* mutations were found in 67% of the patients with a disease onset ≤ 20 years old, in 14% and 6% of the patients with AAO between 21-30 years and ≥ 31 years, respectively, showing that the earlier the AAO, the higher the probability of finding *parkin* mutations.

Recently, a more severe disease status was found in carriers of one missense mutation compared to carriers of two truncating mutations [20]. No relationship with AAO was observed. However, we observed a significantly lower AAO in patients with point mutations (missense and splicing) compared to patients with exon deletions or those without *parkin* mutations, suggesting a relationship between the nature of the mutation (point mutation vs. exon deletion) and the AAO.

It has also been reported that missense mutations within the functional domains of the parkin protein might lead to earlier AAO [20]. It is difficult to disentangle the effect of the localization from those of the nature of the mutation. For example, missense mutations located within the RING1 finger domain of parkin might lead to an abnormal distribution of the protein within large cytoplasmic and nuclear inclusions in cellular models [40].

On the other hand, exon deletions can lead to in-frame or frameshift mutations. The stability of the shorter proteins or protein carrying missense changes is difficult to predict.

When the patients carrying at least one in-frame deletion (exon 2-3, 3-4, and 5) were removed from the analysis, the difference in AAO between the groups with point mutation (n=7) or exon deletions (not in frame, n=8) disappeared. However, due to the small number of patients it is difficult to establish conclusions. Exploring this potential relationship in larger and independent sample sets will shed more light on the topic.

Previous studies have suggested that a single *parkin* mutation might sometimes cause EOP or represent a risk factor for late-onset PD [11, 12, 14, 20, 21, 39]. However, whether a single *parkin* mutation is sufficient to cause EOP or whether the available methods are unable to detect a second *parkin* mutation, is not clear.

Final considerations

The analysis of this newly ascertained series of cases confirms that *parkin* mutations are a relevant cause of EOP. An early age at onset and a slower disease course differentiate the group of patients with *parkin* mutations from the rest of the EOP cases. Among the *parkin* mutations, heterozygous exon rearrangements represent 28% of the mutations, confirming the importance of exon dosage analysis when studying the *parkin* gene. We implemented a novel method to investigate gene dosage, which can be applied to the study of other genes where a dosage effect matters, including *alpha-synuclein*/PARK1 and *DJ-1*/PARK7.

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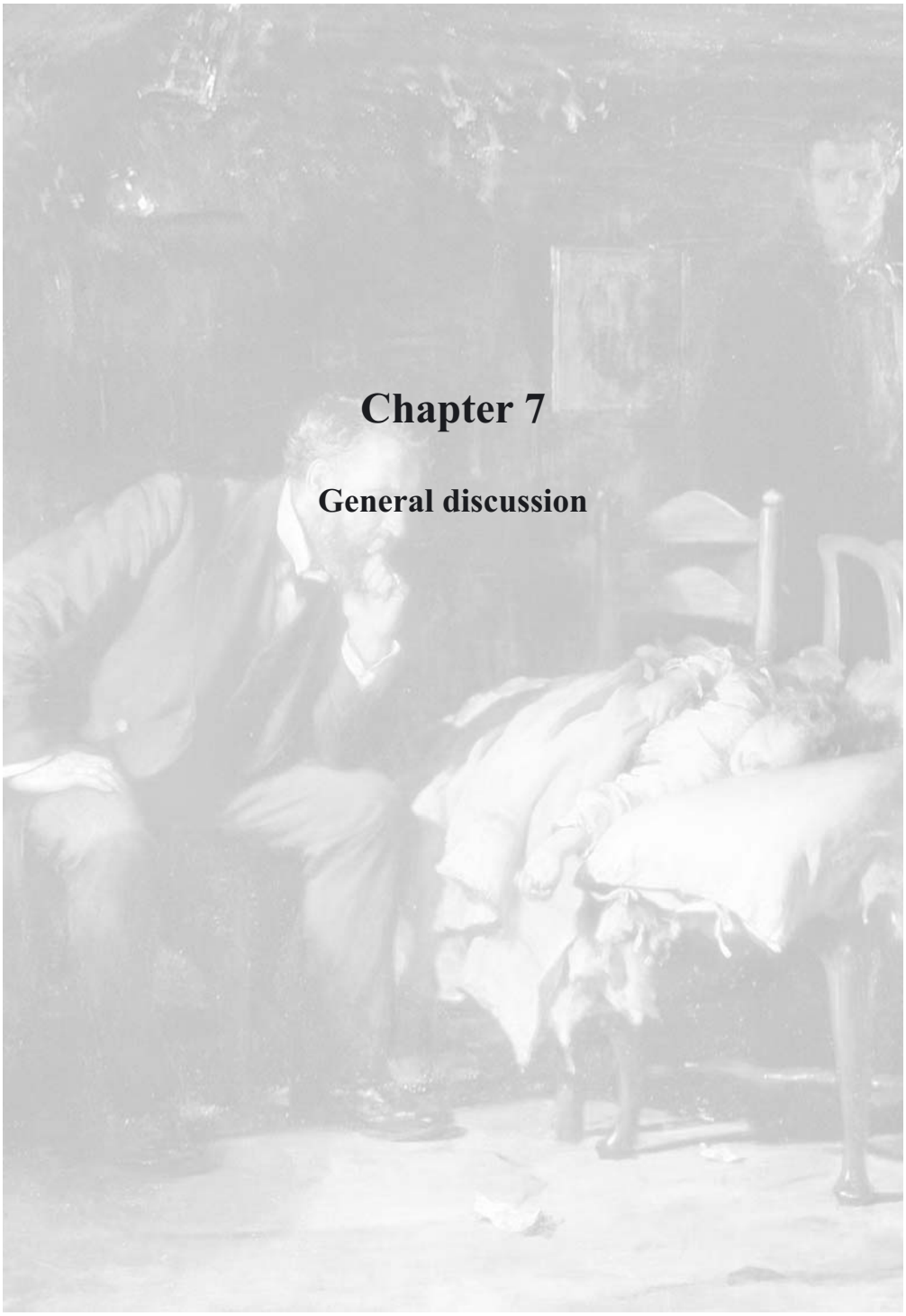

Chapter 7

General discussion

General discussion

The modern era of genetics started 135 years ago when Gregor Mendel described the "limpid" segregation of characters or traits trough generations based on his observations and experiments on the garden pea. Today, five "Mendelian" patterns of inheritance are recognized in humans, when transmission of the defective gene (although mutations not always localize in a gene) is followed in a particular pedigree.

Resembling Mendel whose success describing the modes of gene segregation was based on the appropriate selection of the trait to study [1], the success of gene mapping in human diseases has been determined by the genetic component underlying the targeted phenotype. In that way, genes involved in disorders following a recognizable (Mendelian) pattern of inheritance has been firstly identified, generating a wave of gene discoveries in the past 20 years.

However, finding and characterizing families with a sufficient number of available patients distributed through several generations implies a great effort. Moreover, in the case of late-onset neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's disease (AD), the scenario is more complicated: patients from previous generations are usually deceased, there is age-related penetrance and an enormous phenotypic complexity. Here the first difficulties emerge, large families powerful enough for linkage studies are difficult to obtain.

In *Chapter 2* and *4* of this thesis, we presented genetic linkage studies in two large families of Cuban origin with AD and PD, respectively. In both cases, extensive clinical investigations were performed. Both families exhibited a clinical picture compatible with the common forms of these disorders; additionally, the segregation of the disease in the families suggested an autosomal dominant pattern of inheritance with high penetrance.

Furthermore, the early onset of dementia in the AD family (59 years, 48-76) suggested the involvement of one of the known AD genes, especially *PSEN1* or *APP.* Clinical investigation showed progressive deterioration of cognitive functions, and evidence of a vascular component in the phenotype, some patients presented transient ischemic cerebral attacks, motor deficits, presence of hypodense regions at brain CT scan examinations, which is indicative of vascular lesions, or cerebral amyloid angiopathy at the neuropathological examination. Haplotype and linkage results (LOD=3.79) were compatible with *PSEN1* involvement (chromosome 14q24.3). Indeed, direct sequence analysis revealed a novel missense mutation (a single base substitution that results in an aminoacid change) located within exon 6 of the *PSEN1* gene. The mutation co-segregated with the identified haplotype and was not found in ethnically matched controls. One patient of the family presented late-onset AD (76 years of age) and did not carry the identified disease-associated haplotype suggesting that this patient had dementia due to other causes.

The second study was performed in a family where several members presented PD, with a mean age at onset of 61 years (45-76). The clinical study revealed the presence of not only typical PD (10 patients, from whom 6 were alive) but also essential tremor and atypical parkinsonism in 3 and 2 patients, respectively. Two more patients presented signs compatible with a prodromal clinical phase (possible PD). Evidence suggesting the presence of a novel PD locus on chromosome 19 was found. In the 19p13.3-q12 region, a common haplotype was identified in 6 out of 7 PD patients.

In addition, the patients with atypical parkinsonism or essential tremor were not sharing the same haplotype.

During both studies we encountered problems such as complex phenotypes, wide variation in the age at onset, coexistence of phenocopies and difficulties related to age dependant penetrance. How do we solve such complications?

The clinical diagnosis

For the clinical diagnosis a careful approach is recommended. Close collaboration between clinicians and researchers is essential, as well as the cooperation between different clinical specialties, that will enable a better assessment of the phenotype of the patients. Clinical follow-up with repeated evaluations of the patients and at risk family members over a longer period of time is often the only method to establish a definite diagnosis. To this end, the application of modern neuroimaging technologies provides data complementary to the clinical evaluation and allows temporal assessment of neuronal changes. Recent advances in functional imaging using single photon emission computerized tomography (SPECT) and positron emission tomography (PET) as well as structural imaging using magnetic resonance imaging (MRI) have provided useful clinical-research tools [2]. The recent design of radiopharmaceuticals that target the dopamine system in PD [3-5] or amyloid in AD [6, 7], has allowed preclinical detection of the diseases as well as monitoring the disease progression. One disadvantage is that they are not available in all countries and the costs are usually very high, limiting their general application.

For a clinician the ultimate task is to establish the patient's diagnosis with the consequent etiological search and corresponding treatment. However, from the genetic perspective the diagnosis has a different meaning that goes beyond the patient extending to the family. For a geneticist (who is hunting for genes) a "question mark" on the pedigree drawing is always better than an erroneous diagnosis. The consequences of having one patient less in the analysis are far preferable than the consequences of a misdiagnosis, which can ruin the complete research.

A conservative approach that consider as affected only the individuals that beyond doubt fulfil the criteria established for the diagnosis is therefore recommended. Individuals with unclear or incomplete phenotypes must be considered as "status unknown". In this way one should start the analysis with the narrowest phenotype to ensure the finding of the true genomic location and subsequently proceed to expand the boundaries of the disease definition, once a genomic region has been identified.

As example, we can refer to the finding of essential tremor in families with PD, which is not an infrequent scenario. Essential tremor (ET) is a nosographic entity characterized by postural or action tremor that frequently behaves as an autosomal dominant trait. An increased prevalence of ET among first-degree relatives of PD patients has been reported [8, 9], supporting a relationship between PD and ET. In a large pedigree (the Iowa kindred) with parkinsonism, several family members suffered also from essential tremor. Initially, suggestive linkage to chromosome 4p15 (PARK4) was reported, and a common haplotype in PD and ET patients was described [10]. The cause of parkinsonism in this family was recently identified, a triplication of the α-*synuclein* gene (PARK1, 4q21) with consequent protein over-expression [11]. It turned out that the essential tremor phenotype is due to a different aetiology.

In the Cuban family described in *Chapter 3,* the chromosome 19 haplotype was only

segregating with the PD phenotype. The coexistence of related phenotypes in a single family is intriguing and is difficult to explain on the bases of a monogenic model of inheritance. After many years of extensive gene search, only one PD gene (and 3 mutations) acting in an autosomal dominant manner has been found (PARK1/α-*synuclein*) [12, 13]. One might wonder whether a monogenic model fits the familial PD cases with suggestive autosomal dominant inheritance or whether a digenic or trigenic inheritance would explain better the phenotypic variability observed in most of the PD families.

The recent identification of a novel PD locus, PARK8, in a large Japanese family [14], and further independent confirmation in two families (German-Canadian and western Nebraska) with large neuropathological variability[15], and eventually the identification of the PARK8 gene may help to clarify the issue.

The study design

Family sampling should be preceded by evaluation of the pedigree and simulation studies performed to assess the statistical power of the family under study. In this way, the contribution of different patients (depending on the position they have in the pedigree structure) can be assessed. Threshold values can be then defined according the specific family structure and the number of available patients. In that way, when a LOD score of 2 is obtained in a family where only a small percentage of the replicates exceed such a value, then the result is probably corresponding to true linkage even when the value is not strictly significant.

When large number of families are available, segregation studies may allow us to explore the best fitting genetic model helping to its specification. In the case of a single extended pedigree the situation is more complicated and designing a genetic model is often based on the single family characteristics and the existing published data (gene frequencies, age dependant penetrances, phenocopies). Simulation calculations will also help investigating the loss of statistical power due to the specification of a wrong model [16].

Finding genes involved in complex disorders has shown to be complicated and difficult to accomplish. Several approaches have been applied in AD and PD: (1) linkage-based studies that mostly involve a collection of small families with two or more patients, affected sib-pairs (ASP) and affected relative pairs (ARP) or multiplex families; (2) association-based studies that attempt a candidate gene approach in samples of patients and controls. The appropriate selection of both the choice of sample to collect (extended pedigrees, ASP, ARP) and the method of analysis is also a crucial step. In addition, the number of subjects included in the study and the origin of those individuals (population isolate, admixed population, ethnic group) are essential factors.

Sibling pairs are expected to share 0, 1 or 2 parental haplotypes with a frequency of ¼, ½ and ¼, respectively. When ASPs are genotyped, it is expected to identify regions where the sharing of alleles exceeds the random probabilities. ARPs are expected to share less DNA fragments, according to the position they occupy in the pedigree structure. However, a large number of patient pairs is required to find the disease loci. The existence of genetic heterogeneity and the observed clinical variability complicate the task of obtaining a homogeneous sample set. Studies aimed to find genetic factors involved in AD [17-20] and PD [21-23] have identified candidate genomic regions, some of them showing overlap among studies. However, methodological problems as multiple testing and poor study design are challenging the identification of the already evasive genes *(Chapter 1).*

Inaccuracy in genotyping can also generate false results in linkage studies. These types of errors can lower or inflate the LOD scores, mostly introducing false recombinants. Extreme care and precision is needed when genotypes are determined. The use of adequate controls (samples with known genotypes such as the CEPH controls) and negative controls, in addition to the verification of the segregation of alleles through the family, are strongly recommended. They allow the detection of incompatibilities and also of rare double recombinants or infrequent haplotypes; programs such as PEDCHECK and MERLIN are useful tools, especially when a large group of families or extended complex pedigrees are assessed.

It is not a rare practice in linkage studies to assume equal allele frequencies for the marker loci. The explanation is that true allele frequencies are often not available, either because of poor characterization of the marker or absence of assessment in a specific population. However, assuming equal allele frequencies for the marker locus can lead to false positive evidence of linkage [24]. In the case of large families it is sometimes possible to estimate the allele frequencies based on founder individuals. For smaller pedigrees a solution would be to test a group of unrelated controls originating form the same population. If actual allele frequencies are difficult to obtain for all markers used in a genome scan, estimating those frequencies for the "positive regions" may help.

Genetic heterogeneity, variability of the clinical phenotype and the lack of reliable disease markers are affecting the success of gene mapping in complex disorders. Strategies such as stratification of the sample set based on covariates such as age at onset, exclusion by linkage and sequencing analyses of known genes, and study of more homogeneous populations have been applied with promising results [25-27].

In recently founded population isolates, extensive linkage disequilibrium might be expected. Using the last approach, we performed a genome-wide search in a genetic isolated population in The Netherlands (*Chapter 5*). Forty-four apparently unrelated patients displayed late-onset PD resembling the "sporadic" form of the disease; genealogical research linked 40 of them to a common ancestor 16 generations ago.

Several STR markers were identified for which one allele showed higher frequency in PD cases compared to controls originating from the same population ($p<0.01$). Some of these markers (on chromosomes 9q, 5q, 1p) overlap with regions detected during previous genome screenings [22, 25, 28], suggesting that some of them may indeed bear genes influencing the susceptibility to PD. Specifically for the chromosome 9q region, a common haplotype was observed in 23% of the patients and only 4% of the controls. In addition, two markers were located close to the α-*synuclein* and *tau* gene. For each gene "risk haplotypes" has been previously reported as associated with PD [29-31]. It will be interesting to elucidate whether these risk haplotypes are also present in these PD patients and whether they have a joined or independent effect. Fine mapping of the identified regions will enable further haplotype analyses and eventually the identification of a genomic region(s) commonly inherited from an ancestor bearing a PD susceptibility factor.

Genetic testing: *PARK2* and *PSEN1*

We carried out a multi-centre based study of early-onset PD and developed a novel method for gene doses quantification for *parkin* (PARK2). A multiethnic group of 46 index patients with early-onset PD were clinically and genetically characterized. Fifteen different mutations were identified, four of which are described for fist time.

The study confirmed the worldwide distribution of *parkin* mutations, its high frequency among early-onset cases (33%) and the importance of exon dosage assessments that represent 28% of the mutations found. We also assessed phenotype-genotype relationships and confirmed the earlier age at onset, symmetric onset, slower disease progression and longer disease duration that characterize the "parkin disease".

Our results (*Chapter* 6) confirm previous reports on *parkin* mutation frequencies in early-onset cases with sporadic (15 to 18%; 21%, this thesis), or familial compatible with autosomal recessive inheritance (49%; 53% this thesis) [32, 33]. Due to its high frequency especially among early-onset cases, testing for *parkin* mutations can be recommended for the diagnosis workup of the early-onset cases and for prognosis evaluation.

An overview of the reported *parkin* sequence alterations (n=71) is presented in Table 1. Yet the pathogenicity of several of these changes has not been investigated, making difficult their interpretation, especially concerning the penetrance of the mutations.

Although the contribution of *parkin* mutations to PD (overall) seems to be rather small (0.4 to 0.7%) [34], only a few studies have conducted sequencing and exon dosage assays in late-onset PD cases [35] or in population-based series of patients. In a recent study, *parkin* mutations (mainly in heterozygous state), were found in patients with late-onset familial PD suggesting that *parkin* could act as a susceptibility factor for late-onset PD [35].

The contribution of other "early-onset genes" seems to be smaller than *parkin* [36]: initial estimates of around 1% for PARK7 (*DJ-1*) [36, 37] and 15% for PARK6 [34] have been reported. Since the last is based on linkage reports, the contribution of PARK6 may be actually smaller. While genetic testing of PARK7 and PARK6 for research purposes is certainly fruitful, their application to the diagnosis workup of PD is expected to be limited.

Table 1. **Sequence variations reported in the *parkin* (PARK2) gene. Nucleotides are numbered according to GeneBank accession number AB009973 (ATG is at position 102)**

Exon	DNA change/ position	Type of mutation	AA change	Frequency in controls	References
1	102A>T	Missense/Frameshift	Met1Leu	0%	Rawal et al. 2003 [38]
2	144A>G	Missense	Val15Met	0%	Munoz et al. 2002 [39]
2	198C>T	Nonsense	Arg33Stop		Maruyama et al. 2000 [40]
2	199G>A	Missense	Arg33Gln	0%	Oliveira et al. 2003 [35]
2	202 del A	Frameshift/Nonsense	Gln34Stop43		Nisipeanu et al. 2001 [41]
2	202-203 delAG	Frameshift/Nonsense	Gln34Stop37	0%	Abbas et al. 1999 [42]
2	211C>T	Missense	Pro37Leu		Kann et al. 2002 [43]
2	226G>C	Missense	Arg42Pro	0%	Terreni et al. 2001 [44]
2	255 del A	Missense/Nonsense	Asn52Stop81	0%	Abbas et al. 1999 [42]
2	237G>C	Missense	Ala46Pro	0%	Xu, Liu et al. 2002 [45]
2	268T>A	Missense	Val56Glu	0%	Hoenicka et al. 2002 [46]
3	321-322 insGT	Missense/Nonsense	Trp74Stop81	0%	Abbas et al. 1999 [42]
3	336G>T	Nonsense	Glu79Stop		Foroud et al. 2003 [47]
3	346C>A	Missense	Ala82Glu		Hedrich et al. 2001 [48]
3	401G>C	Polymorphism	Gln100His	45%	Chen et al. 2003 [49]
3	438-477 del(-40bp)	Frameshift/Nonsense	Leu112Stop	0%	Farrer et al. 2001 [50]
3	474C>T	Polymorphism	His124His	50%	Chen et al. 2003 [49]
4	584A>T	Missense	Lys161Asn	0%	Abbas et al. 1999 [42]
4	601G>A	Polymorphism	Ser167Asn	1%	Hattori et al. 1998 [51]
4	620G>A	Silent	Thr173Thr		Bertoli et al. (this thesis)
5	636delG	Frameshift/Nonsense	Gln178/Stop187		Hattori et al. 1998 [51]
5	675A>G	Missense	Met192Val		Foroud et al. 2003 [47]
6	734A>T	Missense	Lys211Asn	0%	Lucking et al. 2000 [32]
6	733A>G	Missense	Lys211Arg		Periquet et al. 2001 [52]
6	736G>A	Missense	Cys212Tyr	0%	Pineda et al. 2001 [53]
6	820C>T	Missense	Thr240Met	0%	Foroud et al. 2003 [47]
6	820C>G	Missense	Thr240Arg		Hattori et al. 1998 [54]
7	859G>A	Missense	Cys253Tyr	0%	Oliveira et al. 2003 [35]
7	867C>T	Missense	Arg256Cys	0%	Abbas et al. 1999 [42]
7	884G>A	Silent	Leu261Leu		Oliveira et al. 2003 [35]
7	905T>A	Nonsense	Cys268Stop		Lucking et al. 2000 [32]
7	914A>T	Polymorphism	Arg271Ser	14%	Chen et al. 2003 [49]
7	924C>T	Missense	Arg275Trp	0%	Abbas et al. 1999 [42]
7	939G>A	Missense	Asp280Asn	0%	Lucking et al. 2000 [32]
7	951G>C	Missense	Gly284Arg		Wang et al. 2003 [55]
7	966T>G	Missense	Cys289Gly		Lucking et al. 2000 [32]
7	972delG	Frameshift/Nonsense	Ala291/Stop297		Munoz et al. 2000 [56]

Table 1 continued

Exon	DNA change/ position	Type of mutation	AA change	Frequency in controls	References
8	1032C>T	Nonsense	Gln311Stop		Hattori et al. 1998
9	1072 del T	Frameshift/stop	Val324/Stop434		Klein et al. 2000[57]
9	1084G>A	Missense	Gly328Glu		Lucking et al. 2000 [32]
9	1101C>T	Missense	Arg334Cys		Lucking et al. 2000 [32]
9	1116G>T	Polymorphism	Ala339Ser	29%	Chen et al. 2003 [49]
9	1142-1143 delGA	Frameshift/Nonsense	Gln347/Stop368		Lucking et al. 2000 [32]
9	1147-1148 delAA	Frameshift	Lys349/Stop368	0%	Hedrich et al. 2001 [48]
9	1152A>C	Missense	Thr351Pro		Kann et al. 2002 [43]
10	1197C>T	Polymorphism	Arg366Trp	4%	Wang et al. 1999 [58]
10	1239G>C	Polymorphism	Val380Leu	16%	Abbas et al. 1999 [42]
11	1276-1277 delGA	Frameshift/stop	Arg392/Stop394	0%	Alvarez et al. 2001 [59]
11	1281C>A	Polymorphism	Asp394Asn	7%	Abbas et al. 1999 [42]
11	1293G>A	Missense	Ala398Thr	0%	Periquet et al. 2003 [33]
11	1305C>T	Missense	Arg402Cys	0.2%	Bertoli et al. (this thesis)
11	1326G>T	Nonsense	Glu409Stop		West et al. 2002 [60]
11	1345C>A	Missense	Thr415Asn	0%	Abbas et al. 1999 [42]
11	1353T>C	Missense	Cys418Arg		Bertoli et al. (this thesis)
11	1385insA	Frameshift/Nonsense	Asn428/Stop568	0%	Rawal et al. 2003 [38]
12	1390G>A	Missense	Gly430Asp		Lucking et al. 2000 [32]
12	1393G>T	Missense	Cys431Phe	0%	Maruyama et al. 2000 [40]
12	1411C>T	Missense	Pro437Leu	0%	Nichols et al. 2002 [61]
12	1422T>C	Missense	Cys441Arg		West et al. 2002 [60]
12	1436G>A	Nonsense	Trp445Stop	0%	Rawal et al. 2003 [38]
12	1459G>A	Nonsense	Trp453Stop	0%	Abbas et al. 1999 [42]
IVS2	IVS2+25 T>C	Polymorphism	-	19%	Abbas et al. 1999 [42]
IVS3	IVS3-20 C>T	Polymorphism	-	10%	Abbas et al. 1999 [42]
IVS3	IVS3-21 T>C	Polymorphism	-		Oliveira et al. 2003 [35]
IVS5	IVS5+2T>A	Splicing	Splicing/Stop		West et al. 2002 [60]
IVS7	IVS7-1G>C	Splicing	Splicing/Frameshift	0%	West et al. 2002 [60]
IVS7	IVS7-35 A>G	Polymorphism	-	27%	Abbas et al. 1999 [42]
IVS8	IVS8 +35A>G	Polymorphism	-		Oliveira et al. 2003 [35]
IVS8	IVS8 -21 to -17del	?	?		Lucking et a. 2000 [32]
IVS9	IVS9 +4G>T	Splicing	Splicing	0%	Oliveira et al. 2003 [35]
IVS11	IVS11-3C>G	Splicing	Splicing/Frameshift	0%	Bertoli et al. (this thesis)

From the perspective of the families the situation might be different. In *Chapter* 3, we investigated the genetic knowledge and attitudes towards the presymptomatic diagnosis of presenile AD in 56 relatives of AD patients from a large Cuban family, in which a novel *PSEN1* mutation was identified afterwards *(Chapter 2)*. All participants would use presymptomatic testing to know their own risk of developing the disease, a controversial issue for diseases where no prevention or effective treatments are available, being their main reason to be prepared for the disease. The study showed a clear need for genetic counselling for those individuals at high risk of developing the disease.

The frequency of *PSEN1* mutations in presenile AD has been estimated in 5 to 8%, but it rises to 18% in the cases with a positive family history (suggestive of autosomal dominant inheritance) [62-64]. Thus, in familial cases investigating for *PSEN1* mutations could be helpful for diagnostic considerations. A difficulty here is that most of the 133 reported mutations (AD mutation database, http://molgen-www.uia.ac.be/admutations/) are restricted to single families ("private mutations"). When two or more patients are available, haplotype analysis can help in the decision whether to proceed or not with further sequence analysis.

Concerning other known AD genes, mutations in *APP* (n=16) and *PSEN2* (n=9) are very rare, only a limited number of families have been described. However, testing and exclusion of the known genes is useful for further genetic research. Remarkably, the majority of the AD families do not have mutations in any of the known genes indicating the involvement of yet unidentified genes.

Where to move?

Early disease detection-diagnosis confirmation: Biomarkers

A major goal for clinical research is to improve the early detection of neurodegenerative disorders by developing tools to move diagnosis in the neurodegeneration temporal course [2]. For genetic research, early disease detection will provide means to corroborate clinical diagnosis, occasionally before this is actually made, helping researchers in obtaining a large and homogeneous group of patients.

Difficulties concerning the clinical diagnosis are a well-known problem not only in clinical practice but for genetic research as well, because clinical phenotypes frequently overlap, i.e. clinical distinction is often difficult to achieve between AD, Lewy body disease, frontotemporal dementia, vascular dementia, and others. The lack of adequate tools or reliable biomarkers that facilitate precise disease recognition is largely obstructing the identification of genes, whose variants predispose to neurodegenerative disorders. Functional neuroimaging is one of the emerging strategies orientated to obtain a better disease evaluation. They might offer further phenotype delineation and enable the confirmation of the clinical observations, providing additional power for genetic mapping.

Quantitative outcomes of disease biomarkers could also be used for quantitative trait locus mapping (QTL), a promising approach that is beginning to give insights about complex phenotypes. Due to the high number of susceptibility loci involved in determining complex disorders, looking at intermediate endpoint phenotypes might help the identification of some of the genes and pathways [65]. Finally, QTL mapping in animal models may predict gene localization in the human genome [66].

Accurate phenotype definition seems more than ever to be a necessary prerequisite to establish reliable genotype-phenotype relationships in the study of complex disorders [67].

Genomic expression profiles

Genomics, a recently introduced term, is the study not just of genes but also of their functions and interactions [68]. Gene expression profiling measures the expression levels of many genes at once [69]. The combination of gene mapping methods with gene expression assays might assist the "gene discovery" in complex disorders. Gene expression data can point towards specific positional candidates identified by linkage and association analysis, facilitating the identification of the causative genes variants.

Once mutations in a gene are identified as causing a certain disease, investigating the genes or proteins involved in the same pathway might identify novel functional candidates. Looking back to the genes/proteins already identified for AD, an earlier recognition of the APP processing, would probably have led to the easier identification of the *presenilins*.

Grouping related genes into functional categories increases the explanatory and statistical power of gene expression. Furthermore, identifying analogies among biological processes in diverse organisms by comparative analysis of gene expression patterns [70] can be used to recognize genes that are important in processes such as aging. Genes whose functions are conserved among orthologous might be targeted as candidates related to the phenotype under study.

Final remarks

The identification of genes involved in the monogenic forms of PD and AD has orientated the research on the pathological pathways. Because of the identified genes are mainly responsible for the rare monogenic forms, genetic testing is rarely used as disease predictor, although it can be useful as a diagnostic tool and to evaluate prognosis.

The genes involved in the common forms of the diseases are not yet recognized: a poorly understood interplay of genes with minor effects and the presence of genetic heterogeneity seem to be the main difficulties. The causal genes identified until now represent the peak of a mountain seen from the distance, as we get closer we start to realize its immense magnitude.

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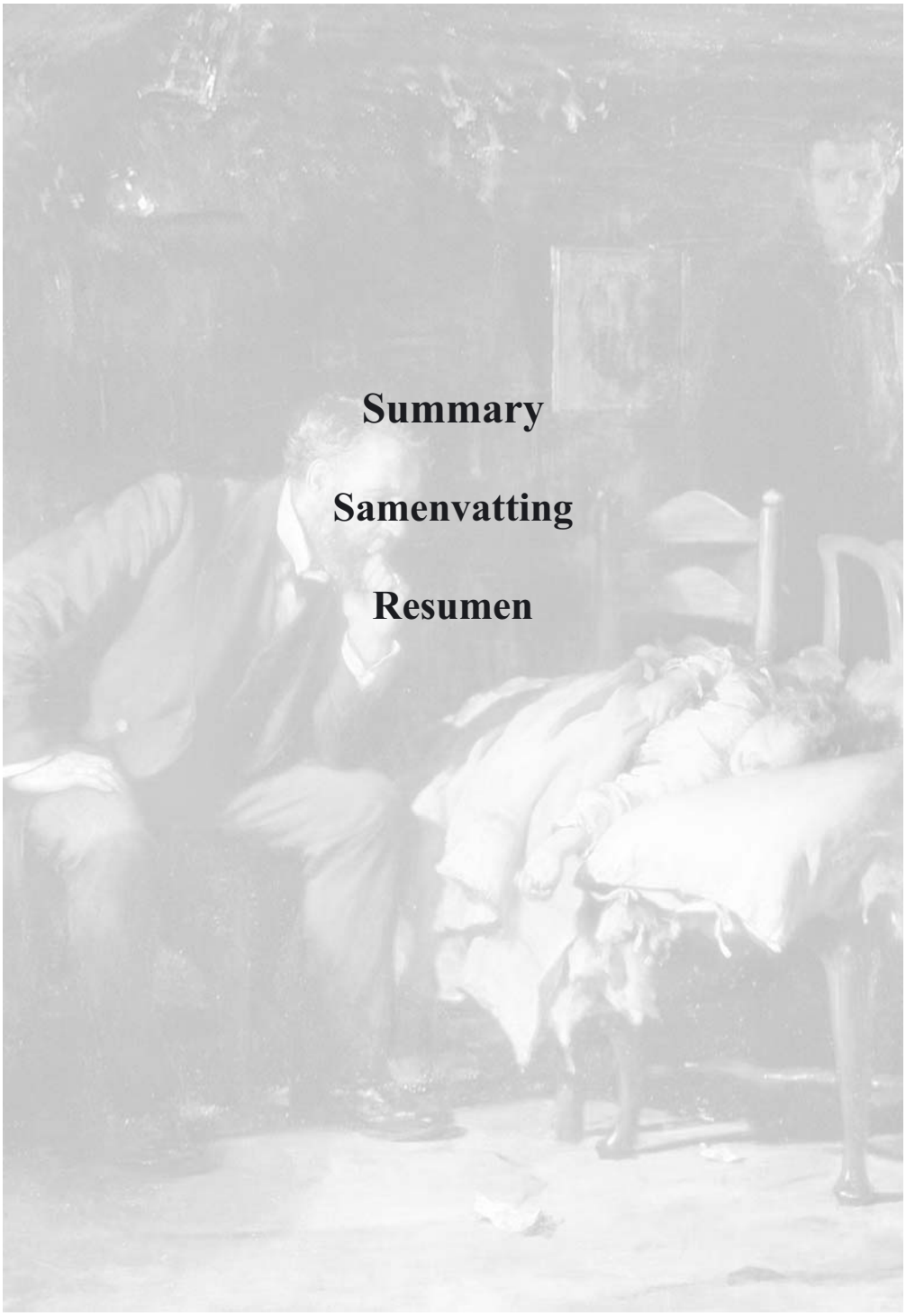

Summary

Samenvatting

Resumen

Summary

In *Chapter 1,* the genetic aspects of Alzheimer's and Parkinson's disease are reviewed. The efforts and progress made in the mapping of genes involved in the genetic forms of Alzheimer's and Parkinson's disease and the ways in which "gene hunting" strategies have evolved are described. The significance of the identification of genes such as the *presenilins* (*PSEN1* and *2*), α-*synuclein (PARK1), parkin* (*PARK2*) and *DJ-1 (PARK7)* are discussed. In addition, possible links between these two neurodegenerative disorders are reviewed. The clinical, pathological and genetic presentation of Alzheimer's and Parkinson's disease suggests the involvement of limited number of overlapping, interrelated pathways. Their features point to a spectrum of neurodegeneration (tauopathies, synucleinopathies, amyloidopathies) that will need further intense investigation to find the missing links.

The first part of the experimental work described in this thesis concerns family studies on Alzheimer's disease. In *Chapter 2,* the study of a Cuban family with autosomal dominant presenile dementia consisting of 281 members within six generations is described. Neuropathological studies confirmed the clinical diagnosis of Alzheimer's disease. Moreover, a significant LOD score was obtained for marker D14S43, located in a 9 cM interval in which all patients shared the same haplotype. Sequencing of the *PSEN1* gene revealed a novel heterozygous base substitution (C520A) that is predicted to cause an amino acid change from Leucine to Methionine in the transmembrane domain III of the presenilin 1 protein. The C520A change was not found in ethnically matched controls. The Leucine at position 174 of the presenilin 1 protein is highly conserved among different species and homologous proteins suggesting that it is important for the normal protein function. This is the first genetic study on Alzheimer's disease that identified the involvement of the *PSEN1* gene in Cuba.

Yet genetic testing in Alzheimer's disease remains controversial. In *Chapter 3,* the clinical and genetic knowledge about Alzheimer's disease and attitudes toward the possible use of presymptomatic genetic testing in the previously described family are explored. Fifty-six first-degree relatives of familial cases with Alzheimer's disease were interviewed. The individuals had only limited knowledge about their personal risk of developing the disorder. All 56 family members would use presymptomatic testing to know their own risk of Alzheimer's disease. Confronted with a hypothetical reproductive choice, 50% would choose not to have children if they themselves had the mutation. The decision to undergo genetic testing for diseases such as early-onset Alzheimer's disease, where no cure or adequate treatment is available, is very complex. The present study suggests a need for education and genetic counselling of family members at high risk of the disease.

The second part of the thesis is focused on Parkinson's disease. In *Chapter 4,* a large Cuban family with typical, late-onset Parkinson's disease and autosomal dominant inheritance is studied. The mean age at onset was 61.2 years (±12.53, 45-76). Other phenotypes such as essential tremor and atypical parkinsonism were observed in the family. We performed a genome-wide scan and linkage analyses. Multipoint analyses yielded a maximum LOD of 2.26 between markers D19S221 and D19S840. Furthermore, haplotype analysis showed a region on chromosome 19 shared by six out of seven patients with Parkinson's disease. The essential tremor phenotype and the atypical parkinsonism do not segregate with this haplotype, suggesting a different aetiology. These findings suggest the presence of a novel locus for Parkinson's disease on chromosome 19p13.3-q12.

Chapter 5 focused on a population-based approach. The study of genetically isolated populations, which are thought to be more genetically homogeneous than the general population, has proven to be a successful strategy for disease gene mapping, including the monogenic variants of complex disorders such as Parkinson's disease. In order to identify genomic regions containing genes that influence the susceptibility to the disease, 44 patients from an isolated community in the Netherlands with the diagnosis of idiopathic Parkinson's disease were studied. Forty patients could be connected to a common ancestor.

We performed a genome wide scan and single-marker association analysis. Four markers showed evidence of association ($p \leq 0.01$): D2S2333, D4S405, D9S158, D13S153. Furthermore, for D9S158 (p=0.006) a neighbouring marker, D9S290, also showed evidence of association. A common haplotype was observed in this 9q region for 10 patients (23%), and only one control individual (4%). This region overlaps with a previously identified interval that showed evidence of linkage with Parkinson's disease. In addition, marker D2S2333 (p=0.009) maps to the same location of a PD locus, PARK3, implicated in disease susceptibility and age at onset. The results of the study of this genetic isolate are consistent with previous studies suggesting the presence of genes contributing to Parkinson's disease susceptibility on chromosomes 2p and 9q. Further investigation of the identified regions will allow the confirmation and refinement of these candidate PD loci.

In *Chapter 6,* a large, multiethnic series of patients with early-onset Parkinson's disease were studied in order to assess the frequency and nature of *parkin* gene mutations, and to investigate phenotype-genotype relationships. Forty-six probands with early-onset Parkinson's disease and 14 affected relatives were ascertained from Italy, Brazil, Cuba, and Turkey. The genetic screening included direct sequencing and exon dosage analyses using a new real-time PCR method. Mutations were found in 33% of the index patients overall, and in 53% of those with family history compatible with autosomal recessive inheritance. Fifteen *parkin* alterations were identified, including 10 exon deletions and five point mutations. Four of the mutations are novel: Arg402Cys, Cys418Arg, IVS11-3C>G, and exon 8-9-10 deletion. Heterozygous genomic rearrangements represented 28% of the mutations found, confirming the importance of exon dosage analyses when screening *parkin*. The patients with *parkin* mutations showed significantly earlier onset, longer disease duration, slower disease progression and more frequent symmetric onset than the patients without mutations. This study confirms the frequent involvement of *parkin* and indicates the importance of genetic testing in the diagnostic work up of the early-onset forms of Parkinson's disease. The novel, cost-effective exon dosage method developed here can also be applied to the study of other genes.

Finally, in *Chapter 7,* the different methodologies and associated pitfalls that are encountered in the search for genes involved in neurodegenerative disorders such as Alzheimer's and Parkinson's disease are discussed.

Samenvatting

In *Hoofdstuk 1* (Introduction) wordt een overzicht gegeven van de genetische aspecten van de ziekte van Alzheimer en de ziekte van Parkinson. We beschrijven de pogingen tot en de vorderingen van het in kaart brengen van genen die betrokken zijn bij de ziekte van Alzheimer en de ziekte van Parkinson. In dit overzicht beschrijven we op welke manier "de jacht op genen" zich gedurende de laatste jaren heeft ontwikkeld en de relevantie van de genen die gevonden zijn, zoals *presenilins, α-synuclein, parkin* en *DJ-1*. Bovendien bespreken we hier ook de mogelijke relatie tussen de twee neurodegeneratieve aandoeningen. De klinische, pathologische en genetische presentatie van de ziekte van Alzheimer en de ziekte van Parkinson suggereert mede de aanwezigheid van een aantal elkaar overlappende en gerelateerde oorzaken. De kenmerken van deze ziekten wijzen op een spectrum van neurodegeneratieve aandoeningen (tauopathieën, synucleinopathieën, amyloidopathieën), waarvoor verder intensief onderzoek noodzakelijk is om gemeenschappelijke oorzaken te kunnen vinden.

Het eerste deel van het experimentele werk uit dit proefschrift heeft betrekking op de ziekte van Alzheimer. *Hoofdstuk 2* beschrijft de studie van een Cubaanse familie, bestaande uit 281 leden uit zes generaties, met autosomaal dominante preseniele dementie. Neuropathologische studies bevestigden de klinische diagnose van de ziekte van Alzheimer. Bovendien werd een significante LOD score verkregen voor marker D14S43, welke is gelokaliseerd in een 9 cM interval, waar bij alle patiënten hetzelfde haplotype werd gevonden. Het analyseren van het *PSEN1* gen liet een nieuwe heterozygote base substitutie zien die een aminozuur verandering tot gevolg heeft, waarbij in het TMIII van het presenilin 1 eiwit Leucine wordt vervangen door Methionine. Wij denken dat de nieuwe mutatie L174M zal leiden tot een abnormale N-terminus en waarschijnlijk ook tot abnormale C-terminal fragmenten en tot het slecht functioneren van het eiwitcomplex. Dit is de eerste genetische studie naar de ziekte van Alzheimer in Cuba, waarbij kan worden aangetoond dat het *PSEN1* gen betrokken is.

In *Hoofdstuk 3* onderzochten we de klinische en genetische kennis met betrekking tot de ziekte van Alzheimer en de standpunten ten opzichte van de mogelijkheid van het presymptomatisch genetisch testen van de ziekte in de hierboven beschreven familie. We interviewden 56 eerstegraads bloedverwanten van familiaire gevallen met de ziekte van Alzheimer, waarbij bleek dat de individuen een beperkte kennis hadden over hun eigen risico voor het ontwikkelen van de ziekte van Alzheimer. Alle 56 personen gaven aan dat zij gebruik zouden maken van de mogelijkheid van presymptomatisch testen om te weten te komen wat hun eigen risico is op het krijgen van de ziekte van Alzheimer. Wanneer ze geconfronteerd worden met de hypothetische keuze van reproductie, zou 50% van deze groep ervoor kiezen geen kinderen te krijgen in het geval ze zelf de mutatie zouden hebben. Het presymptomatisch genetisch testen bij de ziekte van Alzheimer blijft echter controversieel.

Het tweede deel van het proefschrift betreft de ziekte van Parkinson. In *Hoofdstuk 4* wordt het onderzoek beschreven binnen een grote Cubaanse familie met de typische laatbeginnende vorm van de ziekte van Parkinson en autosomaal dominante overerving. De gemiddelde leeftijd waarop de ziekte begon was 61.2 jaar (± 12.53, 45-76). Verder werden binnen de familie nog andere phenotypes gezien, zoals essentiële tremor en atypisch parkinsonisme. We verrichtten een scan van het genoom en koppelingsonderzoek. Analyse van verschillende locaties liet een maximum LOD score zien van 2.26 tussen de markers

D19S221 en D19S840. Verder zagen we bij haplotype-analyse een gebied op chromosoom 19 dat bij zes van de zeven Parkinson patiënten overeenkomt. De essentiële tremor en de atypisch parkinsonisme phenotypes komen niet overeen met dit haplotype, hetgeen suggereert dat hieraan een andere etiologie ten grondslag ligt. Onze bevindingen suggereren de aanwezigheid van een nieuw locus voor de ziekte van Parkinson op chromosoom 19p13.3-q12. Wij veronderstellen dat een oligogenetisch model met een gematigde betrokkenheid van twee of drie genen, in plaats van een "puur" monogenetisch model, de oorzaak zou kunnen zijn van het grote verschil van leeftijd waarop de ziekte zich manifesteert, het gereduceerd voorkomen en de phenotypische gevarieerdheid in de Parkinson families.

Hoofdstuk 5 is gericht op populatieonderzoek. Uit de studie van genetisch geïsoleerde populaties, waarvan wordt gedacht dat ze genetisch meer homogeen zijn dan de gemiddelde populatie, is gebleken dat dit een succesvolle strategie is voor het in kaart brengen van genen welke betrokken zijn bij ziekten, inclusief de monogenetische varianten of complexe aandoeningen zoals de ziekte van Parkinson. Om het genomisch gebied te identificeren waarbinnen zich genen bevinden die de aanleg voor de ziekte van Parkinson beïnvloeden, bestudeerden we 44 patiënten met de idiopathische vorm van de ziekte van Parkinson, waarvan 40 patiënten konden worden teruggekoppeld naar een gezamenlijke voorouder uit een geïsoleerde populatie in Nederland. We verrichtten een scan van het genoom en single-marker associatie analyse. Bij vier markers werd bewijs gevonden voor associatie ($p \leq 0.01$): D2S2333, D4S405, D9S158, D13S153. Verder was D9S290, (p=0.006) een nabijgelegen marker van D9S158, ook geassocieerd. Bovendien werd bij 10 patiënten (23%) een gemeenschappelijk haplotype gezien in dit 9q gebied die maar bij 1 controlepersoon (4%) voorkwam. De overlap van deze regio met een eerder geïdentificeerd gebied laat bewijs zien van koppeling met de ziekte van Parkinson. Tevens bevindt marker D2S2333 zich op dezelfde locatie als een tot nu toe niet geïdentificeerd gen, PARK3, dat wordt geassocieerd met de aanleg voor de ziekte van Parkinson en de leeftijd waarop de ziekte zich manifesteert, hetgeen suggereert dat zich in deze gebieden genen bevinden die bijdragen aan de aanleg voor de ziekte van Parkinson. Nader onderzoek van de ontdekte gebieden met andere, dicht bij elkaar gelegen markers zal het mogelijk maken de bevindingen te bevestigen en de genomische gebieden te verfijnen.

Hoofdstuk 6 presenteert een grote groep multi-ethnische patiënten met de vroegbeginnende vorm van de ziekte van Parkinson die werden bestudeerd om vast stellen wat de frequentie en de aard van *parkin*-gen mutaties zijn. Tevens werd de phenotype-genotype relatie bestudeerd. Hierbij werden 46 patiënten met de vroegbeginnende vorm van de ziekte van Parkinson en 14 aangedane verwanten uit Italië, Brazilië, Cuba en Turkije onderzocht. De genetische screening bestond uit een directe volgordebepaling en uit een exon-dosage bepaling, waarbij gebruik werd gemaakt van een real-time PCR methode. Globaal werden in 33% van de indexpatiënten mutaties gevonden, en in 53% van de personen met een familiegeschiedenis overeenkomend met een autosomaal recessieve overerving. We hebben 15 *parkin* veranderingen geïdentificeerd, inclusief 10 exon deleties en 5 puntmutaties. Vier van deze mutaties zijn nieuw: Arg402Cys, Cys418Arg, IVS11-3C>G, en de exon 8-9-10 deletie. Van de gevonden mutaties bestaat 28% uit heterozygote exon deleties, waarbij het belang van exon-dosage bij het screenen van het *parkin*-gen wordt bevestigd.

Bij patiënten met *parkin*-gen mutaties werd een aanzienlijk vroegere aanvang van de ziekte van Parkinson gezien met een langere duur van de aandoening, een meer frequente symmetrische aanvang, en een langzamere progressie van de ziekte dan van de patiënten

zonder de mutatie. Deze studie bevestigt het frequente voorkomen van mutaties in het *parkin*-gen en laat het belang zien van genetisch testen bij de diagnostiek van de vroegbeginnende vorm van de ziekte van Parkinson. De nieuwe kostenbesparende exon-dosage methode die hier is ontwikkeld kan ook worden toegepast bij het onderzoek naar andere genen.

Tenslotte, in *Hoofdstuk 7* (General discussion), bediscussiëren we verschillende methoden en valkuilen die zowel wij, als andere onderzoekers tegen zijn gekomen bij het zoeken naar genen betrokken bij neurodegeneratieve aandoeningen zoals de ziekten van Alzheimer en Parkinson.

Resumen

En el *capítulo 1*, dedicado a la introducción, se presenta una revisión completa de los aspectos genéticos conocidos en la enfermedad de Alzheimer y en la enfermedad de Parkinson. También se describen desde una perspectiva histórica los esfuerzos y progresos alcanzados en el mapeo y búsqueda de genes implicados en las formas hereditarias de estas dos enfermedades neurodegenerativas y como las estrategias y métodos usados han evolucionado en la última década, así como el significado del hallazgo de genes como las *presenilins (PSEN1 y PSEN2)*, *alfa-sinuclein (PARK1)*, *parkin (PARK2)* y *DJ-1 (PARK7)*. La presentación clínica, genética y patológica de estas enfermedades sugiere la intervención de mecanismos comunes o interrelacionados, sin embargo queda aún mucho por investigar para encontrar la manera y los mecanismos por los cuales estas enfermedades estan relacionadas.

La primera parte del trabajo experimental de esta tesis está dedicada a estudios familiares en la enfermedad de Alzheimer. En el *capítulo 2* se describe el estudio de una extensa familia cubana (281 miembros en 6 generaciones) con demencia presenil y una herencia autosómica dominante. El estudio anatomopatológico en un paciente permitió confirmar el diagnóstico clínico de enfermedad de Alzheimer. A través del análisis de ligamiento genético se obtuvieron resultados significativos en la región genómica correspondiente al gen *presenilin 1*, y la posterior secuenciación de este gen llevó a la identificación de una nueva mutación, localizada en el exon 6. Esta mutación tiene como consecuencia un cambio de aminoácido (Leucina por Metionina) en la secuencia de la proteína. Como resultado los fragmentos N-terminal y C-terminal de la proteína podrían estar alterados y esto determinaría una función defectuosa del complejo proteínico. Por primera vez mutaciones en este gen son identificadas en pacientes cubanos.

En el *capítulo 3* se presentan los resultados de una investigación que evaluó los conocimientos y actitudes de los familiares de pacientes con enfermedad de Alzheimer hacia el diagnóstico presintomático de la enfermedad. En total se entrevistaron 56 miembros de la familia estudiada en el capítulo anterior. En general todas las personas entrevistadas tenían un conocimiento limitado sobre su riesgo personal de desarrollar enfermedad de Alzheimer. A la vez, todos los participantes en el estudio aceptaban realizar los estudios genéticos, una vez que estuvieran disponibles, orientados a obtener un diagnóstico presintomático.

La segunda parte de esta tesis está dedicada a la enfermedad de Parkinson. En el *capítulo 4* se describe el estudio de una extensa familia cubana, con enfermedad de Parkinson y herencia autosómica dominante. El promedio de edad de comienzo de la enfermedad en la familia fue similar al de la forma "idiopática" de esta patología (61 años). La completa caracterización clínica de pacientes y familiares permitió hacer el diagnóstico de temblor esencial y parkinsonismo atípico en otros miembros de esta familia. Con el objetivo de localizar la alteración genética responsable de la enfermedad de Parkinson, se realizó una búsqueda genómica completa y análisis de ligamiento genético. Con el análisis de ligamiento de múltiples puntos se obtuvo un resultado de LOD máximo de 2.26, entre los marcadores D19S221 y D19S840. Además el análisis de haplotipos permitió detectar un haplotipo común en el cromosoma 19 que estaba presente en 6 de los 7 pacientes con enfermedad de Parkinson. A su vez este haplotipo no se encontró en los pacientes con tremblor esencial o con parkinsonismo atípico o en personas no afectadas. Estos hallazgos sugieren la presencia de un nuevo locus para enfermedad de Parkinson en el cromosoma 19p13.3-q12. Un modelo oligogénico con moderada contribución de dos o tres genes podría explicar mejor la gran variación en edades de comienzo, penetrancia reducida y variabilidad fenotípica observada en familias afectadas por la enfermedad de Parkinson.

El *capítulo 5* está basado en estudios poblacionales. El estudio de poblaciones aisladas genéticamente, las cuales se consideran más homogéneas que la población general, ha demostrado ser una estrategia exitosa en el mapeo de genes, incluyendo las variantes monogénicas de enfermedades complejas como la enfermedad de Parkinson. Con el objetivo de identificar regiones genómicas que contengan genes implicados en la etiología de la enfermedad de Parkinson, se estudiaron 44 pacientes que padecían esta enfermedad. La mayoría de estos pacientes (n=40) pudieron ser conectados a un ancestro común con origen en esta comunidad aislada, localizada en el sur de Holanda. Se hizo una búsqueda genómica completa y análisis de asociación con cada marcador. Para 4 marcadores se obtuvieron evidencias de asociación ($p \leq 0.01$): D2S2333, D4S405, D9S158, D13S153. Además para D9S158 ($p=0.006$), otro marcador (D9S290) localizado muy cercano en el cromosoma, se encontró también asociado ($p=0.006$). En esta región del cromosoma 9q se identificó un haplotipo común en 10 de los pacientes (23%) y el mismo haplotipo sólo se encontró en un control (4%). Además, el marcador D2S2333 ($p=0.009$), está situado muy cerca del locus *PARK3*, el cual ha sido relacionado con una mayor susceptibilidad y con la edad de comienzo de la enfermedad. Estos hallazgos indican que posiblemente genes localizados en esta región genómica determinan una mayor susceptibilidad a la enfermedad de Parkinson.

El *capítulo 6* presenta una extensa serie de pacientes con enfermedad de Parkinson de comienzo temprano, que fueron estudiados clínica y genéticamente con el objetivo de determinar la frecuencia de mutaciones en el gen de *parkin* y para investigar posibles correlaciones fenotipo-genotipo. Se estudiaron 46 casos índices con edad de comienzo menor de 45 años, y 14 familiares de primer grado afectados; los pacientes provenían de diferentes países: Italia, Brasil, Cuba y Turquía. Los estudios genéticos incluyeron secuenciación directa del gen (*parkin*) y estudios de dosis de exones para lo cual se utilizó un nuevo método desarrollado durante esta investigación. En 33% de los pacientes en general y en 53% de los casos con historia familiar positiva se detectaron mutaciones en el gen de *parkin*. En total se encontraron 15 alteraciones diferentes en el gen, incluyendo 10 deleciones de exones y 5 mutaciones puntuales. Cuatro de las mutaciones se describen por primera vez en este trabajo, ellas son: Arg402Cys, Cys418Arg, IVS11-3C>G y la deleción consecutiva de los exones 8-9-10. Las alteraciones heterocigotas de exones representaron el 28% de todas las alteraciones génicas encontradas, confirmando la importancia de realizar estudios de dosis de exones además de la secuenciación directa del gen.

Los pacientes con mutaciones en este gen mostraron de forma significativa un comienzo más temprano de la enfermedad, el cual ocurrió más frecuentemente de forma simétrica, un curso clínico más prolongado, y progresión lenta de la enfermedad comparado con los pacientes sin mutaciones en el gen *parkin*. El presente estudio confirma hallazgos anteriores con respecto a la relación fenotipo-genotipo, y a la alta frecuencia de mutaciones en *parkin*, indicando la importancia de los estudios genéticos en el diagnóstico de la enfermedad de Parkinson de comienzo temprano. El método para determinación de dosis de exones desarrollado durante esta investigación puede ser aplicado al estudio de otros genes como *alfa-sinuclein* y *DJ-1*.

Finalmente, en el *capítulo 7* dedidado a la discusión general, se consideran las diferentes metodologías aplicadas y problemas encontrados durante la búsqueda de genes implicados en la etiología de enfermedades neurodegenerativas como la enfermedad de Alzheimer y la enfermedad de Parkinson.

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Mi "paraninfo", Dr. Fernando Rivadeneira Ramírez (uit Colombia): Our friendship started when we met in the year 2000, shortly after you and Carolina came to the Netherlands. Since then, we have shared many important things (a wedding, the birth of your first daughter and many, many dinners, parties, holidays…). Thanks for your advice on statistics and for translating the writings of my father. After all this, I'm happy that besides good epidemiology groups, Holland has good football teams and high quality cheese, your main reasons to come to this country! Fernando y Carolina (y Grabriela por supuesto): Muchas gracias por todo! Nunca los olvidaremos.

And now my second paranimph, Bianca de Graaf. Beste Bianca: I remember when we met in

1997 (in the Genetic Epidemiology Unit), at the end of my first year in Holland. Later I went back to Cuba and you moved to the Clinical Genetics department. At present we are working together again and I enjoy that. You always helped me with many, many things, especially the introduction to the Dutch culture. Thanks for everything and especially for all your help as paranimph… I'm glad you found "the painting".
Dr. Jose Luis Giroud, the Cuban neurologist! Thanks for all your work in the clinical characterization of the Cuban families. Since we met in Havana in 1998, I realized your enormous enthusiasm and scientific curiosity would bring you far in the field. Gracias por todo y mucha suerte con tu tesis!
From the Department of Clinical Genetics: my gratitude goes to Guido Breedveld ("the lab master"), who instructed me in the lab arts. Oh! Those radioactive acrylamide gels!! Thanks to Esther* (always helpful), Erik (so many genotypes!), Onno (all those sequences), Alice (LOD of 5!!), Fred, Herma (Real-time PCR?), Annemieke, Li Li, Christan, and to the people from the Fragile X group; Edwin, Lau, Ingeborg, Sandra, Surya, Bart, Maria, Cathy, Marianne, Lies-Anne (thanks for the wonderful immunohistochemistry work). And Pietro, how to forget him! My special thanks to Rob Willensen who picked me up at Schiphol Airport (January, 1997), bringing a warm coat (coming form 30 degrees to 4 graden onder nul). Beste Rob, thanks for all the help and support during that year and thanks also to your wife, I will be always grateful.
Dr. Yolanda de Diego (uit España): Querida Yolandita: Many thanks for your valuable friendship, for sharing with us a very important day (21-Jan-2002), thanks for all your invitations to Spain, and for the pleasure of meeting your family and friends (aquí recordamos especialmente a nuestra amiga Almudena, gracias guapa!). Ahora solo falta David, con el que esperamos seas muy feliz. Te debo un viaje a Cuba, esperemos que pueda ser pronto…
Thanks to Jeannette Lokker for the valuable help filling in the infinite numbers of forms, and for all the letters and translations into Dutch…and especially for the translation of the Summary of this thesis. Dank je wel!
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I would like to acknowledge a great person and teacher who is sadly no longer with us: Lodewijk Sandkuijl.
From the Genetic Epidemiology Unit: A special acknowledgment to Leon Tester (the other master), your help and support were very important. It was a pleasure working with you. Leon, you are a great person! You also have a great ability in organizing salsa parties and canoeing trips at midnight! Succes met je nieuwe beroep!
Many thanks to Marieke Dekker, my roommate on the 22nd floor, we spent many long, long days together (noodle soups and thee!). Dear Marieke, it was a pleasure working with you in the GRIP-PD project. As a person, your kindness impressed me. I wish you the best together with Marco, Ida, and the young Willem and in your future career.
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Gracias a nuestros amigos, Magdalena (de Chile) y Stefan (futuros papas!), Nelly (de Mexico), Maria y Fermin (Lucía y Constanza), por compartir tantas fiestas y cenas Oye che! que todavía nos deben el asado argentino. Muchas gracias por su apoyo y compañía.
Going back to the tropics, thanks to my former colleges from the National Center of Medical Genetics: Ramiro Chaves Guevara, thanks for helping me during my initial probe isolations and Southern blots for my first linkage studies, the nice trips and conversations while crossing Havana from west to east (22 km), and for sharing with me the family photos and writings of your famous uncle. Manuel Goméz from the DNA lab, who taught me how to PCR, although at that time it was a bit different (3 water baths, and ambient temperature of 35°C!). Gracias Manolo, y también por los chistes! Thanks also to Dr. Aracely Lantigua and Dr. Estela Morales (great clinical geneticists), Dr. Hilda Granda, and the people from the DNA lab: Anita, Teresita, Blanca, Yadira, and from the Cytogenetic lab: Carlos, Elena, Viviana. Gracias a todos!
Many thanks to my old friends from Cuba, a Enrique Montero (el más viejo de todos), Niurka Sotto (contigo aprendí a bailar salsa!), Odalis, Zoilita, Rosa Maria, y tantos otros amigos y compañeros de la Vocacional. También a mis amigas Mechy y Eneida (+Elio y los niños), todavía extraño aquellos días juntas en la Universidad!
Gracias a todos los vecinos del Reparto Peralta por su ayuda y cariño en todos estos años.
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Finally, my deep gratitude goes to my family. A mis tías y tíos, y a la más experimentada (Tía Toña). A mis abuelos: mis Tata y Papa, que ya no están con nosotros pero vivirán siempre en mi memoria. Ustedes me dejaron tantas cosas, la pasión por la vida, los mejores ejemplos de bondad, amor y entrega hasta el significado de "palabra de honor". Gracias por su amor, immenso e incondicional. Mis queridas hermanas y mi pequeñita Maria Lucia, nuestro amor es mayor que el océano que nos separa. La esperanza que un día estaremos juntas de nuevo, me da fuerzas para seguir adelante. Mamá y papá, a ustedes les debo todo. Una vez más, gracias por su amor, ese que me dió la vida y que hasta siempre me acompaña. Y a mi esposo, gracias por estar ahí, siempre a mi lado. Citando a Benedeti, "Con tu puedo y con mi quiero, vamos juntos, compañero".

Aida M. Bertoli Avella

Curriculum Vitae

Date and place of birth: March 10, 1965, Holguin, Cuba.

1977-1983	Secondary and High school at the Vocational Jose Marti, Holguin, Cuba.
1983-1989	Faculty of Medicine, Institute of Medical Sciences, Santiago de Cuba. Research projects on Histology, Paediatrics and Clinical Genetics. Degree of Doctor in Medicine, *summa cum laude*.
1989-1993	Residency on Clinical Genetics at the National Centre of Medical Genetics, Higher Institute of Medical Sciences, Havana. Research project "Autosomal Dominant Polycistic Kidney disease: a clinical and genetic study of two families" (Prof. Dr. Luis Heredero). Degree of Specialist on Clinical Genetics.
1993-1999	Clinical geneticist, researcher and teaching staff member at the National Center of Medical Genetics, Havana, Cuba.
1997-1998 1999-2000	Fellowship at the Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands. Research projects on Fragile X syndrome, Post-axial polydactily and Alzheimer's disease (Prof. Dr. Peter Heutink and Prof. Dr. Ben Oostra).
2000-2003	PhD research on genetics of Parkinson's and Alzheimer's disease at the Genetic Epidemiology Unit (Prof. Dr. Cornelia van Duijn), in close collaboration with the Department of Clinical Genetics (Prof. Dr. Peter Heutink and Prof. Dr. Ben Oostra) Erasmus Medical Center, Rotterdam.
2003-present	Research position at the Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands.

Nuestra hija mayor

Por Gilberto B. de Bertoli de Armas y Aida V. Avella Iglesias

10 de marzo de 1965. Recibimos este dia, llenos de alegría, a la primera de nuestras 3 hijas, a la que pusimos el nombre de su mamá y su abuela paterna: Aida María.

Su primera infancia la recordamos como una niña alegre y muy saludable, de un notable desarrollo y viveza. Cuando tenía alrededor de 3 años, paseando un día cerca de un prado donde pastaba una vaca y con intención de enseñarle que no debía temer a los animales, nos acercamos a ella e intenté pasarle la mano por el lomo, en ese momento la vaca hizo un movimiento brusco de la cabeza y muuuu!!, yo di un pequeño salto hacia atrás, mi hija se quedó mirándome y admirada me dijo: "pipo, tú también le tienes miedo a la vaca".

Disfrutaba mucho los juegos, sobre todo al aire libre. A los 5 años le regalamos su primera bicicleta que fue para ella muy especial. Su incorporación a la escuela primaria fue fácil, aprendía rápidamente, despertando la estimación y admiración de sus profesores. En esta época de su vida nos impresionaban sus cuidados con sus materiales escolares, la limpieza y pulcritud con sus libros y su vestuario, nos llamaba la atención que su uniforme escolar se mantenía limpio mucho tiempo, el orden y cuidado para con sus juguetes y libros de cuentos. Era apasionada de la literatura infantil adquiriendo desde esta temprana edad hábitos y habilidades que nos asombran, con sus hermanas organizó una biblioteca usando códigos y etiquetas con los que marcaba cada libro.

Terminó la escuela primaria con magníficos resultados e hizo la enseñanza secundaria y preuniversitaria en un internado especial para estudiantes destacados, por primera vez dejó nuestra casa y solo ella sabe lo que le costó adaptarse. Un día con los ojos llenos de lágrimas nos dijo "Yo los extraño mucho, pero de aquí no me voy". Por su carácter abierto hace amistades fácilmente, le gustan las fiestas, el baile y la buena cocina, es una persona alegre y dócil, generosa y modesta, aparentemente frágil, sin embargo nada ni nadie logra apartarla de sus obligaciones y tareas, por lo que desarrolla una gran autodisciplina que ha perfeccionado a través de los años. Es muy firme en el momento de tomar decisiones y mantenerlas. Es desprendida y solidaria, cuida y quiere mucho a sus hermanas, de las cuales se ha sentido siempre responsable, disfruta regalando y ayudando a los demás.

Desde pequeña mostró su predilección por la Medicina. De sus primeros días de la Universidad recuerdo que estoy al tanto de sus estudios para ayudarla, como médico sabía que era una carrera difícil, y cuando observé que además de su libreta de notas y su libro de texto básico, busca otros autores para ampliar la información, comprendí que mi presencia no era tan necesaria, pues sabía que ya era de los que no se conformaban con una sola opinión.

El tiempo me daría la razón, terminó su carrera de Medicina con Título de Oro y fue la más destacada en investigación en su curso. Escoge la especialidad de Genética Clínica, y le gusta el trabajo de laboratorio e investigación. A los 23 años se fue a la Habana donde definitivamente aprendió a ser independiente, terminó la especialización y obtuvo su primer empleo en el Centro Nacional de Genética Médica. Ahora vive en un país lejano del que supo por primera vez cuando leyó un cuento infantil sobre un niño que salva a su país taponenado con su dedo un dique que los protegía del mar.

Para su abuela materna nació con muy buena estrella, para nosotros brilla tanto como ellas.

Our oldest daughter

By Gilberto B. Bertoli and Aida V. Avella. English version by Fernando Rivadeneira

March 10, 1965. There was the rejoicing day that the first of our three daughters was born. We named her Aida Maria in remembrance of her mother and grandmother.

We remember her early childhood as that of a happy healthy girl, smart and finely developed. Once, when she was about three years old, we were walking through a field close to a herd of cows. I brought her close with the intention of teaching her not to fear the animals. While I was attempting to pet one of the cows, the animal made a sudden move and mooed. I jumped back while my daughter stared at me and exclaimed: "Pipo, you are also afraid of the cow!"

She always enjoyed playing, especially outdoors. When she was five we gave her her first bicycle, which would be very special for her. Her first days at school went smoothly; she was a fast learner and drew the sympathy and admiration of her teachers. In that time, we would be impressed by how cautious she was with her school materials, the neatness of her books and attire. Her school uniform was clean for unusually long times, and she was always very delicate with her toys and story books. She was passionate for children literature in a way that would amaze us: together with her sisters she organized her own library with labels and codes for all her books.

She finished her primary school successfully and went to an intern school for outstanding children. There she completed her high school and pre-college education. This was the first time she was away from home and only she knows how difficult this was. One day, with her eyes full of tears she told us: "I miss you so much, but I am not leaving". Under a fragile appearance lies a determined person who will achieve all her duties and enterprises and is firm about making and committing to her decisions. She is a happy person, always generous and humble. Being such an open person makes her easy to get to know; she enjoys parties, dancing and magnificent cooking. She is generous, careful and always willing to support others; she loves and cares for her sisters and has always felt responsible for them.

Very early she was attracted to medicine. Being a physician myself and knowing what a difficult career it was, during the first days at Medical School I was always looking forward to help her. Soon I learned this was not necessary when I noticed that for her, the information in the textbook was never enough. She would always search the literature thoroughly for other author's opinions. In time I was proved correct: she finished the medical school with honours and in research she was the most outstanding student. She chose to specialize in clinical genetics, where she showed enormous affinity for research and lab work. This way, when she turned 23 years old she went to Havana where she definitely became independent. After finishing her residency she was employed at the National Centre of Medical Genetics.

Today, she lives in a country faraway that she first learned from a children's story: the tale of a boy who saved his country by placing his finger in the hole of a breaking dike that protected the land from the sea.

Her maternal grandmother use to state she was born under a good star, for us she shines just like one.

List of Publications

Linkage disequilibrium in young genetically isolated Dutch population.
Yurii S. Aulchenko, Peter Heutink, Ian MacKay, Aida M. Bertoli-Avella, Jan Pullen, Norbert Vaessen, Tessa A. M. Rademaker, Lodewijk A. Sandkuijl, Lon Cardon, Ben Oostra, and Cornelia M. van Duijn.
European Journal of Human Genetics; In press, 2004.

Polymorphisms in the prion protein gene and in the doppel gene increase susceptibility for Creutzfeldt–Jakob disease.
Esther A. Croes, Behrooz Z. Alizadeh, Aida M. Bertoli-Avella, Tessa Rademaker, Jeannette Vergeer-Drop, Bart Dermaut, Jeanine J. Houwing-Duistermaat, Dorothee P.W.M. Wientjens, Albert Hofman, Christine Van Broeckhoven, Cornelia M. van Duijn.
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Chasing genes in Alzheimer's and Parkinson's disease.
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Human Genetics; 114(5):413-438, 2004.

A study of gene-environment interaction on the gene for angiotensin converting enzyme: a combined functional and population based approach.
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A new locus for postaxial polydactyly type A/B on chromosome 7q21-q34.
Galjaard R.J., Smits A.P., Tuerlings J.H., Bais A.G., Bertoli Avella A.M., Breedveld G., Graaff Ed E., Oostra B.A., Heutink P.
European Journal of Human Genetics; 11(5):409-415, 2003.

A novel presenilin 1 mutation (L174 M) in a large Cuban family with early onset Alzheimer's disease.
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Neurogenetics 4:97–104, 2002.

Clinical and molecular characterization of the Fragile X Syndrome in a Cuban family.
R.A. Llaurado Robles, V.J. Tamayo Chang, Y. Perez Gonzalez, N. Santos Falcon, AM Bertoli Avella, and V. Gonzalez Gomez.
Revista Española de Pediatría; 56(5):437-441, 2000.

Mutation G1138A in the *FGFR3* gene is present in Cuban patients with Achondroplasia.
Thelvia I. Ramos Gomez, Estela Morales Peralta, Teresa Collado Mesa, Suany Ojeda Fernandez, Aida Bertoli Avella, and Luis Heredero Baute.
Biotecnología Aplicada; 16:109-111, 1999.

Brillaron los muros blanqueados de cal, cuadrados y simétricos; brillaron las rosas.
Y ella también brilló en un espesa claridad de espejos.
Y así de pronto, la luna empezó a temblar con un temblor cada vez más apresurado, más violento cada vez...
La luna se desprendía, desgarraba las nubes y se precipitaba sobre la tierra dando volteretas por el espacio.
Pasó un minuto y pasó un siglo. La luna en el alero del mirador, rebotó con un sonido de cristales y fue a caer despedazada en el jardín a los pies de ella.
Astillas de la luna saltaron sobre su cara, y ella pudo sentir todavía un frio desconocido.
Se arrodilló en el sendero, recogió de entre la yerba la luna rota y la envolvió en su chal de encaje. La tuvo un rato entre las manos, dueña por unos segundos del secreto de la noche.
Luego hizo un hoyo muy hondo en el lugar en que la tierra era mas tibia... Y así enterró la luna en el jardín.
Arriba plantó un gajo de almendro, y se fue con las manos húmedas embarradas de tierra y de luna.

Dulce María Loynaz, "El jardín", 1935