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Genetic Epidemiology of Parkinson disease

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To my family

Publications

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[14] **Manu Sharma**, Claudia Schulte, Jakob C Mueller, Peter Lichtner, Daniela Berg, Bertram Müller –Myhsok, and Thomas Gasser. Comprehensive association study and meta analysis of BDNF gene with Parkinson disease ***(Manuscript submitted)***

[15] **Manu Sharma**, Jakob C Mueller, Peter Lichtner, Daniela Berg, Thomas Illig, Bertram Muller-Myhsok and Thomas Gasser. Role of Fibroblast Growth Factor 20 gene polymorphisms with Parkinson's disease in the German population. ***(Manuscript submitted)***

Workshops and Poster Presentations

1. Poster Presentation in "9th Annual Meeting and 10th Worshop on Neurogentics Deutsche Gesellschaft für Neurogentic e.V". Tübingen Germany (21st – 23rd Sep 2003)
2. Workshop on "Population aspects of Complex diseases" held in Kiel on 9th Jan 2004.
3. Workshop on Design and Analysis of "Genetic-Based Association studies" held at the Wellcome Trust Genome Campus, Hinxton, Cambridge from 3-7th October 2006

1. Introduction

1.1 Parkinson disease and genes

Parkinsonism is a clinical syndrome characterized by resting tremor, bradykinesia, rigidity and postural instability. Parkinson disease (PD) is the major cause of parkinsonism (1). Clinically, it begins insidiously, asymmetrically and is steadily progressive due to loss of dopamine neurons in the substantia nigra pars compacta. The majority of the patients with parkinsonism receive a clinical diagnosis of “possible or probable” PD after follow up over time (2). There is lack of availability of diagnostic tests or markers for PD. Current criteria for diagnosis of PD requires at least two of the symptoms: resting tremor, bradykinesia, rigidity or postural imbalance (3). Asymmetric onset and a good response to levodopa are supportive for the diagnosis of PD and are most commonly used features to discriminate PD from other diagnosis (4). Pathologically, presence of the cytoplasmic inclusions known as Lewy bodies is widely recognized as the hallmark of PD (5). Still, whether the presence of Lewy bodies are pathogenic or confer a protective effect remains controversial (6).
There is marked variability in estimating the prevalence of PD. Estimates of the prevalence as well as incidence may vary according to applied methodology which further complicates the comparison across different studies (7, 8). The prevalence of PD in industrialized countries is approximately 0.3% to 1% in people over 60 years of age. The prevalence of PD in different population based surveys showed that PD is an age related disease (fig 1) (9). PD is rare before age of 50 years and the prevalence increases with age up to 4% in the 8^{th} and 9^{th} decade of life (9, 10).

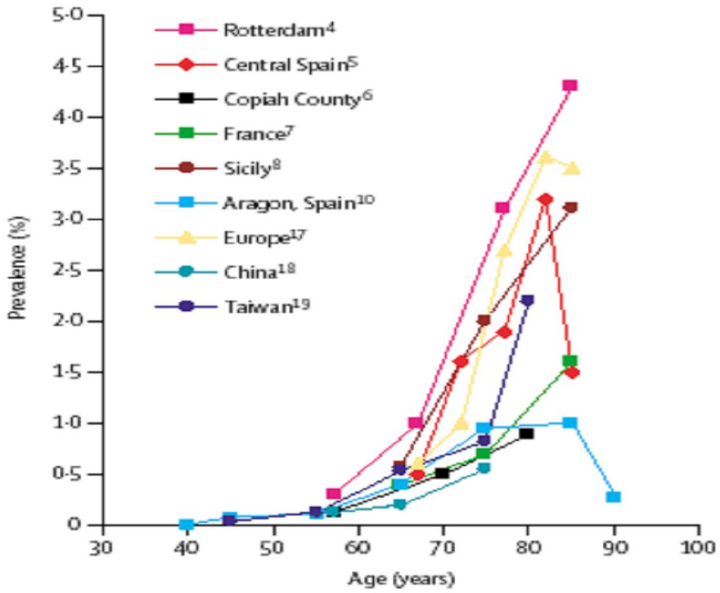

Figure 1: The prevalence of PD in different population based surveys.

Besides genetic factors, many environmental risk factors for PD have been proposed which are thought to play an important role in the pathogenetic mechanism of the disease (10, 11) Putative environmental and demographic factors that may predispose to idiopathic PD include age, sex, estrogen status, race or ethnicity, exposure to pesticides, head trauma, smoking, alcohol and coffee (12). Of all factors, age is the most highly linked to PD, the incidence of PD increases steeply with age in both men and women (13).

Currently, there is no treatment available that can halt the neuronal degeneration of PD. At present L-dihydroxyphenylalanine (L-dopa) is the most commonly and effectively used agent for PD (14). Administration of levodopa in combination with an extracerebral dopa-decarboxylase inhibitor (carbidopa in the United States and benserazide in Europe) is commonly used in practice (14). Initially, patients respond well to the treatment but later on these drugs fail to halt disease progression (15). Moreover, intake of medication leads to side effects in the form of dyskinesia (16). So, there is urgent need to develop new therapeutic approaches which will help in treating the PD patients.

To develop a therapeutic strategy for PD, further insight in the pathogenetics is essential. In recent years, the discovery of the monogenetic forms of PD has generated considerable interest. However, these mutations explain only 10% of

all PD cases and 90% of cases are sporadic. Despite these encouraging developments in the genetic epidemiology of PD, it is clear that research on the genetics of PD is far from completion.

The aim of this thesis was to identify new susceptibility genes for PD. To meet this objective, two strategies were followed. First, a case–control based approach using a candidate gene study was performed. Candidate gene(s) were selected because of their potential involvement in the pathophysiology of the disease. The likelihood of their involvement is further increased if the candidate gene(s) were in the regions which were already identified by genome screens (8).

Secondly, a family–based approach using sib pairs was used to locate a new gene(s) and confirm the previous linkage region(s) which were described by previous studies. The rationale of using this approach was co-segregation of the genetic markers with disease in all affected sibs. Linkage studies so far have been successful in identifying the monogenetic forms of PD. So, far this approach has led to the identification of the mutations in the SNCA, Parkin, PINK1, DJ1 and LRRK2 genes.

In chapter 2, the current status on the genetic epidemiology of PD is reviewed. Retrospective studies on several candidate genes for PD are described. In Chapter 3, case control studies on FGF20, NOS2A gene, BDNF are presented. In chapter 4, family based studies are described. Firstly, the potential involvement of the sepiapterin reductase gene (SPR) within the *PARK3* locus was assessed. Secondly, *PARK11* was tested to confirm the linkage in a European sib pair population. Finally, in Chapter 5 a new methodology is discussed to conduct a meta analysis of genome wide linkage scans of PD which incorporated the data uncertainty. Meta analysis is used to synthesize the information from all published studies which can be then analyzed together to assess the validity of different chromosomal regions which were shown by different studies across different population. Finally, the implications of the findings of the thesis are discussed in chapter 6.

Chapter 2: Genetic Epidemiology of Parkinson disease.

Parkinson disease is the second most common neurodegenerative disorder. Prevalence is age dependent and affects approximately 1.5% to 3.4% of the population over the age of 60 years in Europe (7, 8). The main clinical phenotype of PD is parkinsonism, a movement disorder that is characterized by tremors, rigidity and bradykinesia (17). PD is due to selective loss of dopaminergic neurons in the substantia nigra pars compacta which leads to profound depletion of dopamine in striatum (18). Pathological features include the formation of lewy bodies in the substantia nigra (5). Still, whether the formation of lewy bodies are protective or confer a risk is very much debatable.

2.1 Clinical Phenotype

There is ambiguity in properly classifying PD patients based upon clinical diagnosis alone. To clinically define PD, many researchers widely use the British Brain Bank criteria (3) as shown in table 1.

Table 1: Diagnostic criteria for PD.

Features	Diagnosis
Group A: Resting tremor Bradykinesia Rigidity Asymmetric onset	Following conditions should be met for the POSSIBLE diagnosis of PD. 1) 2 of the 4 features from group A should be present or 2) There should not be any feature present from group B. or 3) Substantial and sustained response to levodopa or use of dopamine agonist has been documented. PROBABLE diagnosis At least 3 of the 4 features in group A should be present. There should be substantial and sustained response to levodopa. DEFINITE diagnosis: All criteria for possible Parkinson disease are met.
Group B: Postural instability in the first 3 years after symptom onset. Freezing phenomenon in the first 3 years. Hallucinations unrelated to medications, Dementia preceding motor symptoms. Supranuclear gaze palsy. Severe symptomatic dysautonomia unrelated to medications.	

Broadly, clinical phenotype of PD patients can be divided into two categories.

1. Motor clinical features
2. Non motor clinical features

Motor Clinical Features

Tremor

Tremor may be defined as more or less regular rhythmic oscillatory movement produced by alternating or irregularly synchronous contractions of antagonistic muscles (3). Tremor at rest is one of the cardinal symptoms of PD. Electromyographically, it is characterized by a burst of activity that alternates between opposing muscle groups (19). Tremor is relatively gentle and limited to the distal muscles in PD. It is often asymmetrical and may be unilateral at the outset. Although tremor, particularly resting tremor, is common in PD patients. It is also reported in other disease with parkinsonian features (20). It was reported that 80% of autopsy diagnosed multiple system atrophy patients had tremors and interestingly resting tremor was explicitly noted in 34% of patients (20).

Rigidity

Rigidity is defined as an increased resistance of a joint to passive movement. PD patients sometimes experience rigidity along with resting tremor that has been named cogwheel –like rigidity. Rigidity is a consequence of an enhanced response of muscles to stretch. Indeed, the stretch reflex has long latency components named M2 component, which at least in part represent the output of transcortical reflex loop, and is enlarged in parkinsonian subjects (21). The gain in transcortical reflex is altered because of abnormal output of basal ganglia. Abnormal output of basal ganglia was also reported in Huntington disease as well (22). Besides abnormality in reflex loop, spinal mechanism also contribute to parkinsonian rigidity (23).

Bradykinesia

Among the above two mentioned symptoms of parkinsonism, bradykinesia is the most important symptom that mainly contributes to disability associated with parkinsonism. It affects all voluntary and involuntary movements (3, 4). PD patients experience less mobility of the face and there is less use of expressions during talking. As the disease progresses, patients do experience problems in performing daily routine chores. In particular, patients do have difficulties in moving and turning their body axis. A number of studies have assessed the pathophysiology of bradykinesia (24, 25). They suggested that PD patients have a problem in starting the movement as well as executing the movements. The effect is much more pronounced when patients have to self-initiate movements as compared to when they respond to external stimuli (25).

Non motor clinical features

Depression

Depression is one of the most common psychiatric disturbances developed in PD patients. Depression may occur at any stage of Parkinson disease (26). Patients with PD experience mild to moderate depression. Psychomotor slowing and sleeping difficulties occur frequently in PD patients (26). The cause of depression in PD is attributed to abnormalities in neurotransmitters mainly in dopamine and serotonin and norepinephrine (27). A study by Mossner et al. suggested an involvement of functional polymorphisms in the promoter region of the serotonin gene as risk factors for depression in PD patients (28).

Proper management of depression in PD is to optimize dopaminergic therapy for improved motor symptoms. Furthermore, levodopa, dopamine agonist and selegiline are considered to have mild antidepressant effects and have been studied as antidepressants (29).

Psychosis

Psychosis is defined as a disturbance of perception and thought and commonly includes hallucinations, delusions, agitation and sleep disorders (26, 30). These symptoms are common in PD patients and occur in 20-40% of medication

treated patients with PD (30, 31). The majority of patients observe various perceptual disorders including sense of a presence and hallucinations (32). Paranoid delusions, often pathological jealousy, can develop and cause distress and reduce quality of life (32). Cognitive impairment and dementia have been reported as most frequent risk factors for psychotic symptoms in PD (32). The underlying pathophysiological mechanism of psychosis in PD patients is still unclear. There are several lines of evidence that support the central role of dopaminergic systems in the development of psychotic symptoms in PD (33).

Dementia

The prevalence of dementia is approximately in between 28-44% of PD patients (34). The cognitive decline can present early in the development of the disease and sometimes precedes the development of memory deficits (35). Neuropsychiatry symptoms are more common in patients having Parkinson disease with dementia (PDD) (34). A number of studies have found association of psychosis with poorer scores on a variety of cognitive function tests (31, 32, 36-38). A recent study compared the neuropsychiatry symptoms between non demented PD patients, PDD and Dementia with Lewy bodies (DLB) patients (38). The authors noted that delusions and hallucination occurred with increasing frequencies in PDD and DLB patients. They did not report any significant differences over the type of hallucinations and delusions across the three groups. However, they observed that psychotic symptoms with cortical lewy bodies and cholinergic deficits were present in both PD and DLB (38).

2.2 Pathology

Neurodegenerative diseases such as PD, Alzheimer disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) have abnormal deposition of protein aggregation in the brain, which can be cytoplasmic, nuclear or extarcellular as shown in table 2.

Table 2: Neurodegenerative diseases: protein and pathology

Disease	Brain region	Pathology	Protein deposit
ALS	Spinal motor neurons and motor cortex	Axonal spheroids and Marinesco bodies	SOD1
HD	Striatum , cortex	Intranuclear inclusions	Huntington
AD	Cortex, hippocampus	Neuritic plaques and tangles	Aβ peptide
PD	Substantia nigra, cortex	Lewy bodies	Synuclein

Protein aggregation can be formed either by mutation in a disease related gene or can occur in the absence of genetic alterations, triggered by environmental stress (39). In neurodegenerative diseases large intracellular or extarcellular accumulation of aggregated proteins known as inclusion bodies are formed (40). Although inclusion bodies represent the distinct pathological features of neurodegenerative diseases, still there is continuous debate regarding the role of aggregation in a disease process.

Synucleinopathies

Synucleinopathies represent a group of disorders that have pathological lesions composed of fibrillary aggregates of insoluble alpha synuclein protein in selective populations of neurons and glia (41). These disorders include dementia with Lewy bodies (DLB), multiple system atrophy (MSA) in which α-synuclein constitutes characteristic inclusions predominantly in oligodendrocyte cell bodies (42). Although the exact mechanism of these overlapping pathologies in PD is poorly understood. However, recent studies do suggest that PD in elderly individuals can also arise due to different pathologies involved in dopamine pathways (43, 44). Several studies showed that deposition of cortical β-amyloid is more common in DLB than in PD and may contribute to this clinical syndrome (45-47). The involvement of abnormal accumulation of α-synuclein in these groups of disorders suggests that the selective disruption of

diverse intracellular events, utilizing α-synuclein, lead to distinctive anatomical and cellular patterns of pathology.

Tauopathies

Tauopathies represent a group of disorders in which neurofibrillary degeneration of neurons occurs along with the deposition of tau proteins (48). It includes progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Parkinson–dementia complex of Guam and Pick disease (PiD) (48). They have the propensity to damage the globus pallidus and substantia nigra (49). Cortical damage and glial pathology is more variable and allows the distinction to be made between these conditions (50). CBD and PiD represent asymmetric frontoparietal and frontotemporal atrophy, as compared to PSP where more mild involvement of cortical layers were seen (51). Glial inclusions have been reported to be associated with compartmentalization of tau within glial cells (52). In this context, in PSP tau accumulates in the cell body producing the tufted astrocyte appearance; in CBD tau accumulates at the distal astrocytic processes producing astrocytic plaques and in PiD tau is distributed more diffusely (52). Although they share similar changes, the cellular mechanisms by which this or other potential detrimental processes interact to produce the features of any form of tauopathies remain purely speculative.

2.3 Braak's staging of PD

There is considerable overlapping with which idiopathic PD is associated with other degenerative conditions, notably Alzheimer's disease, particularly in advanced age even though the clinical spectrum of multiple pathologies remain poorly defined (53). Lewy bodies represent the pathological hallmarks of the sporadic form of PD (5). Neuropathological diagnosis of PD involves alpha synuclein immunopositive Lewy neurites and Lewy bodies (54). To properly classify the neuropathological diagnosis, Braak's "pathological staging" of PD represents a landmark in our understanding of PD (55) as shown in table 3.

Table 3: Braak pathological staging of PD-related pathology.

Stage	Areas affected
Stage 1	Affects the olfactory bulb, lesions in the dorsal IX and X cranial nerves in the medulla.
Stage 2	It includes the pathology of stage 1 and also lesions in caudal raphe nuclei, coeruleus-subcoeruleus complex and magnocellular parts of reticular formation.
Stage 3	Affects the substantia nigra pars compacta , amygdala, hippocampus and the pathology of stage 2.
Stage 4	Affects the temporal mesocortex and allocortex (CA2-plexus). The neocortex is unaffected.
Stage 5	Affects the high order sensory association areas of the neocortex and prefrontal neocortex.
Stage 6	Affects the entire neocortex. Cortical pathology extends upto first order sensory areas.

The pathology begins in the dorsal IX/X motor nucleus and finally reaches to cerebral cortex as shown in fig 2.

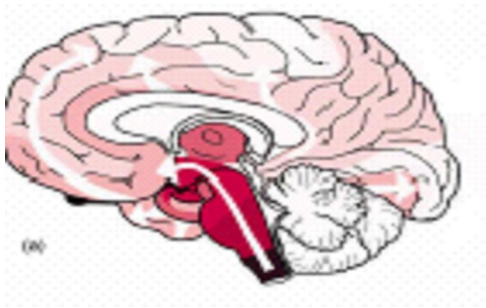

Figure 2: The progression of PD pathology

Stage 1 and stage 2 is confined to the lower brain stem. At this stage conspicuous changes were observed in Lewy neurites (LN) (56). In more advanced cases, LN are abundantly present as compared to Lewy bodies as shown in fig 3 a and b (57).

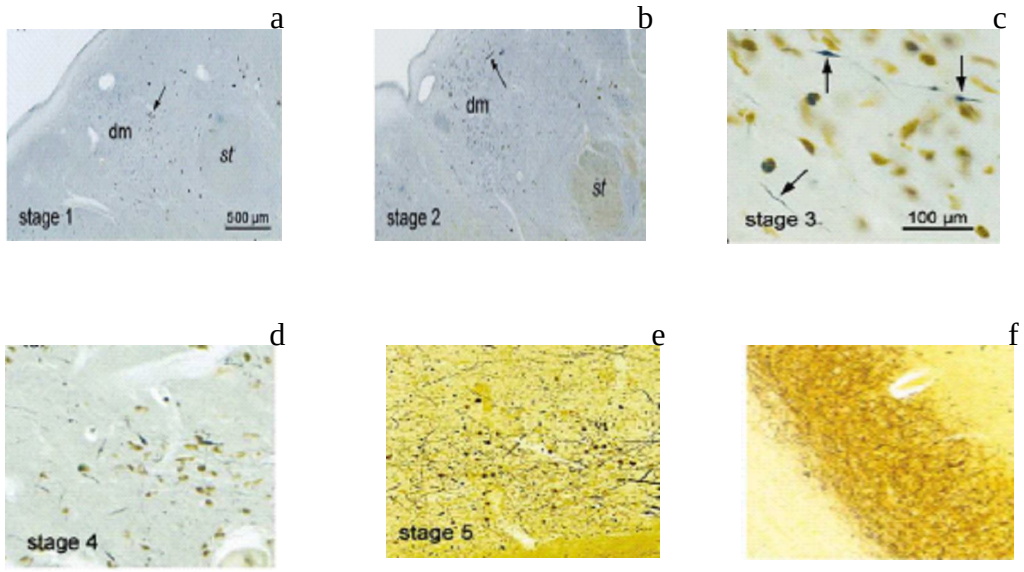

Figure 3: Development of brain stem pathology. (a-b) LN are shown by arrows;. dm= dorsal IX/X motor nucleus; st = solitary tract. (c)The elongated LNs (as indicated by arrows) showed spindle shaped enlargement in the brain stem. (d) Stage 4 presents the marked reduction of melanin neurons in substantia nigra. (e) Increased densities of LNs and LBs shown by black granules like structures.(f) LNs reaches maximum density in stage 6.

Stage 3 and stage 4 covers the lower as well as upper brain stem. Main characteristics of stage 3 are the subset of melano neurons in substantia nigra and the involvement of lipofuscin –laden projection neurons in the magnocellular nuclei of the basal forebrain are affected as shown in fig 3c (58). Stage 4 presents the marked reduction of melanin neurons in substantia nigra (59). LNs appears to be slightly reduced in thickness and there is a local accumulation of extarneuronal neuromelanin granules as shown in fig 3d (58, 60). In stage 5 more conspicuous damages are observed in subcortical and mesocortical structures (55). In this stage pathology extends from temporal mesocortex into adjoining sensory association areas of the mature neocortex as shown in fig 3e. Finally, in stage 6, cortical pathology extends further into first order sensory association areas, premotor fields and occasionally primary sensory and motor fields (53). This lead to a decline in intellectual faculties as well as impaired cognition; a frequent phenomenon in the final phase of PD (53). The number of cells containing LBs increases considerably from stage 5 to the stage 6 as shown in fig 3f.

2.4 Genetic epidemiology of PD

Familial clustering of PD was first observed by Gowers, who reported approximately 15% of his patients had a family history of PD (61). Later on Mjones described in his patients that the disease was inherited as autosomal dominant pattern with high penetrance (62).

Numerous studies reported a high frequency of PD among relatives of probands when compared with relatives of controls (63-70). These studies reported a relative risk varied from 2.3 to 14.6. Recently, two studies, using different study designs, assessed the clustering of PD in families. In the first study, using family history, authors concluded that the overall risk was modestly increased (71). Futhermore, the relatives of probands with younger onset had significantly increased risk. The results were confirmed in another study and suggested that referral and ascertainment bias might be influencing the increase in risks as reported by previous studies (72).

Twin studies were used to compare the frequency of concordance for disease in monozygotic twin pairs to that in dizygotic twin pairs. A number of studies estimated overall low heritability in monozygotic as well as dizygotic twins (73-75). Within twin pairs, the risk of developing PD was inversely associated with the dose of cigarette smoking as measured by pack-years (76). However, the protective effect of smoking in twins was less marked when comparing cases with PD to controls (77).

Lack of consistencies among twin studies result from the fact that these studies are underpowered to detect genes with incomplete penetrance. A study by Simon et al using theoretical models assessed the effect of mutations ranging from 1 to 5% present in population (78). The authors argued that at least 2000 twin pairs are required to detect genetic effect with incomplete penetrance.

What can we learn from these studies? Do these studies underestimate the genetic factors, which are thought to play an important role in PD genetics? It is quite probable that these studies are underpowered to detect the genetic effect as PD is not a fully penetrant homogeneous disease even within those families which are known to be affected by monogenic forms of PD (79).

Despite this scenario, six genes for monogenically inherited forms of PD, which account for a small fraction of all PD cases, have been identified.

2.4.1 Autosomal dominant forms of Parkinson disease

To date, two genes for autosomal dominant forms of PD have been identified. They appear to play a major role in late onset sporadic form of PD as discussed below.

α Synuclein (PARK1):

The first PD locus was mapped on chromosome 4q21-22 in a large Italian-American family, famously called Contursi family (80). So far, three point mutations have been identified in a few families with autosomal dominant PD (80-82). The first missense G209A mutation which resulted in a change from alanine to threonine at amino acid position 53 (A53T) was identified in the Contursi kindered (81). A second missense mutation which resulted in an amino acid change from alanine to proline at postion 30 (A30P) was identified in a

single German family and finally, a third mutation E46K was identified in another family with an autosomal dominant parkinsonism (82, 83). Several clinical features may distinguish patients having A53T mutations from idiopathic PD including younger onset, lower prevalence of tremor and presence of dementia and myoclonous earlier in the disease course (81, 84). By contrast, patients having A30P mutations clinically resembles to idiopathic PD. To date, no neuropathological details on patients with A30P have been published. However, neuropathological examination of the A53T brains revealed extensive substantia nigra degeneration and an abundance of alpha-synuclein Lewy bodies and Lewy neurites (84). Interestingly, patients having E46K mutations showed the clinical features of dementia and visual hallucinations besides parkinsonism (82). Neuropathological examination also revealed the extensive presence of Lewy bodies in the substantia nigra as well as in the cortex (85). Besides that a large number of PD patients were screened for mutations in the α synuclein gene (86-88). Although α synuclein mutations represent a rare cause of PD, this protein is the major component of Lewy bodies and Lewy neurites in the familial as well as in sporadic forms of PD (85).

The importance of α synuclein was further revealed when Singleton et al showed in his seminal paper that a genomic triplication of SNCA can cause parkinsonism with or without dementia (89). This was replicated in other families as well (90, 91). The importance of this finding was that it showed an increase of wild type SNCA is likely to be a cause of dopaminergic neuronal loss in PD. Interestingly, SNCA multiplication results in a doubling of the amount of alpha synuclein in blood as well as in the cortex and in the cerebellum (89). All these evidences suggested that alpha synuclein aggregation can be promoted by presence of increased expression of the wild type of alpha synuclein (92). Finally, the findings of kindered with duplications as well as triplication of the alpha synuclein gene locus implicates the dosage effect of the gene in the pathogenesis of PD (90, 91). All these evidences suggest that even a subtle variation in the expression of SNCA gene might play a role in the pathogenesis of PD. Alleles at NACP –REP1, polymorphic marker located 10kb upstream of the SNCA gene, were found to be associated with PD. A study by Chiba-Falek

and Nussbaum using luciferase reporter assay, identified two segments, few hundred base pair in length, which flanked the NACP-REP1, acted in additive manner to increase the promoter function whereas NACP-REP1 itself was shown to have negative effect on expression (93). Futhermore, Chiba-Falek et al identified a PARP1, a DNA-binding protein and transcriptional regulator, binds to NACP-REP1 and reduced the transcriptional activity of SNCA promoter in luciferase reporter assays (94). The authors suggested that association of different NACP-REP1 alleles with PD may be, in part, associated with PARP1 on SNCA expression.

The findings of functional studies are in agreement with association studies. In this context, a number of studies suggested a common variability located 4kb upstream of the transcriptional start site of SNCA associated with sporadic PD ((95-97). This was confirmed in a recent meta analysis which showed the same results, although exact relationship between promoter variability and α synuclein expression requires further studies (98).

To further delineate the signal, a study by Müller et al showed a strong association of a haplotype comprising exon 5 and 6 and the 5` untranslated region (UTR) with PD: conferring a risk of 1.4 in heterozygote carriers of the risk haplotype and about 2 in homozygote carrier (99). Taken together data from expression studies as well as from association studies clearly established a mechanistic link between neurodegeneration and alpha synuclein abnormalities.

Leucine rich repeat kinase 2 (PARK8)

LRRK2 was another gene to be included in the list of known genes in PD pathogenesis (100). The PARK8 locus was originally mapped as a dominant trait in a Japanese family with late onset of PD (101). Subsequently, a study by Zimprich et al further delineated the region harboring common haplotypes segregating in the affected family members (102). This led to the identification of multiple mutations in the LRRK2 gene. The encoded protein was also called dardarin (103). The gene consists of 51 exons, encoding 2527 amino acids and approximately covers a genomic region of 144kb. The discovery of LRRK2 was subsequently confirmed in several families and subsequent sequencing of the gene revealed multiple mutations (104, 105).

In silico experiments have predicted five conserved C –terminal domains, which are thought to be involved in multiple functions. LRRK2 is highly conserved among vertebrates and shares homology to the ROCO protein family (100).

One common mutation, Gly2019Ser, is the most common in Caucasian population which explains up to approximately 1-2% of patients with PD and approximately 5% of patients with familial PD (106-110). Recently two studies showed that the Gly2019Ser mutation is the most common cause of parkinsonism in Ashkenazi Jews as well as in the North African Arab population (111, 112).

The Gly2019Ser mutation is associated with a common haplotype that is smallest in the Arab population indicating that the mutation might have originated in the middle east (112).

Patients with LRRK2 mutations show a remarkable variability in pathology. These pathological changes include typical Lewy body disease that is consistent with *post mortem* diagnosis of definite PD (113). However, other cases showed nigral degeneration without distinctive histopathology and also progressive supranuclear palsy –like tau aggregation (114). To date, functional data is still very limited. However, LRRK2 is expressed in most brain regions including substantia nigra pars compacta, caudate nucleus and putamen (100). Intriguingly, LRRK2 is postulated to be a member of the RIP kinase family of proteins, which are essential sensors of cellular stress (115). Further genetic and functional studies are required to identify the LRRK2 modifiers; either genetic or environmental that influences the age of onset or the progression of the disease.

2.4.2 Autosomal recessive forms of PD

Early onset parkinsonism is used to define patients with onset of parkinsonian syndrome before age of 40 years. Early onset parkinsonism is further subdivided into cases with onset before age 21 years defined as juvenile parkinsonism and those with onset above 21 years referred to young onset parkinsonism (YOPD). To date, mutations in parkin, PINK1 and DJ1 and ATP13A2 are known to cause the early onset of parkinsonism.

Parkin (PARK2)

The first mutations in the parkin gene were identified in a Japanese families with an autosomal recessive form of juvenile parkinsonism (ARJP) (116, 117). The gene consists of 12 exons with a very large intronic segment, and approximately covers a distance of 500kb (116). It encodes a protein of 465 amino acids with an N terminal ubiquitin like domain and two RING finger domains (116). Parkin is perceived to function as an E3-ligase, conjugating ubiquitin to proteins to target them for degradation by the proteosomal pathway (118). From a clinical perspective, the phenotype of the patients having parkin mutations is comparable with patients without parkin mutations. A study by Lohman et al assessed the phenotypic effect of parkin mutations in a large series of patients with and without parkin mutations (119). It was concluded that patients with point mutations have only a partial loss of parkin function and tend to be more mildly affected than patients with deletions (119).

Point mutations in the parkin gene are the most common cause of the juvenile form of PD, although exonic rearrangements, deletions and duplications are also common (68). Clinically, homozygous and compound heterozygous mutations in the parkin gene account for approximately half of the cases of autosomal recessive young onset PD, especially in juvenile patients having an age of onset under 21 years of age (120-122). However, mutations in the parkin gene are rare cause of PD in individuals over the age of 40 years.

The role of parkin mutations, in a heterozygous state, is still a controversial issue. A study by Khan et al reported heterozygous mutation carriers, in a large family with parkin mutations, presenting with minor parkinsonian symptoms (123). On the other hand, a study by Lincoln et al did not show any significant differences, when comparing the frequency of parkin mutations in patients with late onset of PD with elderly healthy individuals (124). Similarly another study reported 12 asymptotic heterozygous carrier of a particular parkin mutation (ex3delta40) in a large family (125). To further assess the role of hetrozygous mutations in the Parkinson pathogenesis, a recent study showed reduced uptake of fluorodopa on position emission tomography (126). At present, the

data is still insufficient to assess the role of single heterozygous parkin mutations in the development of PD (127).

Autosomal recessive parkinsonism due to deletions and mutations in the parkin gene are associated with degeneration of pigmented neurons in the substantia nigra, similar to that seen in Parkinson disease, but Lewy bodies are not observed. A study by Farrer et al reported a patient having compound heterozygous parkin mutation with synuclein positive Lewy bodies suggesting that parkin may play a role in typical lewy body PD ((128, 129). Moreover, parkin mutations have been found to be associated with tau pathology, indicating that parkin may be involved in multiple pathogenic routes to parkinsonism (130).

Novel functions of parkin are now being identified which strengthens the importance of the involvement of parkin in the pathogenesis of PD. In this regard a recent study reported the involvement of the parkin gene in oxidative stress and mitochondrial damage (131). This evidence indicates that proteosomal dysfunction may not be the sole mechanism contributing to neurodegeneration in parkin related disease.

PTEN-induced kinase1(PINK1) (PARK6)

Homozygous mutations in the PINK1 gene have been identified as responsible for another form of autosomal recessive young onset PD (132, 133). The gene consists of 8 exons and contains 581 amino acids. PINK1 encodes a putative protein kinase (132). The gene is ubiquitiously expressed and contains a mitochondrial targeting motif and a highly conserved protein kinase domain, which is also found in the Ca /Calmodulin family of serine threonine kinases (132).

So far limited epidemiological data are available on population frequencies of PINK1 mutations. Valente et al identified 2 homozygous mutations affecting the PINK1 kinase domain in 3 consanguineous families with Parkinson disease: a missense mutation at a highly conserved amino acid and a nonsense mutation (132). Similarly, another study by Hatano et al identified 6 pathogenic mutations in 6 unrelated families in the PINK1 gene (134). All these evidence suggests

that PINK1 may be the second most common causative gene next to parkin in early-onset autosomal recessive Parkinson disease.

Mutations in the PINK1 gene have special significance within the context of linking PD with mitochondrial dysfunction. Valente et al reported that in cell culture studies wild type PINK1 seems to protect the neurons from stress induced mitochondrial dysfunction as well as stress induced apoptosis (132). A recently published study generated and characterized the loss of function mutants of Drosophila Pink1 (135). They showed that Pink1 mutants exhibit dopaminergic neuronal degeneration accompanied by locomotive defects (135). Furthermore, transmission electron microscopy analysis and a rescue experiment with Drosophila Bcl2 demonstrated that mitochondrial dysfunction accounts for the degenerative changes in all phenotypes of Pink1 mutants (135).

DJ1(PARK7)

Mutations in DJ1 are another cause of autosomal recessive parkinsonism. DJ1 contains 8 exons that span 24kb and is located 25cM telomeric of PINK1 on chromosome 1 (136). Recessively inherited deletions and missense mutations have been identified in family based linkage studies (137, 138). These mutations are rare, causing overall <1% of early onset parkinsonism (137, 138).

The DJ1 protein is a member of the ThiJ/Pfpi family of molecular chaperones, which are induced during oxidative stress (139, 140). In humans, clinically unaffected DJ1 mutation carriers seem to be normal in brain neuroimaging studies, indicating that a complete loss of protein function is required for the disease (141).

Still, the functional data on DJ1 are preliminary but there is evidence linking DJ1 to oxidative stress response and mitochondrial function. A study by Caniet – Aviles et al reported that in the presence of oxidative stress wild type DJ1 is associated with neuroprotection (142). Interestingly, DJ1 is expressed mostly in astrocytes, stressing the importance of glial –neuronal interaction in PD (142). This hypothesis was also supported in another published study who argued that although DJ1 protects against neuronal stress, however the loss of DJ1 alone may not be sufficient to cause parakinsonism (143).

ATPase type 13A2 (ATP13A2) PARK9

This locus was described in a Jordanian family with features closely resembling to idiopathic PD (144). Hampshire et al identified a region of 9 cM between markers D1S436 and D1S2843 on chromosome 1p36 likely to contain the gene associated with this phenotype (145). A maximum multipoint lod score of 3.6 was obtained between markers D1S1592 and D1S199. The locus showed an autosomal recessive mode of inheritance (145). The locus was named Kufor-Rakeb syndrome, after the community from which the affected individuals originated. Recently, Ramirez et al identified a loss of function mutation in the predominantly neuronal P-type ATPase gene ATP13A2 as the cause of Kufor-Rakeb syndrome (146). The gene consist of 29 exons encoding 1180 amino acids and covers approximately 26kb. Mutations were identified in a large non consanguineous Chilean family with early –onset Parkinson disease resembling the original Kufor-Rakeb family (146). They identified two compound heterozygous mutations in all affected individuals. The first mutation is a one base pair deletion in exon 26 leading to a frameshift and a stop codon and the second is a splice site mutation affecting exon 13. Northern blot analysis detected ubiquitous expression of a 3.8-kb transcript, with strongest expression in brain (146). Dot blot analysis confirmed predominant expression in adult human brain and demonstrated high expression in fetal brain and all tested sub regions of the adult central nervous system, including substantia nigra (146). The involvement of lysosomal ATPase encoding gene in the pathogenesis of PD is intriguing. Recently, lysosomal system has been implicated in the degradation of alpha synuclein (147). Moreover, the discovery of heterozygous mutations in the glucocerebrosidase gene (GBA) which cause the Gaucher disease have also been implicated as a risk factor for PD (148).

Besides the above described genes, other genes have been implicated in the pathogenesis of PD.

Ubiquitin carboxy terminal hydrolase (UCHL1)

UCHL1 is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer (149). Expression of UCHL1 is highly specific to neurons. UCHL1 represents 1 to 2%

of the total soluble brain protein. Its occurrence in Lewy bodies and its function in the proteasome pathway make it a compelling candidate gene in Parkinson disease (150). Leroy et al identified a missense mutation (I93M) in a small German family (150). They suggested the mutation lead to aberrations in the proteosomal pathway and aggregation of proteins. The role of UCHL1 gene has been controversial. To further explore the involvement of UCHL1, a study by Osaka et al observed that UCHL1is associated and colocalized with monoubiquitin and elongated ubiquitin half-life (151). In the gad mouse, in which function of UCHL1 is lost, was demonstrated the reduced level of monoubiquitin in neurons. In contrast, overexpression of UCHL1 caused an increase in the level of ubiquitin in both cultured cells and mice. The authors suggested that UCHL1, with avidity and affinity for ubiquitin, may insure ubiquitin stability within neurons (151).

To further confirm the involvement of the UCHL1 in PD, Lincoln et al sequenced the entire coding region of the UCHL1 gene in 11 families with a pattern consistent with autosomal dominant inheritance (152). Although they found polymorphisms in noncoding regions, the only amino acid change was S18Y (152). The S18Y allele was found in approximately 20% of chromosomes in a Caucasian population, suggesting that it is unlikely to be pathogenic (152). A number of studies showed inconsistent results regarding the involvement of UCHL1 in PD pathogenesis (153, 154). Recently, Maraganore et al performed a collaborative pooled analysis of data from 11 published studies of the UCHL1 S18Y variant and Parkinson disease (155). From a total of 1,970 cases and 2,224 controls, they found an overall inverse association of S18Y with PD. Carriers of the variant allele (Y/Y plus Y/S compared to S/S) had an odds ratio (OR) of 0.84, and homozygotes for the variant allele (Y/Y compared to S/S plus Y/S) had an OR of 0.71. There was a linear trend in the log OR consistent with a gene dosage effect (155). The inverse association was most apparent for young cases compared with young controls. However, these findings should be interpreted with caution as the possibility of publication bias can not be excluded.

Omi

Strauss et al performed a mutational screening of the HTRA2 gene in 518 German patients with PD (156). They described the phenotype of 4 German PD patients with a mutation in the HTRA2 gene (156) . They also reported that a novel polymorphism (A141S) is associated with PD. Clinical symptoms of patients having mutations resemble with idiopathic PD ((156). All patients responded well to levodopa therapy. Immunohistochemistry and functional analysis in stably transfected cells revealed that S399 mutant HTRA2 and to a lesser extent the S141 risk allele polymorphism induced mitochondrial dysfunction associated with altered mitochondrial morphology. They concluded that their results provided a novel link between mitochondrial dysfunction and neurodegeneration in PD (156).

Nurr1

Mutations have also been described in the Nurr-1 gene (157). Several studies fail to confirm mutations in different population suggesting that the Nurr-1 gene is unlikely to play an important role in the pathogenesis of PD (158, 159).

Glucocerebrosidase gene (GBA)

Recently, mutations in the GBA gene have been reported to be associated with PD in Ashkenazi Jews (148). A total of 99 Ashkenazi patients with idiopathic Parkinson's disease, 74 Ashkenazi patients with Alzheimer's disease, and 1543 healthy Ashkenazi Jews were screened for the six GBA mutations. Among 99 Ashkenazi patients with PD, 28 were heterozygous and 3 were homozygous for one of these mutations. Moreover, among 74 Ashkenazi patients with Alzheimer disease (AD), 3 (4.1%) were carriers of Gaucher disease and among 1,543 controls, 95 (6.2%) were carriers of Gaucher disease. They reported that patients with PD had significantly higher risk of being carriers of Gaucher disease than did patients with Alzheimer disease (OR = 10.8) or controls (OR = 7.0) (148). They suggested that some GBA mutations are susceptibility factors for Parkinson disease.

Besides that studies on fibroblast growth factor 20 (FGF20) and Brain derived neurotrophic factor (BDNF) genes provided inconsistent results regarding their

involvement in PD pathogenesis (160-163) (see chapter 3.2 and chapter 3.3 for details).

2.4.3 Parkinson disease locus

PARK3

This locus was described by Gasser et al on 2p13 in familial parkinsonism with clinical features closely resembling those of sporadic Parkinson disease, including a similar mean age of onset: 59 years in these families, 59.7 years in sporadic PD (164). The maximum lod score of 3.96 was obtained in all the six families which were under study (164). Still, the gene responsible for the disease has not been identified. Destefano et al using variance component methodology conducted a genome wide linkage scan to identify genes modulating the age of onset in PD (165). They reported a maximum lod score of 2.08 in the PARK3 locus. They concluded that allele 174 of marker D2S1394 is associated with age of onset. Closest gene in this vicinity is SPR (165) . Karamohamed et al genotyped 23 SNPs around the marker D2S1394, which covers the SPR gene in 281 sibpairs. They reported that one SNP, rs1876487, is significantly associated with age of onset (166). These results were further confirmed in a recently published study (167). Taking together these evidence suggest that SNP rs1876487 in the SPR gene might be responsible for modulating the progression of the disease. However, expression studies are now warranted to confirm this association.

PARK10

Hicks et al performed a genome wide linkage scan on 117 Icelandic patients with classic late-onset Parkinson disease (mean age of onset 65.8 years) and 168 of their unaffected relatives from 51 families (168). They found linkage to chromosome 1p32, and further analysis yielded a lod score of 4.9 near marker D1S2652 within a 7.6-cM segment. The Hugo committee designated this locus PARK10.

PARK11

Pankratz et al reported linkage to 2q in a sample of sib pairs with Parkinson disease. They further expanded the sample to include 150 families meeting their strictest diagnostic definition of verified Parkinson disease (169). To delineate further the chromosome 2q linkage, they performed analyses using only those pedigrees with the strongest family history of PD. Linkage analyses in this subset of 65 pedigrees generated a lod score of 5.1, which was obtained using an autosomal dominant model of disease transmission. They designated this locus as PARK11(169). Recently, Prestel et al performed a linkage analysis in 45 European sib pair families with strong familial history of PD. This study did not show any evidence of linkage in the PARK11 locus (170). It is, therefore, unlikely that PARK11 may play a major role, at least in European population. Nevertheless, the gene responsible for this locus has not been identified yet (170).

PARK12

Pankratz et al performed a genome wide linkage analysis for PD in 160 multiplex families (171). The families were screened for parkin mutations. After excluding the families with parkin gene mutations they obtained a lod score of 2.1 on the X chromosome (171). They observed that the same region was also providing evidence of linkage in other samples as well. Nevertheless, the gene responsible for this locus has also been not identified yet.

2.5 Animal Models

Long before the discovery of genes involved in the familial form of PD, investigators used animal models in order to dissect the underlying pathology. Animal models relied upon administration of toxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone or paraquat to destroy dopaminergic neurons. The use of toxins, to understand the mechanism, is based on the observation by Langston who reported that the use of MPTP can kill dopaminergic neurons and produce a severe parkinsonian phenotype (172). Although, MPTP destroy dopaminergic neurons, still it is not clearly understood the mechanism by which MPTP works (173). Since the discovery of genes (as

discussed in the above section) involved in PD, researchers have used these mutations by expressing them in animal models, to understand the mechanism that causes the sporadic form of PD (174). It has been shown that mutations in Parkin and UCHL-1 affect proteasomal function, which has also been implicated in sporadic PD. PINK-1 and DJ1 are mitochondrial proteins. Finally, α-synuclein accumulates in central and peripheral neurons of patients with sporadic PD. Several lines of mice were generated either by over expressing wild type or mutant α-synuclein (175, 176) . Several of these mice show progressive motor deficits, abnormal dopamine function, and in some cases, even reproduce non-motor deficits observed in PD patients and delayed loss of striatal dopamine and/or tyrosine-hydroxylase positive neurons in the substantia nigra pars compacta (175). These models are useful to understand the mechanism by which α-synuclein accumulation leads to cell death in vivo. It has been shown that dopaminergic neurons can also be killed with toxins in flies or in worms. The new addition of animal models opened a whole new avenue for researchers to do rapid genetic manipulations to decipher the pathophysiology of the disease in the brain even before the actual nigral degeneration takes place (177, 178).

Chapter 3.1: Comprehensive association analysis of NOS2A with Parkinson's disease

Schulte C, **Sharma M,** Mueller JC, Lichtner P, Prestel J, Berg D, Gasser T. Comprehensive association analysis of the NOS2A gene with Parkinson disease. **Neurology 2006;67(11):2080-2.**

Inducible nitric oxide synthase (iNOS) is an enzyme that produces nitric oxide (NO), a free radical serving as a messenger molecule. Changes in the activity of iNOS have consequences on the amount of NO production. Therefore, overproduction contributes to oxidative stress, which might lead to devastating effects on cellular mechanisms. NO combines with superoxide anions and forms peroxynitrite. This can nitrate or nitrosylate proteins, lipids and DNA, leading to an impairment of mitochondria and an overload of the degradation pathway. Alterations like these are also observed in the development of Parkinson's disease (PD) (179).

Involvement of iNOS in the pathology of PD is further demonstrated by an iNOS deficient mouse model being protective against loss of dopaminergic neurons when treated with the neurotoxin MPTP as a model of PD (180).

NOS2A is the gene encoding iNOS and spans over 40kb on chromosome 17. A previous study showed a linkage peak for PD risk in this chromosomal region encompassing *NOS2A* (181). Two studies reported a genetic association of *NOS2A* with PD risk. The first study found an association of a single nucleotide polymorphism (SNP rs1060826) in exon 22 of *NOS2A* in French PD patients. A protective effect was reported against PD with genotype A/A (OR=0.5; p=0.01) (182). The second study confirmed this association in Finish PD patients (OR=0.5; p=0.045) (183).

Here, we performed a replication study to assess the previously reported association of the iNOS gene with PD in German patients. We genotyped ten SNPs in the coding region with an average spacing of 3.5 kb and two promoter microsatellites. These microsatellites were chosen because they are

functionally relevant by leading to allele-dependent expression differences. The linkage disequilibrium (LD) structure was determined and common haplotypes were identified to assess association with PD risk.

Methods

Subjects

PD patients were recruited by participating institutions in Munich and Tübingen. Specialists in movement disorders examined the patients, and diagnosed them according to the PD Society Brain Bank criteria. Blood samples were drawn for DNA extraction from 340 German sporadic PD patients, after obtaining informed consent. The median age at onset was 52 years. A total of 680 healthy, age- and sex-matched subjects from the KORA Survey 2000 were used as control individuals. These controls were sampled population-based. They have a median age at examination of 52 years, matched in age intervals of 5 years to the age at onset of the cases. Cases and controls originate from the southern part of Germany.

Genotyping

Ten SNPs in *NOS2A* were selected, comprising seven intronic and three exonic variants. One of the exonic polymorphisms (rs2297518) leads to a change of the amino acid sequence (L608S). The promoter region was analysed by examining two microsatellite polymorphisms. The multiallelic CCTTT pentanucleotide repeat is located 2.6kb upstream of *NOS2A*. The biallelic 4bp deletion / duplication in an AAAT-repeat-motif is located 0.7kb upstream of *NOS2A*. The analysis of these microsatellite polymorphisms were carried out with a PCR-reaction followed by fragment analysis with the ABI 3100 Avant Genetic Analyzer and the Gene Scan Analysis software (ABI). The SNP (rs1060826) in exon 22 was genotyped by restriction enzyme analysis using previously described primers and conditions (182). The genotyping of the remaining SNPs were performed using the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry method (MassArray system, Sequenom, San Diego, CA).

Statistical analyses

Allele frequencies of the multiallelic microsatellite were compared between patients and controls using CLUMP (184). Logistic regression analysis using UNPHASED (185) was carried out to assess single marker association of the biallelic markers. Genotypic association of SNPs with PD was tested under different risk models: no specific risk model and recessive model with each allele as the risk allele. Linear regression analysis was performed to assess the effect of age at onset in our sample. The linkage disequilibrium (LD) structure was determined by HAPLOVIEW ((186). The sample set studied here possesses a power of over 99% to replicate the previously described association (OR 0.5) (182, 183).

Results

Genotype distributions of all polymorphisms were found to be in Hardy-Weinberg Equilibrium. The biallelic polymorphisms (SNPs and AAAT-repeat) had a minor allele frequency higher than 10%. The multiallelic polymorphism (CCTTT-repeat) displayed 13 different alleles with allele frequencies ranging from 0.05% to 33%. All SNPs were in strong LD (high D'), reflecting the fact that little historic recombination has taken place over the generations. Two LD blocks according to the algorithm described by the Gabriel *et al* can be detected (187). One block covered a region of 15kb from rs1962380 to rs4796052 and a second block ranged from rs1137933 to rs3730013 spanning a genomic region of 19kb. The correlation (r^2) among markers, however, is low, as shown in figure 4.

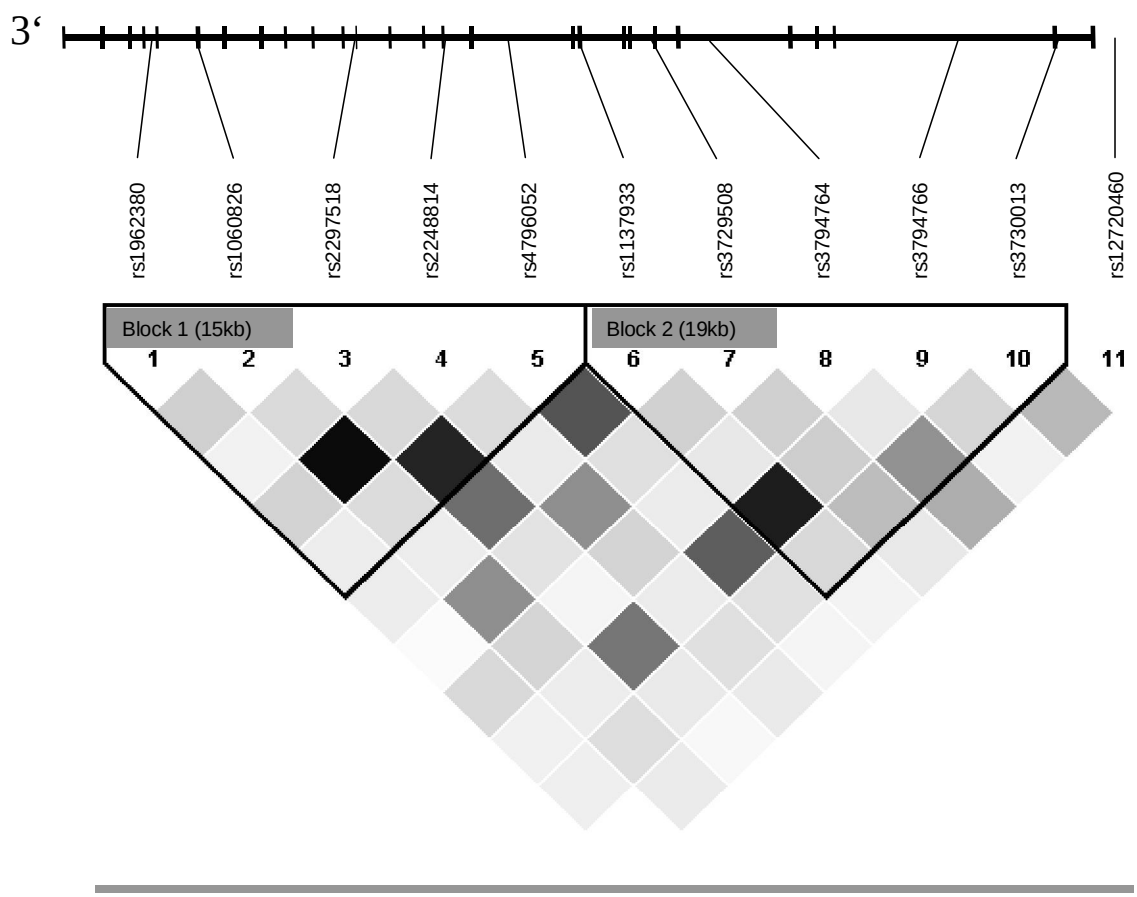

Figure 4: Linkage disequilibrium structure of NOS2A gene. *Shading according to measured r^2 values. black cells: high r^2; white cells: low r^2. D'-blocks are indicated. The gene structure of *NOS2A* is shown on top, with vertical bars indicating exons.

Although only six of the 35 SNPs in the *NOS2A* region genotyped by the HapMap project (the International HapMap Consortim 2003) correspond to our SNP set, the block structure identified in HapMap corresponds well to our structure (188).

We could not observe any significant differences in allele frequencies between cases and controls in our sample population and there was no significant association of the genotypes with PD, as shown in table 4.

Table 4: Association tests with PD for dominant and recessive risk models in the overall data set.

SNP ID	Allele Major/ Minor	Frequency of major allele		Recessive risk model of major allele		Recessive risk model of minor allele	
		Case	Control	p value	Odds ratio (95%CI)	p value	Odds ratio(95%CI)
rs1962380	A/T	0.234	0.233	0.660	1.06 (0.81-1.38)	0.404	1.30 (0.69-2.46)
rs1060826	G/A	0.372	0.373	0.622	0.93 (0.71-1.22)	0.541	0.88 (0.60-1.30)
rs2297518	G/A	0.179	0.206	0.228	0.84 (0.63-1.11)	0.268	1.54 (0.71-3.32)
rs2248814	G/A	0.389	0.379	0.784	0.96 (0.73-1.26)	0.657	0.91(0.63-1.33)
rs4796052	C/T	0.183	0.208	0.298	0.86 (0.65-1.13)	0.240	1.57 (0.73-3.39)
rs1137933	C/T	0.234	0.230	0.654	1.06 (0.81-1.38)	0.591	1.18 (0.63-2.20)
rs3729508	G/A	0.393	0.379	0.609	1.07 (0.81-1.40)	0.638	0.91 (0.63-1.31)
rs3794764	G/A	0.236	0.229	0.471	1.10 (0.84-1.43)	0.455	1.28 (0.66-2.47)
rs3794766	C/T	0.243	0.241	0.862	1.02 (0.78-1.33)	0.854	1.05 (0.59-1.87)
rs3730013	C/T	0.353	0.353	0.656	1.06 (0.81-1.38)	0.553	1.14 (0.73-1.75)
rs12720460	-/+	0.122	0.141	0.273	0.84 (0.62-1.14)	0.433	1.67 (0.45-6.11)

genomic position according to NCBI build 35; MAF = minor allele frequency; CI = confidence interval; -/+ = deletion/duplication

In particular, the allele frequency of the SNP rs1060826 was similar to the frequencies in the cases in the previous studies. The A/A genotype, however, was not protective in our sample (OR=1.064; CI=0.704-1.609; p=0.76). The multiallelic microsatellite was not associated with PD. On stratifying by gender, there was also no significant association (data not shown). We did not observe any influence of an age at onset effect in our sample. Furthermore, an analysis of haplotypes within blocks and all 2 and 3-locus haplotypes in a sliding window approach did not reveal any significant association.

Discussion

We genotyped twelve polymorphisms and characterised the LD structure of *NOS2A*. All of these polymorphisms did not show any association with PD. We therefore have no evidence that common variants in iNOS contribute significantly to PD risk, at least in our sample population.

Previously, two studies reported the association of rs1060826 with PD in two different populations. The association of PD with the A/A genotype of rs1060826 was first described in 209 French PD patients (182). This association was confirmed in 147 Finish patients (183).

Several reasons can explain these discrepancies. An effect of the SNP rs1060826 might be small in our population, suggesting genetic heterogeneity, i.e. a risk factor for only a subgroup of the population. Moreover, the patients from Finland represent an isolated population, thus increasing the chances to detect small risk effects. However, it can also not be excluded that the previous studies, due to smaller sample sizes, report a false positive result.

We genotyped an adequate number of SNPs to cover the coding region as well as the promoter region of *NOS2A.* Other than the previously published two studies, we have saturated the whole gene with a high SNP density, so it is unlikely that we have missed an association even if the reported SNP is in the LD with “true causal” variant. Finally there is a difference between the ages at onset between the French sample population (65 years median age at onset), the Finish sample population (67.2 years mean age at onset) and the present study (52 years median age at onset).

Damage due to oxidative stress, the mechanism proposed for iNOS involvement, might need to accumulate in a longer period of time until clinical symptoms appear. Therefore alterations leading to a higher level of oxidative stress could be risk factors for disease symptoms at an older age, hence only detectable in patients with a late onset. Our population is relatively young, and therefore possibly not able to display association with a susceptibility locus for late onset PD. Hence, although we did not find evidence for it in our population, it can not be excluded that iNOS might still be involved in the development of PD.

Chapter 3.2: Comprehensive association study and meta analysis of BDNF gene with Parkinson disease

Manu Sharma, Claudia Schulte, Jakob C Mueller, Peter Lichtner, Daniela Berg, Bertram Müller –Myhsok, and Thomas Gasser. Comprehensive association study and meta analysis of BDNF gene with Parkinson disease **(Submitted)**

Several studies have reported conflicting results regarding the effect of BDNF gene polymorphisms, including two frequently studied polymorphisms: G196A and C270T, on the risk to develop PD (163, 189-192). A study by Momose et al reported an association of G196A polymorphism with PD in the Japanese population (189). However, another study from the same population reported contradicting results (190). Other studies also reported no genetic association (162, 163, 191, 192). A study by Parsian et al identified two novel polymorphisms at position C1331T and C270T. They concluded that C270T polymorphism was influencing the age of onset in PD in familial cases (162).
Here, we performed an association study to assess the previously reported association of the BDNF gene with PD in German patients. We genotyped five SNPs, including three previously described polymorphisms, rs6265 (G196A), C270T and C1331 polymorphism in our cohort. The linkage disequilibrium (LD) structure was determined and common haplotypes were identified to assess association with PD risk. Furthermore, we performed a meta analysis to assess the risk of G196A polymorphism and PD.

Methods

Subjects

PD patients were recruited mainly by participating institutions in Munich and Tuebingen Specialists in movement disorders examined the patients. Diagnosis was established according to U.K Brain Bank criteria (2). After appropriate informed consent, blood samples were drawn for DNA extraction from 340 German sporadic PD patients. The median age of onset was 55.4 years. A total

of 680 age and gender matched subjects from the KORA (Cooperative Research in the region of Augsburg) Survey 2000 which studied a large population – based sample, were used as controls.

Genotyping

Genotyping of SNPs was done by primer extension of multiplex PCR products with the detection of the allele-specific extension products by matrix-associated laser desorption/ionisation time of flight (MALDI-TOF, Sequenom, San Diego, USA) mass spectrometry. The genotype frequencies of successfully typed SNPs were in Hardy-Weinberg equilibrium (HWE).

Meta Analysis

For the meta analysis, published data were used from previously published studies. In addition our own data were included. The studies were identified with Pubmed/Medline using the following key words: BDNF and Parkinson disease, BDNF and Parkinson disease association studies. The search was restricted to articles in English. Case control studies that assessed the distribution of rs6265 polymorphism is cases and controls were eligible for inclusion in the meta analysis. A total 7 studies were included in the final analysis.

Statistical Analysis

We use the D' and r^2 measure of characterize the LD as implemented in Haploview (186). Genotype association with PD was tested under different assumptions in a logistic regression setup: recessive model with each of the alleles as risk alleles and trend model assuming a dose effect of alleles. For the meta analysis, a pooled odds ratio (OR) was estimated using fixed effects and random effect models. Fixed effect model assumes that risk to disease is same in all studies and that observed differences are due to chance. Random effect models allows that risk to disease might be different due to heterogeneity across different studies. Random effect models take into account the between-study heterogeneity. Heterogeneity between studies were tested using χ^2 based Q statistics. It is quantified with I^2 metric, which ranges from 0% to 100%

(I^2<25% reflects no heterogeneity , 25-50% moderate heterogeneity, 50-70% large heterogeneity and >70% suggest very large heterogeneity). The meta analysis and heterogeneity analysis was performed using Review Manager 4.2.7. (RevMan Analyses version 1.0; Cochrane Collaboration –Winter tree Software Inc). The power of our study to identify an association with odds ratio at least 2.00 was greater than 99% (α =0.05).

Results

High D' was observed among all five SNPs reflecting that there has been little recombination taken place over the generations in the BDNF gene. However, based on r^2 indicate relatively independent SNPs as shown in table 5.

Table 5: Measures of linkage disequilibrium between SNPs

SNP	rs6265	C270T	C1331T	rs7934165	rs12273363
rs6265	…..	0.01	0.06	0.25	0.04
C270T	1.0	…..	0.01	0.06	0.01
C1331T	0.97	1.0	…	0.22	0.72
rs7934165	1.0	1.0	0.92	…	0.20
rs12273363	0.93	1.0	0.97	0.99	…..

r^2 values are given above the diagonal, and D' values are given below the diagonal

In the overall sample, we could not observe any significant differences in allele frequencies between cases and controls in our population. After testing different genetic risk models, there was no significant association of the genotypes with PD, after adjusting for multiple testing as shown in table 6.

Table 6: Association tests with PD for dominant and recessive risk models in the overall data set.

SNP	Allele Major /Minor	Frequency of major allele		Dominant model		Recessive model		P value§
		Case	control	P value	Odds ratio	P value	Odds ratio	
rs6265	G/A	0.786	0.803	0.206	1.18(0.90-1.55)	0.610	1.21(0.57-2.57)	1.0
C270T	C/T	0.940	0.943	0.225	0.25(0.02-2.79)	0.949	1.01(0.61-1.54)	1.0
C1331T	C/T	0.788	0.785	0.816	1.07(0.78-1.45)	0.917	0.98(0.75-1.29)	1.0
rs7934165	G/A	0.525	0.513	0.667	1.07(0.78-1.45)	0.021	0.70(0.52-0.95)	0.087
rs12273363	T/C	0.824	0.829	0.676	1.06(0.80-1.41)	0.867	1.06(0.52-2.14)	1.0

MAF=minor allele frequency; CI = confidence interval; SNP = single nucleotide polymorphism; § p value after bonferroni correction

In the meta analysis, total of 1760 PD patients and 2100 controls free of PD patients were analyzed. The prevalence of allele A of SNP rs6265 was 48.47% and 44.51% was observed in cases and controls respectively. The meta analysis did not show significant association between the polymorphism and PD. Subjects with AA genotype compared with individual with GG genotype had a relative risk of 1.06 (95% CI 0.72-1.56, p =0.76). In fig 5, a carrier of A allele were compared with GG genotype in all studies. There was no significant difference in risk, 1.00 (95% CI 0.83-1.20, p=0.99).

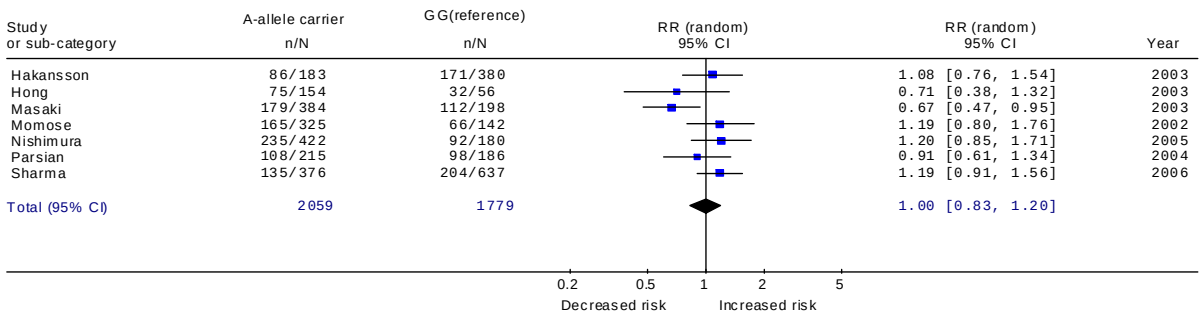

Figure 5: Meta Analysis of PD risk for carriers of A-allele vs the GG genotype carriers

RR= relative risk; CI= confidence interval ; n = number of A and G alleles; N = Total number of alleles

However, there was evidence for heterogeneity (I^2 =46.7% p = 0.08) as shown in table 7. On stratifying the analysis based on different populations, evidence for large heterogeneity was observed in Asian studies (I^2 =70.4% p = 0.02), but we did not find any evidence for heterogeneity in Caucasian population (I^2 =0% p = 0.64).

Table 7: Fixed and random effects odds ratio with different models.

Genetic Model	Population/Subpopulation	Studies	Fixed effect OR (95% CI)	Random effect OR(95%CI)	Q test p-value*
Allelic	All	7	1.02(0.93,1.13)	1.02(0.89,1,17)	0.08
Model	Asian	4	1.00(0.88,1.14)	1.00(0.78,1,28)	0.02
	Caucasian	3	1.06(0.90,1.23)	1.02(0,90,1.23)	0.61
Genotype	All	7	1.04(0.82,1.32)	1.06(0.72,1.56)	0.03
Model	Asian	4	1.03(0.79,1.35)	1.05(0.63,1.75)	0.02
	Caucasian	3	1.07(0.64,1.17)	1.09(0.54,2.20)	0.19
Recessive	All	7	1.09(0.89,1.35)	1.11(0.81,1.52)	0.07
Model	Asian	4	1.11(0.88,1.39)	1.11(0.88,1.39)	0.07
	Caucasian	3	1.05(0.64,1.73)	1.09(0.50,2.41)	0.12
Dominant	All	7	1.01(0.88,1.16)	1.00(0.83,1.20)	0.12
Model	Asian study	4	0.94(0.77,1.14)	0.94(0.77,1.14)	0.05
	Caucasian	3	1.09(0.89,1.31)	1.09(0.90,1.31)	0.54

Allelic Model=A vs G ; Genotype Model= AA vs GG; Recessive model= AA vs AG+GG ; Dominant Model=AG+AA vs GG; OR=odds ratio; CI =confidence interval; Q test = heterogeneity analysis

Discussion

We did not obtain any evidence of an association for the polymorphisms genotyped in the BDNF gene for PD in our population. However, we observed a trend for an association for SNP rs7934165 in our sample. However, it was not significant after adjusting for multiple testing.

We could not replicate the association of rs6265 in our sample as previously reported by two studies. The study by Momose et al (189) which reported an over representation of the AA homozygotes in patients with PD. However, controls included were not in HWE. The departure from HWE indicate false positive signal either due to genotyping error or due to unrecognized population admixture. Therefore, the results should be interpreted with caution (189-192). Another study by Karamohamed et al, using a familial PD sample showed rs6265 to be significantly associated with age of onset (193). The familial

background of PD in the study as compared to the sporadic background in the present study might contribute to the difference. It can be generally assumed that familial cases have a stronger genetic contribution to the development of PD than sporadic cases.

It is important to note here, that none of the published studies, using sporadic and white Caucasian subjects, have reported positive association with PD (162, 163, 194). These results fits well with our results as well as with recently published meta analysis, suggesting that rs6265 is unlikely to play a major role at least in sporadic forms of PD (194).

Another study using a haplotype tagging approach also found no association with any of the polymorphism genotyped in different populations (195). Taken together the results from our study as well the previously published studies suggest that it is unlikely that BDNF plays an important role in the pathogenesis of sporadic forms of PD.

Chapter 3.3: Association analysis of Fibroblast Growth Factor 20 gene polymorphisms with Parkinson's disease in the German population

Manu Sharma, Jakob C Mueller, Peter Lichtner, Daniela Berg, Thomas Illig, Bertram Muller-Myhsok and Thomas Gasser. Role of Fibroblast Growth Factor 20 gene polymorphisms with Parkinson's disease in the German population. **(Submitted)**

FGF20, a neurotrophic factor, expressed in the cerebellum and in the substantia nigra pars compacta, is thought to be involved in neuronal survival (196). It is conceivable that neurotrophic factors that promote survival or differentiation of neurons may play a role in neurodegeneration and are therefore good candidate genes for Parkinson disease (PD) .

A genome scan by Scott et al showed a suggestive evidence for linkage at marker position D8S520 on chromosome 8p21.3-8p22, the chromosomal region harboring the FGF20 gene (181). They further extended the work by combining gene expression data with genetic linkage analysis, and selected the FGF20 gene as a potential candidate for further follow up (160, 197). In their follow up study, van der Walt et al genotyped five SNPs in the FGF20 gene in 644 multiplex families ascertained from North America (160). They reported two SNPs (rs12720208 and rs1989754) within the FGF20 gene to be strongly associated with PD (160). We performed a replicative study in a large cohort of sporadic PD patients ascertained from Germany. We characterized the linkage disequilibrium (LD) structure of the FGF20 gene.

Methods

Subjects

PD patients were recruited by participating institutions in Munich, Tuebingen, Bonn, and Würzburg. Specialists in movement disorders examined the patients. Diagnosis was established according to U.K Brain Bank criteria (2). After appropriate informed consent, blood samples were drawn for DNA extraction

from 653 German sporadic PD patients. The median age of onset was 55.4 years. A total of 981 age and gender matched subjects from the KORA (Cooperative Research in the region of Augsburg) Survey 2000 which studied a large population – based sample, were used as controls.

Genotyping

Genotyping of SNPs was done by primer extension of multiplex PCR products with the detection of the allele-specific extension products by matrix-associated laser desorption/ionisation time of flight (MALDI-TOF, Sequenom, San Diego, USA) mass spectrometry. The genotype frequencies of successfully typed SNPs were in Hardy-Weinberg equilibrium.

Statistical Analysis

Allele frequencies between cases and controls were compared using UNPHASED (185). Genotype association with PD was further tested under different assumptions in a logistic regression setup: dominant model, recessive model and trend model assuming a dose effect of alleles. In addition a sliding window approach was used to test two or three locus haplotype associations. Permutation procedure was used to adjust for multiple testing in each tested models. Furthermore, D' and r^2 measure were used to characterize the LD as implemented in Haploview (186) . The power analysis showed a power of over 99% to replicate the previously described association.

Results

We did not obtain significant allele frequencies differences between PD patients and controls. At the genotypic level, however, a single SNP rs12720208 in the 3' UTR showed weak evidence of genotypic association with PD as shown in table 8.

Table 8: Association tests with PD for recessive risk models in the overall data set.

SNP ID	Allele Major/ Minor	Frequency of major allele		Recessive risk model of major allele		Recessive risk model of minor allele	
		Case	Control	p value	Odds ratio (95%CI)	p value	Odds ratio(95%CI)
rs12718379	C/T	0.531	0.466	0.737	1.03(0.83-1.29)	0.855	1.02(0.80-1.30)
rs1989756	G/A	0.927	0.916	0.556	0.66(0.16-2.65)	0.176	0.82(0.62-1.09)
rs1989754	C/G	0.541	0.446	0.329	1.11(0.89-1.39)	0.792	0.96(0.75-1.24)
rs1721100	C/G	0.725	0.707	0.538	1.12(0.77-1.62)	0.291	0.89(0.73-1.09)
rs12720208	C/T	0.921	0.909	0.388	0.88(0.67-1.16)	0.049	4.01(0.89-18.0)

We could not replicate the association of the second reported SNP rs1989754 in our sample population. However, when stratified by gender, the SNP rs1989754 showed significant association in males suggesting that this SNP may have a higher propensity for PD only in the male sub population as shown in table 9.

Table 9: Association test of dominant risk models in males with PD

SNP	Allele Major/Minor	Frequency of Major allele		Dominant Model	
		Case	control	p value*	odds ratio
rs12718379	C/T	0.547	0.462	0.145	1.27(0.92-1.75)
rs1989756	G/A	0.931	0.916	0.190	0.78(0.53-1.13)
rs1989754	C/G	0.514	0.449	0.027	1.39(1.03-1.86)
rs1721100	C/G	0.739	0.715	0.331	0.87(0.67-1.14)
rs12720208	C/T	0.927	0.909	0.286	0.82(0.57-1.18)

However, none of association remains significant after adjusting for multiple testing. Two and three locus haplotype did not show significant associations. The LD analysis based on r^2 indicate relatively independent SNPs as shown in table 10.

Table 10: Measures of linkage disequilibrium between SNPs

SNP	rs12718379	rs1989756	rs1989754	rs1721100	rs12720208
rs12718379	…..	0.09	0.69	0.08	0.08
rs1989756	1	…..	0.07	0.16	0.008
rs1989754	0.98	1	…	0.31	0.07
rs1721100	0.41	0.68	0.97	…	0.24
rs12720208	0.98	1	0.98	0.99	…..

Note- r^2 values are given above the diagonal, and D' values are given below the diagonal

The LD structure is in agreement with the original study (160). Among all five SNPs high D' values were observed reflecting that there has been little recombination taken place over the generations in the FGF20 gene.

Discussion

We genotyped five polymorphisms to assess the risk to PD in our population. Our results did not provide any evidence of association for these SNPs in our population after adjusting for multiple testing. We therefore have no evidence that common variants in the FGF20 gene contribute to PD risk at least in our population.

To date, two studies reported a conflicting results regarding the involvement of FGF20 gene and risk to PD. Firstly, study Van der Walt reported two most significant SNPs, rs1989754 and rs12720208 in intron 1 and 3'UTR respectively are associated with PD (160). On the contrary, study by Clarimon et al did not lend its support to the findings (161).

Several reasons can explain the discrepancy. Firstly, the study by Van der Walt was performed on familial PD data set. The familial background of PD in the original study as compared to the sporadic background in the present study might also contribute to the difference. It can be generally assumed that familial cases have a stronger genetic contribution to the development of PD than

sporadic cases. The second reason might be the effect of the variants reported by Van der Walt are small in our population suggesting genetic heterogeneity. Genetic or locus heterogeneity among populations could produce different effect strengths between different samples originating form different ethnicities or geographic areas. Indeed, we observed a minor effect for SNP 12720208 in our overall sample and for SNP rs 1989754 only in the male sub population. But it was not significant after correction for multiple testing. The third reason might be the effect of the variants are only specific for late onset of PD. Since our population is relatively young with median age of onset 55 years it is conceivable that the effects of variants reported by the original study may not detectable in our relatively young population.

It might be argued that original study by Van der Walt overestimated the effect size and true effect size conferred by FGF20 gene is much smaller and hence escaped detection in our population.

In summary, our study did not provide any evidence that FGF20 gene contribute to sporadic form of PD at least in our population. Taking together the indications from this study, and from Clarimon et al, we suggest that FGF20 did not play a major role in the sporadic form of PD at least in the European population.

Chapter 4.1: The Sepiapterin Reductase Gene Region Reveals Association in the PARK3 locus: Analysis of Familial and Sporadic Parkinson Disease in European Populations.

Sharma M, Mueller JC, Zimprich A, Lichtner P, Hofer A, Leitner P, Maass S, Berg D, Durr A, Bonifati V, De Michele G, Oostra B, Brice A, Wood NW, Muller-Myhsok B, Gasser T. The sepiapterin reductase gene region reveals association in the PARK3 locus: analysis of familial and sporadic Parkinson's disease in European populations. **J Med Genet 2006;43(7):557-62.**

Gasser et al have mapped a locus for autosomal dominant PD on chromosome 2p13 (PARK3) (164) which was further refined to 2.5 mega bases (198). Three other genome scans confirmed the PARK3 locus (199-201). In a refinement of the locus position, DeStefano et al. found evidence for association between later PD age at onset and allele 174 of marker D2S139 (165). Karamohamed et al extended the work by genotyping SNPs in the vicinity of marker D2S1394 (166). They reported association of one SNP and a haplotype across the SPR gene with age of onset in sib ships of North American origin.

Here, we thoroughly assess the SPR gene region in sporadic PD cases ascertained from Germany as well as in familial PD samples from five different European countries. After characterizing the linkage disequilibrium structure in the SPR gene region, we further refined association signals for PD susceptibility to haplotypes and SNPs within an LD block comprising the SPR gene.

Methods

We conducted our study in two parts. First, we genotyped 5 STR markers and 17 SNPs from the PARK3 core region centered around the SPR gene in a cohort of European affected siblings (122 sib-ships). In the second part of our study, additional 23 SNPs, chosen to cover the SPR gene and flanking regions, were genotyped in 340 German sporadic PD patients and 680 controls.

Recruitment of PD families

122 families from five European countries were ascertained through the European Consortium on genetic susceptibility in PD (GSPD). We included families having at least two affected siblings. A total of 35 families from Germany, 26 families from the U.K, 12 families from The Netherlands, 33 families from France and 16 families from Italy were ascertained. The characteristics of the families are described in table11.

Table 11: Characteristics of the recruited families.

Country	Number of families	Number of sibs		% Male	% Female	Age at onset (Mean ± SD)
		affected genotyped	unaffected genotyped			
Germany	35	100	44	48.8	51.2	57.4 ± 10.4
England	26	65	24	50.6	49.4	61.2 ± 7.3
France	33	84	36	49.4	50.6	56.4 ± 12.8
Italy	16	54	12	42.3	57.7	52.8 ± 16.6
Netherlands	12	23	22	52.1	47.9	58.2 ± 11.1
	122	326	138	48.6	51.3	57.6 ± 11.4

After appropriate informed consent was obtained, blood samples were drawn from the individuals for DNA extraction. Families suggestive for dominant inheritance were screened for known LRRK2 mutations. In families suggestive for recessive inheritance with early onset (< 45 years) in at least one affected, the parkin, PINK1 and DJ-1 genes were completely sequenced and mutations were excluded.

Recruitment of sporadic PD patients

PD patients were recruited mainly from the Dept. of Neurology at the University of Munich and Tuebingen. Specialists in movement disorders examined the patients. Diagnosis was established according to U.K Brain Bank criteria (2).The median age at onset was 55.4 ±19.1 years. A total of 680 healthy, age and gender matched subjects from the KORA (Cooperative Research in the Region of Augsburg) Survey 2000, which studied a large population–based sample, were used as controls.

Genotyping

We genotyped the 5 STR markers D2S2110, D2S1394, D2S2111, D2S145, D2S2109 with an average spacing of 0.2 cM from the PARK3 core region as defined by West et al (148).The new marker order was obtained from the Marshfield Genetic Laboratories Map. Mendelian inconsistencies in the genotypic data were checked by using the program PedCheck (149).

Genotyping of SNPs: Within the PARK3 core region we focused on the SPR gene region (4kb SPR gene plus 94kb flanking regions; in total 98kb; see fig.1). A total of 40 SNPs with an average spacing of 2.5 kb, were identified using public databases. All SNPs showed high genotyping quality and Hardy – Weinberg equilibrium in the control subjects. Genotyping was performed using the matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry method (Sequenom, San Diego, CA).

Statistical Analysis

We used the non-parametric linkage scores (NPL) for the analysis of the sibship data (202). The NPL compares the estimated proportion of alleles shared identical by descent by sibs with the null hypothesis of no linkage. The S_{all} function in Genehunter was used for calculation. The age of onset effect in siblings was tested by the Quantitative Transmission Disequilibrium Test (QTDT) using an orthogonal and monks model (203). It allows to incorporate pedigrees without parental genotypes. Whenever there are more than two siblings in the families, allelic transmission scores were used to assess the association.

The method of Gabriel et al as implemented in Haploview was used to construct linkage disequilibrium (LD) blocks from SNPs with minor allele frequency (MAF) more than 0.05. (186). In the sporadic patients logistic regression was used to test for association between SNP alleles or haplotypes and PD affection status as implemented in UNPHASED (185). A stepwise approach was used to determine which variants independently influence the risk of the disease. This approach allows the effect at a locus to be assessed conditioning on alleles at other loci. A linear regression model was used to assess the age of onset effect.

Results

Analysis of sibship families:

We obtained overall non-parametric LOD scores with affection status of 2.12 and 1.96 at the SPR-neighboring markers D2S2110, D2S1394, respectively. After the exclusion of six families with PARK8 mutations the LOD scores increased to 2.76 and 2.08 at these two markers, respectively, as shown in table 12.

Table 12: Non-parametric LOD scores of STR markers with affection status; sibship data

Marker	Distance(cM)	Germany	England	France	Italy	Netherlands	All
D2S2110	89.76	1.73	2.17*	0.91	1.14	-0.41	2.76*
D2S1394	90.29	1.29	0.97	1.41	0.41	0.20	2.08*
D2S2111	90.29	0.95	0.97	1.04	-0.25	0.17	1.43
D2S145	90.82	0.99	0.08	1.56	0.41	-1.15	1.21
D2S2109	90.82	-0.46	-0.18	1.78	0.82	0.38	1.12

* p values <0.05

Family sets from single countries show the same trend, but are underpowered to obtain significant LOD scores except for the English sample. Seven of the 17 SNPs located between the STR markers D2S2110 and D2S1394 also showed significant LOD scores (table 13).

Table 13: Non-parametric LOD scores of SNPs with affection status; sibship data

SNP	Germany	England	France	Italy	Netherlands	All
rs1876488	0.51	0.25	0.61	0.23	0.45	2.91*
rs1396107	0.37	0.27	-0.25	-0.17	0.53	0.35
rs1508060	0.42	0.00	1.89	0.00	0.00	2.18*
rs1567230	-0.27	0.25	-0.07	-0.10	0.34	0.22
rs2135985	-0.01	0.73	0.76	-0.71	0.41	0.98
rs1876491	-0.02	-0.13	-0.06	-0.16	0.59	-0.14
rs2421095	-0.13	0.48	0.60	-0.08	0.45	2.70*
rs1876487	0.36	0.30	-0.06	-0.15	0.43	0.40
rs1150500	-0.01	-0.01	-0.85	-0.41	0.11	1.41
rs1561244	0.00	0.02	-0.17	-0.27	0.05	-0.16
rs1561245	-0.11	0.39	0.60	0.23	0.45	1.56
rs4852903	0.02	0.49	0.60	0.23	0.45	2.19*
rs989040	1.62	0.48	0.61	0.23	0.45	2.63*
rs1561247	0.12	1.38	0.00	0.00	0.00	2.26*
rs999494	-0.33	0.60	0.64	-0.41	0.72	0.78
rs1465805	1.58	0.00	0.00	0.00	0.00	2.48*
rs3980960	1.49	0.83	0.53	-0.51	0.33	0.74

* p value <0.05

We further analyzed the age of onset effect in our sib ship samples. The STR marker D2S2110 (p=0.04) and the SNP marker rs1876487 (p=0.04) showed significant association with age of onset. D2S2110 is the only genotyped STR marker which is located within the SPR LD block described below. The SPR promoter SNP rs1876487 was also associated with age of onset in the Karamohamed et al. study (166).

Analysis of sporadic patients

17 of the 40 genotyped SNPs had MAF < 0.05 and were excluded from further analysis. A total of 23 SNPs were used in the final analysis. One highly intercorrelated LD block of 45 kb (block A) can be defined around the SPR gene between SNP rs1396107 and rs1161267 (fig 6).

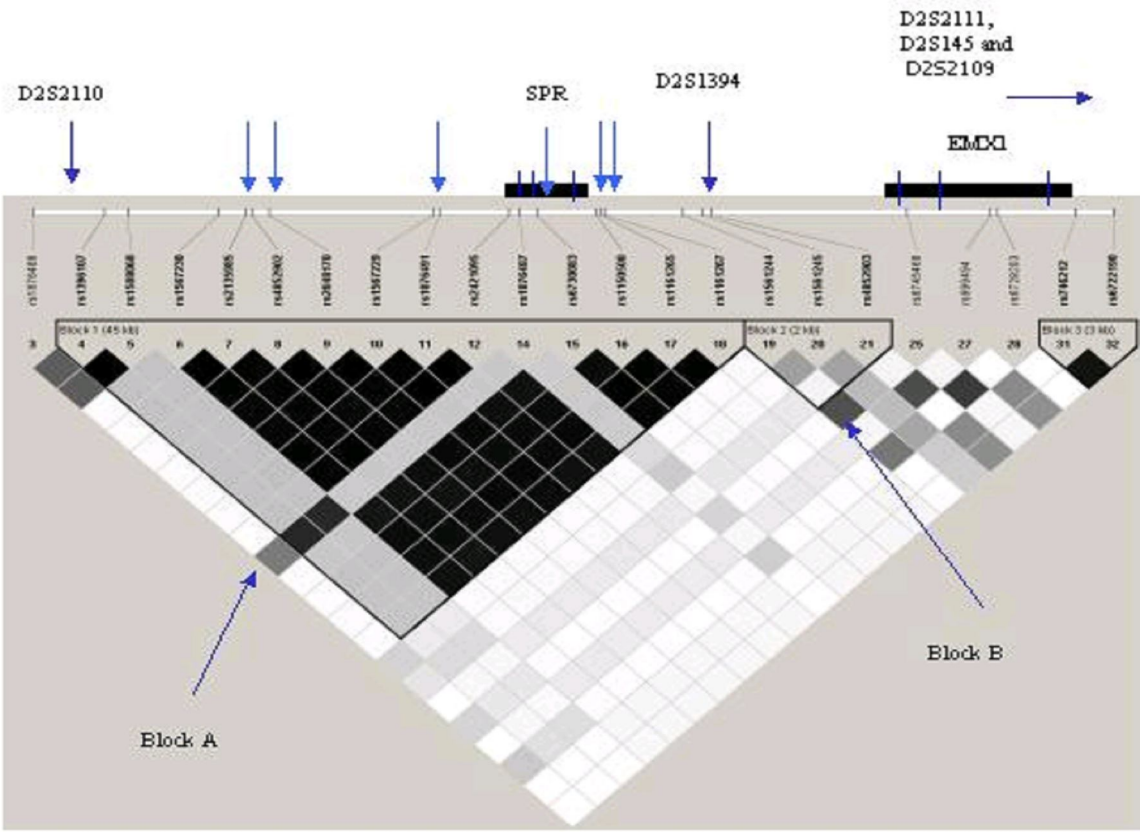

Figure 6: LD structure around the SPR gene region. All markers positions and genes are indicated on the physical map. Black cells= high pair wise r^2 values. White cells= low pair wise r^2 values. Additional SNPs genotyped are indicated by arrows. Exons are indicated by vertical bars.

All 14 SNPs within block A are significantly associated ($p<0.05$) with PD as shown in table 14

Table 14 : Genotypic association tests with each allele as the recessive risk allele; sporadic PD data.

SNP ID	Allele Major/Minor	Distance	Gene position	Frequency of Major Allele		Recessive risk model of major allele		Recessive risk model of minor allele	
				Cases	Controls	p-value	Odds ratio (95% CI)	p–value	Odds ratio (95% CI)
rs1876488	G/T	72981628		0.809	0.825	0.410	0.73 (0.34-1.54)	0.507	1.09(0.83-1.44)
rs1396107	C/T	72988240		0.708	0.751	0.464	0.82(0.50-1.75)	**0.024**	1.35(1.03-1.75)
rs1508060	T/C	72990328		0.716	0.756	0.486	0.83(0.49-1.40)	**0.040**	1.31(1.01-1.71)
rs1567230	T/C	72998602		0.897	0.931	0.817	1.21(0.23-6.29)	**0.004**	1.67(1.16-2.37)
rs2135985	C/A	73001132		0.899	0.928	0.625	1.48(0.29-7.39)	**0.012**	1.56(1.09-2.20)
rs4852902	G/A	73001656		0.898	0.927	0.624	1.48(0.29-7.41)	**0.011**	1.56(1.10-2.20)
rs2048170	A/G	73003323		0.900	0.926	0.590	1.53 (0.30-7.63)	**0.025**	1.49(1.04-2.12)
rs1567229	T/C	73018249	SPR Promoter	0.900	0.927	0.620	1.49(0.30-7.45)	**0.017**	1.52(1.07-2.15)
rs1876491	T/G	73018800	SPR Promoter	0.906	0.926	0.290	2.97 (0.35-24.7)	**0.049**	1.42 (0.09-2.03)
rs2421095	G/A	73025179	SPR Promoter	0.899	0.929	0.812	1.22(0.23-6.32)	**0.011**	1.57 (1.10-2.23)
rs1876487	G/T	73026007	SPR Promoter	0.677	0.731	0.146	0.71(0.45-1.15)	**0.015**	1.38(1.06-1.79)
rs6730083	A/G	73027652	SPR Intron 2	0.905	0.935	0.787	1.25(0.24-6.49)	**0.008**	1.16(1.12-2.32)
rs1150500	C/T	73033098	SPR 3'UTR	0.901	0.930	0.987	0.98(0.18-5.41)	**0.014**	1.54(1.08-2.19)
rs1161265	A/G	73033597	SPR 3'UTR	0.902	0.929	1.000	1.00(0.18-5.48)	**0.025**	1.49(1.04-2.12)
rs1161267	G/A	73033836	SPR 3'UTR	0.904	0.930	0.970	0.97(0.17-5.36)	**0.031**	1.47(1.03-2.10)
rs1561244	G/A	73041041		0.789	0.826	0.174	0.58(0.27-1.27)	0.078	1.28 (0.98-1.70)
rs1561245	C/T	73042822		0.597	0.636	0.473	0.87 (0.60-1.26)	0.060	1.29(0.98-1.70)
rs4852903	G/C	73043640		0.804	0.810	0.909	1.01 (0.77-1.34)	0.468	0.78(0.40-1.51)
rs6745468	G/C	73061450	EMX1 Intron2	0.801	0.836	0.225	0.59 (0.25-1.39)	0.068	1.29(0.98-1.70)
rs999494	G/A	73069050	EMX1 Intron2	0.818	0.829	0.491	1.13 (0.83-1.46)	0.993	1.00(0.48-2.09)
rs6728203	A/G	73069674	EMX1	0.765	0.800	0.532	0.86 (0.40-1.58)	0.061	1.29(0.98-1.68)
rs746212	C/T	7376889	EMX1	0.687	0.705	0.140	0.72 (0.47-1.14)	0.840	1.02(0.78-1.33)
rs6722190	C/G	73080248	EMX1	0.672	0.689	0.090	0.69 (0.46-1.06)	1.000	1.00(0.76-1.30)

* SNP positions are based on human genome assembly (hg17) and NCBI build 35

After testing different allelic and genotypic risk effect models, we only show the recessive risk effect model, which best fitted to the observed genotype data. The associated SNPs comprise variants with risk allele frequencies of either ~7% and ~27% in the control population, and are highly intercorrelated with r^2 values of 0.81 to 0.92. The 11 SNPs with MAF of 7% seem to be stronger associated than the more common SNPs, which include the Karamohamed et al SNP rs1876487. The estimated relative risks range from 1.21 to 1.57. In a stepwise regression procedure, which tested the allelic association of all significant SNPs conditioned on alleles at the other significant loci, we were not able to find an independent signal. It appears that all significant SNPs within block A potentially refer to a single causal variant.

A haplotype within block A with a population frequency of 7.6% was also significantly associated with PD (p = 0.010) as shown in table 15.

Table 15: Association analysis of common (>1%) block A haplotypes with affection status; sporadic PD data.

Haplotype	Case frequency	Control frequency	p value
CTTCGATTAGACAG	0.678	0.731	0.012
CTTCGATTATACAG	0.032.	0.027	0.571
TCTCGATTATACAG	0.189	0.165	0.230
TCCAAGCGGTGTGA	0.097	0.065	0.010

Interestingly, this haplotype is differentiated by 11 mutational steps as compared to the next related common haplotype (frequency: 17.5%) in the same block.

We further evaluated the 3-locus haplotype (rs2421095-rs1876487-rs1561244) described by Karamohamed et al.(166), which extends between block A and B (fig. 6). The haplotype AGG, which was most significantly associated with age of onset in the Karamohamed et al. study, was also most significantly associated in our study (p=0.002; table 16), albeit with affection status.

Table 16: Association analysis of common (>1%) 3-locus haplotype with affection status; sporadic PD data

rs2421095	rs1876487	rs1561244	Haplotype frequency cases	Haplotype frequency controls	p value
A	G	A	0.067	0.062	0.54
A	G	G	0.603	0.670	0.002
A	T	A	0.139	0.111	0.06
A	T	G	0.087	0.083	0.76
G	T	G	0.098	0.072	0.03

We found neither single marker nor haplotype association with age of onset in our sporadic PD sample.

Discussion

There is accumulating evidence that the SPR gene is one of the likely candidates for the PARK3 locus. First, in a large sample of multiplex families (GenePD study), one microsatellite marker D2S1394, which is only 9 kb away from the SPR gene, showed association with PD age at onset (165). The associated allele “174” of this marker is also common to the segregating core haplotype observed in two PARK3 families (164, 198). Second, a haplotype harboring the SPR gene and the promoter SNP rs1876487 was reported to be associated with onset age in the GenePD study (166). Third, we obtained significant LOD scores with PD susceptibility at the two microsatellite markers D2S2110 and D2S1394, which encompass the SPR gene, in an independent large sample of multiplex families (European GSPD study). Interestingly, the English families with the highest mean age of onset contributed most to this linkage signal. This result fits well to the finding that the European genome scan reports a prominent linkage peak at the SPR region only for late age of onset families (201). We also found association with age of onset at the markers D2S2110 and rs1876487 in the GSPD family sample. Fourth, we found

association with PD susceptibility at several intercorrelated SNP markers and haplotypes in an LD block spanning the SPR gene in a German sample of sporadic PD patients.

Given all converging evidence, DNA variant(s) in or around the SPR gene appear to influence PD onset. Coding regions of potential PARK3 candidate genes including SPR have been screened for pathogenic mutations in PD families (198). The failure to detect such mutations could indicate that the functional variant affects expression or splicing regulation rather than the protein structure itself.

A recently published study has revealed a mutation in the 5' UTR of the SPR gene responsible for causing dopa-responsive dystonia (204). SPR is an interesting candidate gene, because it catalyses the conversion of 6-pyrovyl-tetrahydropterin (PTP) to tetrahydrobiopterin (BH4). Previous studies have shown that BH4 acts not only as a cofactor for TH4, and is therefore important for dopamine biosynthesis, but also stimulates NOS isoforms [inducible(iNOS), neural(NOS) and endothelial (eNOS)] (205). It has been suggested that iNOS confers protection to PD.

Across studies, there is some variability of the PD phenotype mapped to the SPR region. Whereas the GenePD studies report associations with PD onset age, (165) the study based on the European GSPD samples reveals associations with PD susceptibility. Of note however, we could also directly replicate the age of onset effect at the promoter marker rs1876487 found by Karamohamed et al (166).

It is obvious that affection status and age of onset are related phenotypes in late-onset disorders that show preclinical and post clinical progression. A factor that influences the preclinical history of PD (e.g. start and rate of the progression of neuronal loss) will affect the onset age of PD if a progression threshold is assumed for the transition from healthy to disease status. As general mortality (independent of PD) increases with age, an age of onset factor may also be seen as a susceptibility factor, because early-onset factors are upweigthed in an association study dealing with affection status. Different age

structures in the analyzed samples may shift the emphasis (detectability) between the extremes of pure age of onset and pure susceptibility effects.

In summary, we show that the SPR gene is likely a PARK3 candidate. The association signal appears to be confined to a haplotype block of 45 kb surrounding the SPR gene. Our data suggest a single risk variant or age-of-onset factor of about 7% frequency with a relative risk of about 1.4 within haplotype block A. Further evidence of this association signal comes from the haplotype pattern, which is indicative for recent natural selection in this genomic region (206).

Chapter 4.2: PARK11 is not linked with Parkinson's Disease in European Families

Sharma M*, Prestel J*, Leitner P, Zimprich A, Vaughan JR, Durr A, Bonifati V, De Michele G, Hanagasi HA, Farrer M, Hofer A, Asmus F, Volpe G, Meco G, Brice A, Wood NW, Muller-Myhsok B, Gasser T. PARK11 is not linked with Parkinson's disease in European families. **Eur J Hum Genet 2005;13(2):193-7. (Contributed equally)**

Evidence for linkage to chromosome 2q36-37 (*PARK11)* was first detected in a sample of 160 families (170 affected sibling pairs) in a genome-wide screen (207). An additional study was performed using a subset of the previous, but expanded sample with a strong family history of PD (169): in an analysis of 65 families (77 sibling pairs) a maximum LOD score of 5.1 at the marker D2S206 on chromosome 2q36-37 was found using an autosomal dominant model of disease transmission. Recently Pankratz et al. confirmed their previous results using an enlarged and overlapping sample of 85 families (113 sibling pairs) with a strong family history of PD(171). They again reported a linkage to the 2q36-37 region (LOD score 4.9).

Methods

We have performed a replication study in a set of European sib pair families to verify the linkage at 2q36-37 in a European population. 45 families were selected for this study. We included families with a strong family history of PD, defined according to the same criteria regarding family history as used by Pankratz et al.: the families had at least four first-, second- or third-degree relatives reported to have PD or they included an affected sibling pair who also had a parent reportedly diagnosed with PD. 15 of our 45 families included at least one affected individual with an age of onset ≤ 50 years. The diagnosis of PD in the index patients was established according to the UK Parkinson's Disease Society Brain Bank criteria (2). After appropriate informed consent was

obtained, blood samples had been drawn from the individuals for DNA extraction. The characteristics of the families are described in table 17.

Table 17: Characteristics of the families

Country	Number of families	Number of sibs		% Male	% Female	Mean ± SD Age at onset (years)
		affected all/ genotyped	unaffected all/ genotyped			
German	19	48 / 39	37 / 18	49.6	50.4	59.4 ± 8.3
English	11	35 / 24	44 / 24	46.3	53.7	58.3 ± 7.3
French	7	21 / 17	10 / 3	47.1	52.9	56.4 ± 12.8
Italian	6	18 / 14	14 / 8	47.8	52.2	52.8 ± 16.6
Turkey	2	8 / 6	18 / 0	52.0	48.0	61.0 ± 4.2
	45	130 / 100	123 / 53	48.2	51.8	57.6 ± 10.5

Pankratz et al. reported a significantly linked region between marker D2S126 and D2S125 spanning a distance of 39.5 cM. We have selected only that region for analysis where the highest LOD score was reported. Six markers (D2S2382, D2S126, D2S396, D2S206, D2S338, D2S125) with an average spacing density of 9.4 cM were used for analysis. These six dinucleotide repeat markers with an average heterozygosity of 82% were genotyped on chromosome 2. Marker order and genetic distances between the markers were obtained from the sex–averaged genetic map from Marshfield Genetic Laboratories. PCR amplification was performed for each marker in a 10-µl reaction using 20 ng of genomic DNA, 2 pM of each primer, 0.2 mM of each dNTP, 1 µl 10x PCR buffer (containing 15 mM $MgCl_2$), 0.5 or 1 mM $MgCl_2$ and 0.3 units of Taq DNA polymerase (Taq PCR Core Kit, Qiagen). Amplification conditions were as follows: preincubation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 sec., annealing at 56°C or 60°C for 30 sec. and extension at 72°C for 40 sec. and final extension for 2 min at 72°C. 1 µl of the PCR product was added to 20 µl of formamide containing the GeneScan-500 ROX size standard. The products were separated by capillary electrophoresis using an ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems). The genotypes were determined by using GeneScan version 3.7. Mendelian inconsistencies in the genotypic data were checked by using the program PedCheck (208). In order to evaluate the power of our sample, a simulation study was performed by using the SLINK programme. It showed that our sample size is sufficient for finding significant

evidence for linkage, with an average maximum LOD score of Z=2.8. Two point LOD scores were calculated using the MLINK programme of LINKAGE software package (209). The mode of inheritance was as autosomal dominant with disease allele frequency of 0.005. Marker allele frequencies are based on all individuals genotyped. The pentrance was set at 40% for <50 years and at 80% for >50 years of age. The phenocopy rate was assumed to be 2%. Multipoint parametric and nonparametric analysis was done by using SimWalk2 (210). The sib transmission/disequilibrium (S-TDT) was used to observe the transmission of alleles among affected sibs (211)

In the 15 families, that included at least one affected individual with an average of onset <50 years, a marker in intron 7 of the parkin gene (D6S305) was genotyped in order to identify families more likely to have mutation in this known PD susceptible gene. Linkage analysis was repeated excluding those families showing possible linkage to D6D305.

Results

We did not obtain any significant LOD score in the parametric analysis: the results are shown in table 18

Table 38: Results of the parametric analysis: two point LOD scores

	LOD score						
Marker	**Recombination fraction θ**						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D2S2382	-4.8	-3.56	-1.82	-0.88	-0.11	0.06	0.04
D2S126	-2.6	-1.72	-0.84	-0.26	0.17	0.17	0.05
D2S396	-1.99	-1.63	-0.83	-0.33	0.07	0.11	0.04
D2S206	-4.33	-3.83	-2.55	-1.63	-0.67	-0.25	-0.07
D2S338	-1.88	-1.6	-0.88	-0.42	-0.04	0.03	0.01
D2S125	-5.5	-4.8	-3.04	-1.84	-0.69	-0.23	-0.05

The results of the nonparametric analysis were not different as shown in table 19.

Table 19: Results of the parametric and non parametric analysis: multipoint LOD scores

Marker	Distance (cM)*	MLOD [a]	NPL [b]	MLOD [a]	NPL [b]
		LOD score of 45 families		LOD score of 36 families	
D2S2382	0	-5.78	0.45	-5.8	0.28
D2S126	7.6	-3.01	0.6	-3.61	0.48
D2S396	19.4	-5.48	0.24	-3.39	0.27
D2S206	27.29	-7.01	0.13	-5.01	0.18
D2S338	37.04	-3.38	0.15	-2.74	0.18
D2S125	47.13	-6.13	0.07	-4.71	0.08

MLOD [a] = multipoint maximum parametric lod score
NPL [b] = multipoint nonparametric linkage score
*Distance in Haldane cM.

We did not observe any significant z score in the S-TDT, which showed that none of the marker alleles is associated with the disease. In our families we could not perform the TDT test, because parental genotypes were not available. There is not much loss of power, when the parents are not genotyped as in our case, given the fact that affected sibs as well as unaffected sibs are genotyped. Linkage to marker D6S305 in the parkin gene could not be excluded in nine of the 15 families, that included at least one affected individual with an age of onset <50 years. Excluding these nine families, we did not obtain any significant LOD score in the parametric and non parametric linkage analysis of the remaining 36 families. Again, the highest LOD score (0.48) was found in the non parametric analysis at marker D2S126

Discussion

The studies by Pankratz et al. showed a significant linkage of PD to 2q36-37 in a North American population. The sample size of Pankratz et al was primarily Caucasian (94%), although Hispanics (5%) also participated (207). Interestingly, the Hispanic families in the sample provided a substantial portion of the linkage evidence (171). We could not find a linkage to this region in our European families nor in any national subgroup of these families. This

discrepancy might be explained by the different population. However, none of the other PD genome-wide linkage studies in the last years have reported evidence of linkage to chromosome 2q ((168, 181, 200): DeStefano et al. analyzed affected sibling pairs mainly from the United States and also from Canada, Germany and Italy. Scott et al. analysed families from the United States and Australia, while Hicks et al. performed a scan on Icelandic families ((168, 181).

Pankratz et al. reported a significant LOD score both in a sample with and without *parkin* mutations. The inclusion of the families with *parkin* mutations resulted in a higher LOD score, but the LOD score remained clearly significant in the sample without *parkin* mutations (169, 171). We repeated our linkage analysis excluding nine families, in which we could not rule out linakge to marker D6S305 in the parkin gene. Excluding these families did not change our overall results, indicating that there is no specific contribution of this subset of families to our results. It is also unlikely that parkinsonism in these nine families is caused by parkin mutations, because we included only families compatible with autosomal dominant inheritance in our study and mutations in the parkin gene cause autosomal recessive parkinsonism (with the exception of very few families, in whom the contribution of the parkin mutation is still somewhat controversial).

A possible linkage of our families to other domiant loci such as PARK3 and PARK8 was not subject of this study and can not be excluded.

The mean age of onset in our PD families (57.6 years ± 10.5) was similar to the mean age at onset in the studies of Pankratz et al.: 58.0 years ± 12.2 (104), 58.3 years ± 12.0 (171). This indicates that the discrepancy of the results between the studies by Pankratz et al. and our study cannot be explained by a different age at onset of PD in the population.

We genotyped the same six markers as Pankratz et al in the region, where the highest LOD score was reported. Thus the discrepancy of the results between the studies can not be explained by a different marker density. Employing a dense set of markers would most probably not affect our overall results.

It may be argued that the original study by Pankratz et al overestimated the linkage, so that the true effect conferred by the PARK11 locus is smaller, and therefore escaped detection in our sample. We did not find a significant LOD score of our study occurred in nonparametric analysis at marker D2S126 (LOD score 0.6) and is far away from significance. The marker D2S126 is nearly 20cM apart from D2S206, where the highest LOD score was reported by Pankratz et al (169).

In summary, our study did not provide any evidence of a susceptibility locus for Parkinson's disease at 2q36-37 in our families. Therefore *PARK11* does not seem to play a major role for familial PD in the European population. A susceptibility locus at 2q36-37 may be a rare form, occurring in special populations.

Chapter 5: Meta analysis of whole-genome linkage scans with Data Uncertainty: An Application to Parkinson's disease

Manu Sharma*, Albert Rosenberger*, Bertram Müller Myhsok, Thomas Gasser and Heike Bickeboller. Meta Analysis of Whole Genome linkage scans with uncertainity of data: An Application for Parkinson Disease. **(Equally contributed)** ***BMC Genetics (2007);2(8):44***.

Genome wide linkage scans have often been successful in the identification of genes for monogenic diseases. However, the chance of success decreases by the multiplicity of genetic and environmental determinants involved in the aetiology of a complex disease. The contribution of each disease gene to overall risk is presumed to be small, and thus large sample sizes are required to detect the effect (212). An ad hoc approach is to look for genomic regions that obtain evidence for linkage across several scans, but this allows no direct statistical assessment. A statistically more rigorous and powerful approach to pool results would be a 'mega analysis' using original genotypes and analyze these as a single dataset as suggested by Lander and Kruglyak (213). Pooling of samples across different studies will increase the sample size and hence help to find loci with small effects. However, one should expect studies to vary in many respects, e.g. ascertainment criteria (multiplex families, sib pair families, and a single large multigenerational family), definition of phenotypes (e.g. diagnostic scheme) and different marker data sets (Marshfield map, Genethon, Decode map). As these marker data sets vary in marker spacing as well as in marker density this leads to further heterogeneity. Moreover, variability in the sample sizes across different studies leads to inconsistencies in the results. Besides that, different ways to incorporate the possible covariates (which are rarely published in detail) are methodological handicaps in a pooled analysis. So, pooling of raw data across several studies needs to be carried out and interpreted with caution. Even though some of these problems cannot be overcome by a meta analysis, pooling of raw data is not necessarily feasible (214).

As the raw genotype data might not always be available to the public, more flexible approaches are required to carry out the meta analysis. In this context Allison and Heo used the Fisher method of combining the p-values across candidate regions in a study of obesity (215). Province suggested a p-value of 0.72 (=1/2ln(2)) (216) to overcome the problem of setting all negative evidence against linkage to zero in nonparametric linkage methods. Recently Badner and Gershon (217) proposed an extended approach of combining the p-values across different studies, further on labelled as Corrected p-value Meta analysis Method (CPMM). In CPMM, each reported p-value of a candidate region needs to be transformed by an equation originally given by Lander and Kruglyak (213). Then, the minimum of these transformed p-values is corrected for the size of the candidate region. Finally Wise et al (218, 219) developed a Genome Search Meta Analysis method (GSMA) specifically to carry out the meta analysis of genome wide linkage searches. GSMA is a nonparametric method based on rank statistics.

If researchers are reluctant to contribute even summary measures like test statistics for linkage (LS), rest assured one may introduce some bias into a meta analysis, similar to publication bias. Additionally, the power of the meta analysis will be decreased. Even if the meta analysis contains an amount of uncertain summary data, the results will provide a higher level of validity than by simply viewing the individual findings. Therefore they are highly valuable in deciding how to proceed next, e.g. which regions to pursue in further studies. That said, one should consider such approaches as preliminary, and the necessity to discuss the impact of data uncertainty onto the findings still remains.

In this study we propose a way to reconstruct test statistics for linkage (LS) and corresponding marker positions as the key summary measure of genome wide scans from condensed materials such as figures in published papers. Furthermore we carry out the meta analysis of all published genome wide scans of susceptibility to PD taking into account the uncertainty of the summary measures by using the GSMA and CPMM.

Methods

We carried out a literature search in MEDLINE for MASH-headings Genetics, Parkinson's disease and genome scan (or screening), restricted from 1998 to 2004 and sourced references of neurological and genetic journals. In total we were able to identify seven genome wide linkage scans of Parkinson's disease (101, 145, 168, 181, 200, 201, 207). Three family samples have been reanalysed and published twice. Recently a genome wide association of PD study was published, that we used only for comparing results (220).

Inclusion / exclusion criteria for genome wide scan

The following criteria for the inclusion of genome wide scans in the meta analysis were defined to ensure the quality of the individual studies and the data to be extracted:

1. Patients are included by status of Parkinson's disease and not being selected e.g. by family history or therapy response.
2. Markers are available across the whole genome.
3. The statistical analysis is carried out by using established genetic epidemiological methods.
4. The analysis concentrates exclusively on the susceptibility to PD, not e.g. to the age of onset. Thus the two genome scans based on other phenotypes are excluded (167).

The study characteristics of the five identified and included genome wide scans on susceptibility to PD are given in table 20.

Table 20: Characteristics of whole genome scans for linkage for Parkinson's disease

Reference	DeStefano et al. [11]	Scott et al. [9]	Hicks et al. [13]	Pankratz et al. [10]	Martinez et al. [12]
Source Population	US	US	Iceland	US	US + Europe
Diagnostic criteria	UKPDS (adapted)	2 cardinal PD-signs + exclusion criteria	2 cardinal PD-signs + Hoen-Yahr crit.	UPDRS (II+III) Diagnosis Check List	3 cardinal PD-signs or 2 signs and at least 30% improvement with levodpoa treatment no parkin mutations
Family Structure	ASP	multiplex families	multiplex families	multiplex families	multiplex families
No. of Families	113	174	51	325 (170 ASP)	199 (227 ASP)
No. of Affected	226	378	117	192	471
No. of Marker	392	344	781	400 SNPs	391
Average distance	11 cM	10 cM		8.6 cM	10 cM
Analysis	MLS / NPL	MLOD	Zlr	MLS	MLS
Significant region / markers	9 – D9S1825	5q –D5S816 8p – D8S520 17q – D17S921	1p32 – D1S231 D1S2652	2 – D2S206	2p - D2S160 5q - D5S471 6q - D6S257 7p - D7S531 11q - D11S4175 19q - D19S902

ASP=affected sib pair; UKPDS=UK Parkinson Disease Society Brain Bank Clinical Diagnosis Criteria; UPDRS= Unified Parkinson Disease Rating Scale; MLS=Maximum Likelihood Score; MLOD= Maximum Parametric LOD Score; NPL=Nonparametric Linkage Score; Zlr= converted LOD score: $\sqrt{[2\ln(10)\bullet LOD]}$

Method of data extraction

Figures presenting LS were copied from the electronic versions of the original papers into a Microsoft Word® document. We electronically enlarged figures to size A4 in order to gauge crude LS and marker positions on the chromosome,

placing arrows from the zero-point to a dot or vertex in the diagram. Length and height values of the arrows were calibrated and rescaled along measurements of the y-axis (linkage statistic) and the chromosome limits plotted along the x-axis (position). More accurate estimates of position could be achieved by placing the arrows at the beginning of each chromosome rather than at the zero-point of the x-axis.

Data extraction was independently accomplished nine times for each study. In order to take into account the uncertainty in position, extractions were matched, clustering the nearest points. The distance between two points i and j was calculated by $d_{ij} = \sqrt{f(LOD_i - LOD_j)^2 + (Pos_i - Pos_j)^2}$, with f as a factor to correct for different scales (LS-units vs. cM). It also can be used to give higher weights to differences of LS than to that of positions, since neighbouring points can be distinguished more easily by LS than by position. A value of f=8 was empirically found useful showing no clear mismatch.

The quality of data extraction was separately checked by visual inspection for each extraction and for the mean of extractions. Finally, the mean and the standard error of matched extracted LS and positions were calculated and used for the subsequent meta analysis. Standard errors for markers extracted only once were defined as equal to the median standard error of all remaining markers. We directly used LS when LS and corresponding marker positions were reported in the articles. In this case standard errors were set to zero.

Methods of meta analysis

CPMM

CPMM is based on p-values for linkage peaks. Badner and Gershon (217) suggested that those nominal p-values per locus have to be corrected for genome wide testing, because evidence for linkage can occur in a region of up to 30 cM away from the disease susceptible locus (217). They refer to Feingold et al. (221) who estimated the probability p* for the minimum p-value being observed within such a linkage region.

The corrected p-value for such a region is $p^* = 1-(1-p)^C + 2\lambda G \cdot Z(p) \cdot \phi[Z(p)] \cdot \nu[Z(p)\sqrt{4\lambda\Delta}]$, where the notation is as follows: p

denotes the Bonferroni corrected point-wise p-value from each scan to take multiple testing into account. C denotes the number of chromosomes. λ denotes the rate of crossovers per Morgan given by Lander and Kruglyak (213); it depends on the analysis method and family structure. G denotes the size of the linkage region in Morgan, here G=60 cM. Z (p) denotes the standard normal inverse of p. $\phi[Z(p)]$ the density function of the normal distribution. Δ denotes the average marker spacing in Morgan. ν(x) denotes the discreteness correction for the distance between markers; for x<2 we have v (x) ≈ exp (-0.583x).

This equation differs from that used by Badner and Gershon (217) and given by Feingold et al. (221). The first term pC was replaced by 1- (1-p)C because observed p-values less than 0.045 (LOD-scores of ~0.89) result in p*>1. Applying CPMM, we proceeded as follows: On each chromosome the most significant marker, defined by the maximum LS, was identified across all scans. A region ± 30 cM around this marker was considered a linkage region if p*<0.01. Hence, all LS of the remaining scans within a linkage region were converted to p-values by using Holman's triangle (222) as implemented in the Nyholt table (223). For the X chromosome we followed the X-linked MLS approach as suggested by Cordell et al. (224). These p-values were further corrected yielding the corresponding p*-values as described in the above equation. The p-values of markers outside the linkage region were set to 0.72 (=1 / 2ln(2)) as suggested by Province (216).

For each region the multiple scan probability $MSP:\ p = p(\chi^2_{1-\alpha,df=1} > Y^2)$ was calculated with $Y^2 = \sum -2\log(p^* \cdot i)\ \ for\, i = 1\, to\, n$. n denotes the number of scans considered, as originally suggested by RA. Fisher in 1932.

According to the criteria for genome scans by Lander and Kruglyak we considered a linkage signal as suggestive following application of CPMM when $p \leq 0.0007$ and as significant when $p \leq 0.00002$. Cross-validation analysis excluding the most significant result was carried out if CPMM analysis yielded $p \leq 0.001$ (166).

GSMA

Briefly, the GSMA (216, 218, 219) method assesses evidence for linkage by splitting all chromosomes into N bins of approximately equal size. For each genome scan included, the most significant LS is recorded. Bins are then ranked in order of significance with the most significant bin assigned rank N. Equal test statistics for several bins within a study were assigned tied ranks. The ranks of bins are summed across the genome scans. This summed-rank-statistic (SR) is compared to the critical values of a summed-rank-distribution (Edgeworth series approximation (225)) under the null hypothesis of no linkage. We also carried out a weighted GSMA analysis. For this each rank was multiplied by it's study weight ($\sqrt{N(affected\ cases)}$, divided by the mean of this value of all studies) before summed up to another summed-rank-statistic SR_{weight} (226). For the analysis we did not consider the X chromosome, since GSMA does not produce reliable results in this situation (101, 145) .

We considered an approximate bin size of 30 cM as recommended by Wise et al.(219). In total 118 bins were used. SR across all 5 studies ranged from 5 to 590.

For each bin we calculated p-values of three kinds of tests. First, p_{SR} gives the probability of an arbitrary bin to achieve the observed SR or a higher value. SR analysis assesses the significance of each bin independently. Applying Bonferroni correction, significant genome wide evidence for linkage of 5%, as defined by Lander and Kruglyak, will be equivalent to $p_{SR} < 0.00042$ for 118 30 cM bins (expected once by chance in 20 meta analyses). Suggestive evidence (expected once by chance per single meta analysis) is given for a $p_{SR} < 0.00857$.

Secondly, p_{OR} gives the probability that the i^{th} most significant result (bin) has the observed-summed-rank (here abbreviated as OR) or higher. OR analysis considers the distribution of SR across all bins simultaneously. Finally, p_{het} gives the probability of heterogeneous ranks across studies for a bin, conditional on the observed rank sum. Therefore we used $Q_j = \sum (R_{ij} - \overline{R}_{.j})^2$ as test statistic, proposed by Zintzaras and Loannidis (227, 228), where R_{ij} is the rank of j-th bin

in the i-th study and $\bar{R}_{\cdot j}$ is the mean rank of the j-th bin across studies. A small p_{OR} indicates consistent evidence for linkage across studies, while a large p_{OR} indicates heterogeneity between the considered searches. We assigned top ranks to known bins and the mean of the remaining ranks to empty bins to overcome the problem of missing values.

Sensitivity analysis

A Monte Carlo (MC) simulation approach was used to determine the change in SR due to data uncertainty caused by the extraction process. The simulations were replicated 333 times (replication number limited by computer time) while randomly drawing a marker position and LS from normal distributions, using mean and standard error from data extraction.

Results

By applying inclusion criteria, the meta analysis is based on all published investigations for genome wide linkage to PD as the phenotype of interest: Scott et al. (181), Pankratz et al. (207), DeStefano et al. (200), Martinez et al. (201) and Hicks et al. (168). We did not include the genome wide scans by Hampshire et al. (145) and Funayama et al. (101), because patients included suffered from the Kufor-Rakeb syndrome or Parkinsonism, respectively.

Quality of extraction

With the methodology proposed above, we were able to extract the same number of markers (n=344) as originally investigated by Scott et al. From the paper by Pankratz et al. the positions and LS of 230 markers could be estimated from figure 2, corresponding to 58% of 400 investigated markers. The corresponding numbers for figure 2 from Hicks et al. are 426 markers (54% of 781 investigated markers), and those for figure 1 from Martinez et al. are 261 markers (67% of 391 investigated markers). DeStefano et al. provided illustrations for only 4 chromosomes, accounting for the reduced number of extracted markers (12% of 399 investigated markers).

Positions and corresponding LSs of a total of 25 markers were provided in the original papers. The extracted LSs were almost similar to those reported

(maximal deviation: 1.15 extracted, 1.24 reported in paper). For two markers the extracted mean positions deviated from those reported by ~12 cM and ~16 cM (corresponding report: table 1,(207)). However, the extracted positions were only 4 cM and 9 cM apart from the sex-averaged locations given by the Marshfield map (229).

Using our method of extraction, markers yielding higher LS are unambiguously identified in a figure. Thus, we may assume that missed markers are exclusively those with a low value of the LS. Moreover, the estimated standard error of LS ranges from approx. 0 to 0.07 LS-units. The largest deviation was 0.29 LS-units. On average LS of a marker was extracted within a range of 0.06 LS-units. This precision is satisfactory for the coarse grid of genome wide scans.

In order to take into account the uncertainty in marker positions, the estimated standard errors of extracted positions for one marker ranges from approx. 0 to 4.15 cM. The largest span between two single extractions is 28 cM. Position uncertainty was exceptionally high on chromosome X, which was printed with an open ended axis in two figures. Hence, locating markers on this chromosome must be regarded as problematic.

CPMM

We found evidence for linkage on chromosomes 1 (p=0.0074) and X (p=0.0015). In a leave-one-(study-)out cross-validation analysis we did not find any significant linkage. This shows the heterogeneity of the scans, because both findings are primarily caused by the results of one single included genome scan each. The results obtained by CPMM did not reach the level of genome wide significance as suggested by the Lander and Kruglyak criteria (213). However, they showed a trend towards linkage.

GSMA

The most significant results by the summed-rank-statistic (SR) could be achieved for the 6^{th} 30 cM bin on chromosome 9 (p_{SR}=0.0145). Furthermore, for the 6^{th} 30 cM bin on chromosome 5 (p_{SR}=0.0363), for the 5^{th} 30 cM bin on chromosome 14 (p_{SR}=0.0363) and for the 4^{th} 30 cM bin on chromosome 1 (p_{SR}=0.0492) locally significant signals were achieved. The individual ranks for

these regions ranged from 51 to 118. Neither for heterogeneity nor for homogeneity between studies evidence was given for any bin (p_{het} from 0.1550 to 0.3320). Adjacent to the significant 6th bin, the 4th (p_{SR}=0.0874) and 5th bin (p_{SR}=0.0940) of chromosome 9 showed some trend towards linkage. Similarly, adjacent to the significant 6th bin of chromosome 5, the 5th bin (p_{SR}=0.0940) showed some trend towards linkage. The summed-rank-statistics of each 30 cM bin are shown in figure 7.

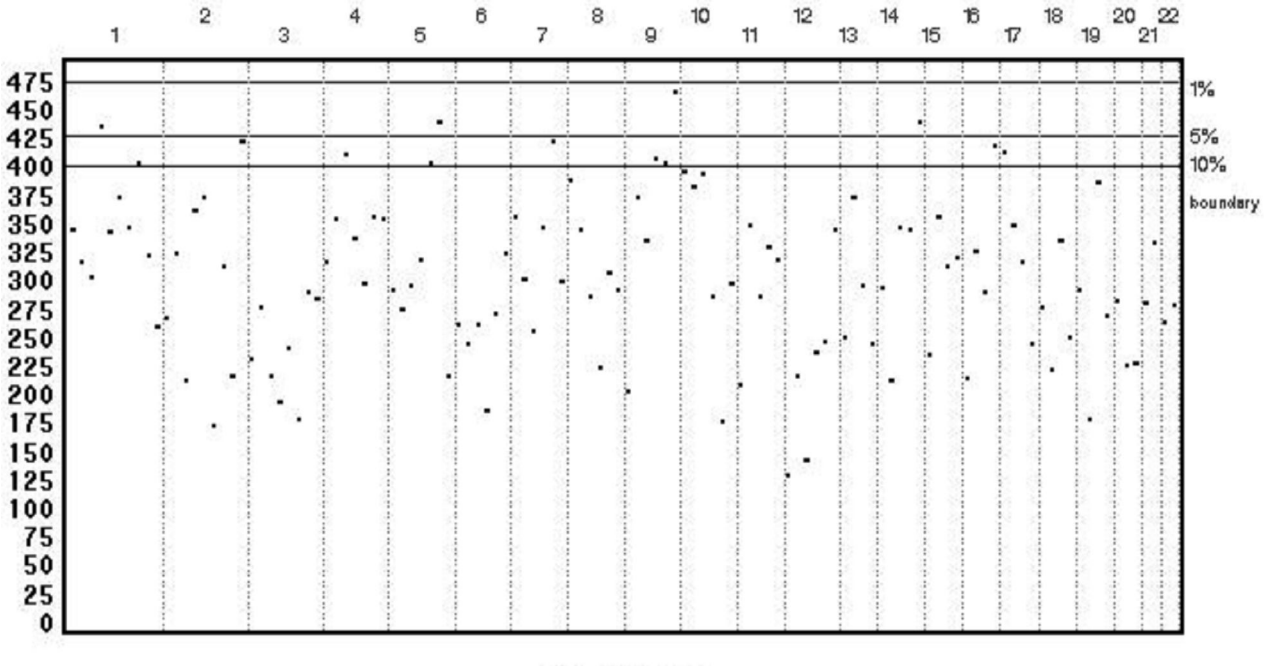

Figure 7: GSMA-results: summed-rank distribution of 30 cM bins chromosome

The p-values of the findings using 30 cM bins of chromosomes 5 and 9 changed slightly when using 60 cM bins, but they did not fall below 0.00875 (suggestive genome wide evidence) as shown in fig 8.

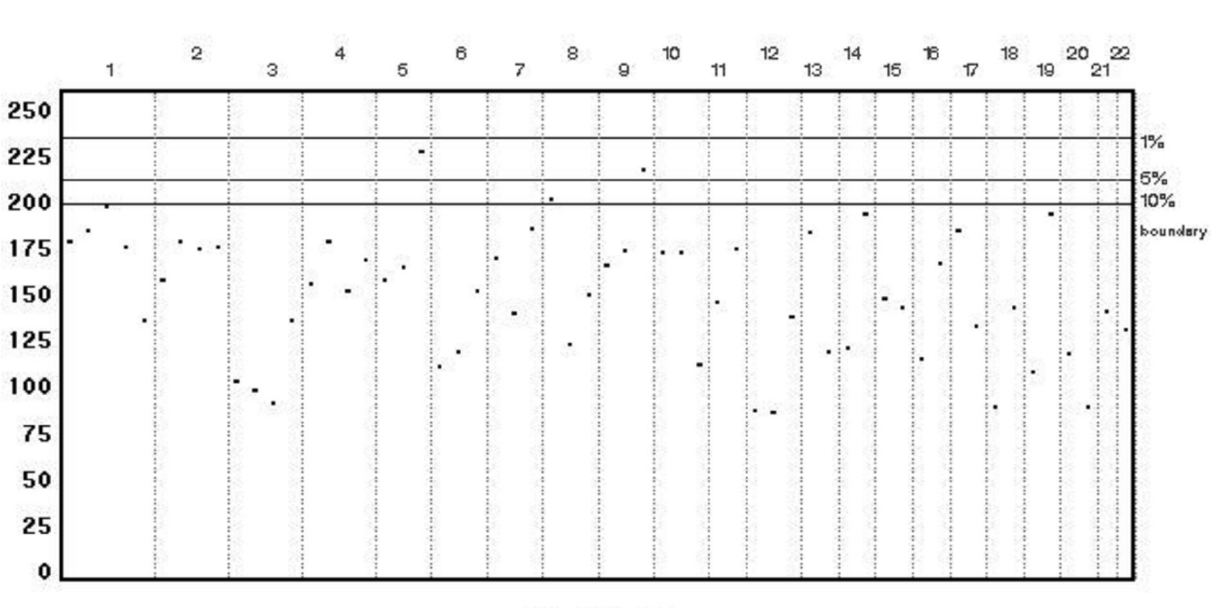

Figure 8: GSMA-results: summed-rank distribution of 60 cM bins

The 3rd 60 cM bin of chromosome 5 (corresponding to the 5th and 6th 30 cM bin) achieved nominal significance by p_{SR}=0.01862. The 3rd 60 cM bin of chromosome 9 (corresponding to the 5th and 6th 30 cM bin) achieved a nominal significance of p_{SR}=0.0353.

All these findings remained significant when accounting for data uncertainty by simulation and achieved p-values less than 0.05 in all 333 replications .No further suggestive evidence for linkage ($p<0.05$) can be seen in any of the 333 replications.

Discussion

We applied data extraction combined with assessing data uncertainty to carry out the meta analysis of genome wide scans of linkage to PD from all published investigations. If known studies without accessible data are not considered, a bias might be introduced in meta analysis so that this problem is reduced by using as much information as possible from published figures.

Both meta analysis methods considered here are robust with respect to design, as they can deal with differences in structure and number of families between

studies, quantitative and qualitative phenotype definition, genetic markers analysed and methods of statistical analysis. In addition, no assumptions on the mode of inheritance or genetic heterogeneity are necessary for the valid application of these two methods. The distribution and interpretation of the linkage test statistics does depend on the statistical method applied. This is no problem for GSMA, since test statistics are ranked within the single scans. The key information used by CPMM is based on p-values, which may be converted from test statistics for linkage by a known relation. But CPMM requires the raw data to produce reliable results. That was one of the reasons for developing GSMA (226).

To our knowledge no extensive comparison between these methods has been published yet. Thus the relative power of these methods is not yet clear. While with GSMA one searches for evidence for linkage across studies in pre-specified genomic segments (termed as bins), CPMM identifies regions of clustered markers with LS-values indicating towards linkage and assessing significance using p-values corrected for the size of the region. In the presence of uncertainty in marker position it remains unclear which of these approaches remains more powerful or robust. Please note, that it could be problematic to combine the lowest p-values from genome scans particularly for smaller scans, because of a severe bias towards linkage (230). Giving for instant full weight to very low p-values, CPMM could better detect linkage in the presence of substantial heterogeneity across samples. GSMA might be more powerful when small genetic effects are present in all samples (226).

Data uncertainty in linkage statistics and marker positions does not deteriorate the strength of the main findings. Since markers are allocated into bins for GSMA, uncertainty in position is reduced to uncertainty of allocation. This allocation is ambiguous only for a small proportion of markers, of which only a small proportion is important for the ranking of bins. Consequently, one might expect less variability in GSMA results due to uncertainty in position. The direct comparison of extracted values of markers to reported values, if available, shows the robustness of the whole approach. The only notable differences appeared from the deviation of original reported marker position to those given

by the Marshfield map. In summary, the extraction process led to tolerable uncertainty in both position and test statistic for linkage.

In meta analysis it is important to consider departures from homogeneity between the included studies. For CPMM, the cross-validation as a test of heterogeneity addresses whether the overall results are primarily affected by one single scan. The test of heterogeneous ranks for a bin might lack power when the number of scan is low. So it does not come as a surprise that we were unable to find evidence of either homogeneity or heterogeneity for any of the major findings.

GSMA appears to be robust towards imprecise data extracted from papers reporting genome wide scans. Setting the analysis into a Monte-Carlo framework and comparing results to those of different meta-analytical approaches is a possible way of investigating the sensitivity to uncertainty. However, GSMA and CPMM lead only in part to concurrent results. GSMA came up with more regions of interest, whereas by CPMM provided stronger evidence for linkage by lower p-values. A similar comparison of methods was reported by Lewis et al. (231) for schizophrenia data. With GSMA it was possible to identify more significant linkage regions than with CPMM. However, this comparison is limping, because different data sets were included into the meta analyses. Subsequently, there is no evidence to generalise this observation in the comparison of the two methods.

Finally, our approach is limited by the use of uncertain secondary data instead of original summary statistics. Hence, a meta analysis based on all real summary values to verify these preliminary results would be desirable both to further validate our approach and to give further support to the results regarding PD.

Linkage to PD

GSMA yields weak evidence for linkage to PD for 30 cM bins on chromosomes 1, 5, 9 and 14. While evidence for linkage on chromosome 1 was also provided by CPMM, the findings for chromosomes 5 and 9 remain stable when enlarging the size of the bin to 60 cM. Additional evidence for linkage was also obtained on chromosome X by CPMM, not detected by GSMA.

We are unable to find a genome wide significant or genome wide suggestive evidence of linkage in our meta analysis based on a total of 1384 affected individuals in 862 families. However, we obtained trends towards genome wide significance.

The conspicuous 30 cM bin on chromosome 1 (87 – 116 cM) overlaps with the PARK10 region designated by Hicks et al. (168). This finding is tally to recently shown genome wide significant associations of SNPs within the PARK10 region (220). However, in our meta analysis we obtained a linkage signal in this region only if the genome scan from the isolated population in Iceland (168) was included. .We could not confirm the evidence of linkage when excluding this most significant single result. Thus, even for the most prominent result we found noteable heterogeneity among genome scans.

The finding on chromosome 5 (132 – 198 cM) was yet suspected before (201) by viewing the results of the genome wide scans. Four of these scans found evidence for linkage within a 10 cM interval. Here we attach a p-value of 0.03 (using GSMA) to this result. This is supported by Maraganore et al. (220), who found 2 of eleven associated SNPs (all genome wide significant) located on chromosome 5.

The finding on chromosome 9 (112 – 169 cM) was highlighted by DeStefano et al. (200) by a maximum lod score of 1.3 at position 136 cM. This finding is supported by a combination of weaker signals (LS between 0.7 and 1.16) located up to 44 cM apart of two single genome scans (201, 207).

The linkage signal on chromosome 14 (110 -138 cM) arises from the combination of weak signals (LS between 0.62 and 1.6) located within a 9 cM distance of three single genome scans(168, 181, 207). Our meta analysis was performed on the basis of only five independent studies. Thus one should regard this finding as an add-in to the list of potential linkage regions.

Conclusion

The aetiology of a complex disease like PD is thought to involve several genetic and environmental components and is characterized by a comparatively low genetic heritability. This complicates the search for new candidate genes by genome wide linkage scans. Here, we showed a methodology to extract

information from published figures to overcome the bias of inaccessible data. We confirm the evidence of linkage on chromosomes 1, 5 and X. Additionally a signal on chromosome 14 was also obtained which needs confirming replication. With the availability of ultra-high-volume genotyping platforms and 500K gene chips genome wide association studies should be regarded as a promising addition to already performed linkage scans (220, 232, 233).

6 Discussion

The goal of genetic epidemiology is to study and enable the understanding of the genetic etiology of diseases. Diseases such as PD, AD, ALS and schizophrenia are considered to be complex traits, in which genetic as well as non-genetic factors seem to play an important role in the pathogenesis of the disease (212). A majority of the patients with complex diseases present with a sporadic form of the disease. Even in these sporadic forms genetic as well as non genetic factors, appear to contribute to the phenotypic expression of the disease (234).

The approaches used for genetic analysis of common multifactorial diseases such as PD are linkage and association analysis (212). Linkage studies aim to obtain a chromosomal location of gene(s) associated with the phenotype of interest, and are more powerful than association analysis for identifying rare high-risk disease alleles. By contrast, the association study design is more powerful to assess the effect of common risk alleles even with modest associated risks (235).

Linkage as well as association analysis are based upon the same basic principles: the co-segregation of adjacent DNA markers. In linkage analysis, the emphasis is laid on the alleles that are identical by descent in affected individuals in a family structure, be it extended or nuclear (236). This approach has successfully been applied in PD, where to date seven genes for monogenically inherited forms of PD have been discovered in large multigenerational families (81, 100, 116, 133, 136, 146, 150). However, the genetics of PD has only been partially explained by these families segregating high-risk alleles. This is because high risk alleles are rare and they explain little of the prevalence of the disease in the general population (237, 238). By contrast, the association study design relies on the determination of population frequencies of marker and risk alleles. Association may result from direct involvement of a genetic variant in the disease development or linkage disequilibrium of marker polymorphisms with the disease gene at the population

level (239, 240). Identification of common risk alleles by an association study design can shed new light on important aspects of disease pathogenesis. Moreover, from a public health perspective, common variants are of much higher importance than the rare ones because they tend to explain a larger attributable fraction of a disease in a population (236).

Both these approaches have their advantages and disadvantages. As stated above, linkage analysis is more powerful than association analysis to detect the rare allele(s) with large effect size in large families. To detect the effect of risk allele(s) of modest effect with linkage analysis, a large number of pedigrees, each containing multiple affected individuals are required which is generally difficult to obtain particularly for late onset diseases like PD. These problems are readily overcome with an association study design as it is easier to recruit a large number of unrelated affected individuals than the families with multiple affected individuals (235). Another major advantage of association analysis is that the region shared identical by descent in unrelated individuals is in general much smaller than the region shared by affected individuals in the families. Thus the process of fine-mapping is often much more efficient in association as compared to linkage studies (237).

In this thesis, we have discussed the findings of PARK3 locus followed by our findings on the PARK11 locus. To overcome the lack of power in linkage studies, a meta analysis was performed (chapter 5.1). Finally, the candidate gene studies are presented and the implications of the association study design are discussed.

We delineated the PARK3 locus in our sib pair cohort obtained from five countries through the European Consortium on Genetic Susceptibility of PD (GSPD) and refined the signal by genotyping densely spaced SNPs in our large cohort of sporadic PD patients ascertained by the Dept. of Neurology, University of Tuebingen and the Dept. of Neurology, University of Munich. It is important to note here that the PARK3 locus was originally identified in families of German descent, segregating PD in an autosomal dominant pattern with reduced penetrance (164). The gene responsible for the PARK3 locus has not been identified yet. However, a number of studies suggested that the PARK3 locus

seems to play an important role in the pathogenesis of sporadic PD as shown in fig 9. First, a study by Destefano et al. showed an association between the age at onset of PD and allele 174 of marker D2S1394 (165). The associated allele "174" of marker D2S1394 is within the 2.5 Mb core haplotype shared by two families (genealogically traced to Southern Denmark and North Germany) that showed linkage to PARK3 locus (198). Secondly, a study by Karamohammed et al fine mapped the PARK3 locus by genotyping SNPs spanning 2.2 Mb region (166). They concluded that a haplotype at the PARK3 locus, harboring the SPR gene, is associated with onset age of PD (166) and suggested that the SPR gene may be involved in both sporadic and familial PD (fig 9).

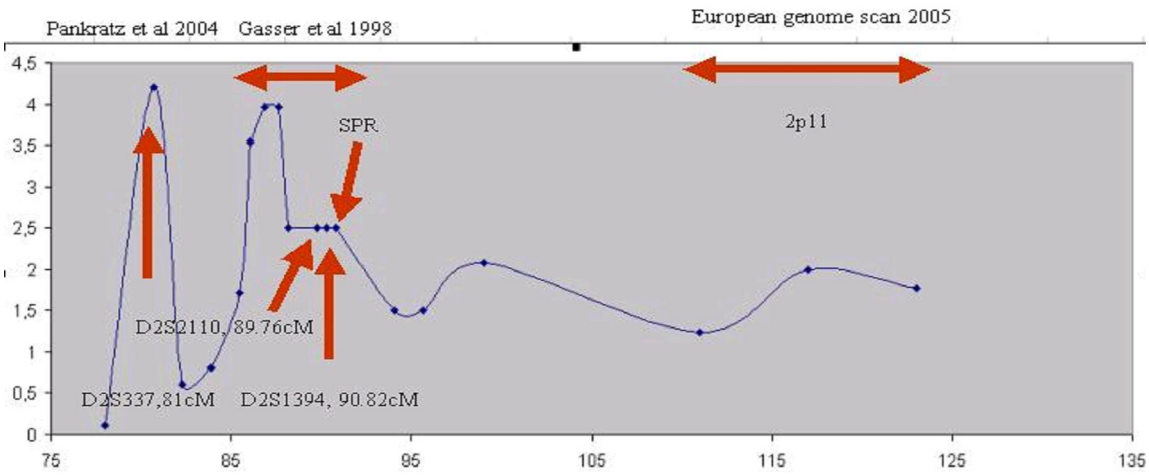

Figure 9: Graphical Illustration of the PARK3 locus. Different studies are highlighted by arrows (marked red). X-axis represents the position in cM (centimorgans) and Y–axis represents the LOD score

Finally, in our own study we obtained significant LOD scores with PD susceptibility at the two microsatellite markers D2S2110 and D2S1394, which encompass the SPR gene, in an independent large sample of multiplex families as shown in fig 6.1 (167). Further refinement of this region showed that DNA polymorphisms in one large highly inter-correlated LD block, again including the SPR gene appears to alter the susceptibility in sporadic as well as familial forms of PD. Moreover, we also found that a haplotype (A-G-G) is significantly associated with affection status in our population (167). Taken together results

from our study as well as from previously published studies suggest that variability in the SPR gene might modulate the progression of the disease.

The original discovery of the PARK3 is based upon linkage analysis of multiplex families but the fine mapping was done by affected sib pair study design (ASP) in large population. By contrast, the delineation of the PARK11 however is based entirely upon the affected sib pair study design (ASP). The ASP design is based upon the simple principle: the pairs of sibs sharing the same phenotype tend to have excessive sharing of alleles as compared to the sibs who do not share the same phenotype. The excessive allele sharing pattern – the probability that the sibs shared zero, one or two alleles identical by descent is then measured by likelihood ratio test under the null hypothesis of no linkage. For a complex disease like PD, Risch showed that ASP has more power as compared to a pedigree study design (241, 242). A study by Pankratz, using ASP study design, showed significant evidence for linkage at marker position D2S206 on chromosome 2q36-37 assuming autosomal dominant mode of inheritance (169). A number of studies, including our own study as discussed in chapter 4.3 failed to replicate the linkage signals on the PARK11 locus (168, 170, 181). This may be because sample size, ascertainment criteria, marker maps or test procedures may vary between studies which in turn leads to inconsistencies across results (243). It is important to note here that the sample used in Pankratz et al study was primarily Caucasian, nevertheless, a substantial portion of the linkage evidence came from the Hispanic families which were also used in the study (169). It is plausible that the effect conferred by PARK11 locus is small and therefore escaped detection in our sample. It is therefore unlikely that PARK11 locus plays an important role for familial PD at least in European population (170).

Apart from confirming/replicating the previous linkage findings, there is a need to develop new strategies to identify new genes for complex diseases like PD. A more effective strategy could be to pool the evidence from multiple studies across different populations, such as in a meta analysis (244). Meta analysis of genome wide linkage scans has in recent years emerged as a powerful approach in the field of linkage analysis (226, 231). The pooling of linkage

results from different studies increases power and allows to have more precision of estimates compared to the original studies (244). A number of methodologies have been proposed to conduct the meta analysis of linkage scans. Allison and Hue used the Fisher method of combining p-values across candidate regions in a study of obesity (215). Badner and Gershon extended the Fisher method of combining the p-values across different studies (217). Finally, Wise et al proposed a non parametric approach, which is based upon the rank statistics, to perform the meta analysis of whole genome wide linkage scans (218, 219).

We used a novel strategy to reconstruct the linkage statistics and corresponding marker positions from published papers (chapter 5.2). We showed evidence for linkage to chromosome 1, 5, 9 and 14. Linkage signals on chromosomes 1, 5 and 9 confirm the signals of previous genome scans. Our meta analysis findings on chromosome 1 as well as on chromosome 5 are further validated by recently published whole genome association scan of PD (220). The evidence of linkage signal on chromosome 14 is noteworthy. The linkage signal on chromosome 14 requires further confirmation in a different population.

In the following section, the implications of association study designs are discussed. A constant decline of genotyping cost and the availability of millions of SNPs in public data bases like HapMap (www.hapmap.org), makes an association study design, as compared to linkage study design, a promising approach to assess the effect of common variants in a population (188, 245). There are primarily two association strategies which are used for identifying complex trait loci: whole genome association scans and candidate gene studies (239).

Genome wide association scans offer a hypothesis free approach as they do not require “a priori” information regarding genes which are thought to play an important role in the disease pathogenesis. Recently, Maraganore et al published a first whole genome scan of PD. They identified 13 SNPs associated with PD (220). Their findings could not however be replicated in different populations indicating that these findings may be spurious (246-248). A large collaborative analysis found no evidence for these 13 SNPs in the pathogenesis

of PD (249). There are a number of reasons which can explain the differences. Firstly, Maraganore et al selected 1862 of 198345 SNPs to be genotyped in a second tier. Assuming the cut- off p- value of 0.01, most of the 1862 SNPs (1862 of 198345 ~ 0.09%) were expected to show an association by chance alone. Furthermore, slightly more SNPs (26 of 1862 ~ 0.01%) were expected to show an association in tier 1 and tier 2. It is therefore likely that most of the selected SNPs for second tier were false positives. Secondly, the sample size (n=332) used in the second tier was small as compared to tier one (n=443). It is important to note here that the power calculations were performed to detect the risk variant with an effect size greater than 2.0. It is likely that their study was underpowered to detect an association of variant(s) having effect size in the lower range. Another genome wide association scan by Fung et al nominate different SNPs for PD(250). The discrepancy between these studies highlight the issue of sample size and power. As discussed above, it is clear that variants that contribute to complex traits such as PD are likely to have modest effect, so large sample sizes are crucial. Since a large number of hypotheses is tested in a whole genome wide association scan, each p-value must be corrected for multiple testing (212). Risch and Merikangas proposed that threshold p-value of $5*10^{-8}$ for declaring significant association in a whole genome wide association scan (212). It is likely that both these studies, because of small sample size were underpowered to detect the expected modest effects.

Although the full description of whole genome wide association scans is beyond the scope of this thesis, nevertheless, it offers a promising approach to disentangle the overall pathogenesis of disease such as PD in near future.

Another approach to dissect the pathogenesis of complex diseases like PD is the candidate gene study design. Failure to replicate associations of candidate genes in different population(s) has however raised concerns about its usefulness (251). Failure to replicate association studies can be attributed to overestimation of effect size, small sample size, population admixture, insufficient SNP density and improper selection of cases and controls as well as overly liberal use of statistics in the first positive study. Nevertheless, by

carefully controlling these sources of errors and problems, rate of false positives can be reduced to a great extent.

In this thesis, we selected three candidate genes: iNOS, BDNF and FGF20 to assess their potential involvement in the sporadic form of PD in our population. A number of studies provided functional evidence regarding the involvement of these genes in the pathogenesis of PD. For example, in HD, the huntingtin protein regulates the transcription of BDNF in the cerebral cortex. Abnormalities in huntingtin protein disrupts the delivery of BDNF to the striatum site, the primary site of neuron degeneration in HD (252). Mutations in the amyloid precursor protein (APP) causes AD. APP has been implicated in anterograde axonal transport of the TrkA receptor for nerve growth factor (NGF). In an animal model of PD, BDNF gene has been shown to play a role in the survival of dopaminergic neurons in the midbrain (253). Fibroblast growth factor 2 (FGF-2) is also known to play a protective role for DA neurons in animal models of PD. FGF20 is a member of a highly conserved growth factor polypeptides that regulate central nervous development and function (196, 254). FGF20 activates the mitogen activated protein kinase (MAPK) pathway: the major intracellular signalling pathway for neurotrophic growth factors. It has been shown that activation of MAPK signalling by FGF20 promotes the survival of dopaminergic neurons in substantia nigra pars compacta (255). This evidence suggests that impairment of neurotrophic factor signalling is a common feature in neurodegenerative diseases (255, 256).

The discovery of PINK1 and DJ1 highlighted the involvement of oxidative stress in PD pathology. One mechanism of oxidative stress is the nitration of protein tyrosine residues, mediated by peroxynitrite, a reaction product of nitric oxide and superoxide radicals. Nitric oxide (NO) combines with superoxide anions and forms peroxynitrite. These free radicals can nitrate or nitrosylate proteins, lipids and DNA, leading to an impairment of mitochondria and an overload of the degradation pathway. The inhibition of two NO synthesizing enzymes, neuronal NOS (nNOS) and inducible NOS (iNOS), showed protective effect in the MPTP models of PD (182). Similarly, in an iNOS deficient mouse model, dopaminergic neurons were protected against MPTP toxicity (180).

Previously published studies reported an association with all three genes (160, 182, 183, 189). In our sample, we found no effect of the polymorphisms studied in iNOS and BDNF genes as discussed in chapter 3.1 and chapter 3.2. We only found a possible effect of the polymorphisms studied in FGF20 gene (chapter 3.3) which did not hold up after corrections for multiple testing.

We tried to control the above mentioned problems in our candidate gene studies by a number of ways. First, our sporadic PD cohort came from the same source population that gave rise to the controls. This is important because if the controls come from a different population, any systematic allele frequency differences between the populations will appear as disease association, even if they only reflect evolutionary or migratory histories (236). Lack of success of replication studies can also be due to the selection of different SNPs as compared to previously published studies. Apart from matching the same SNPs, we further enriched the region by genotyping additional SNPs to overcome any loss of information. Small sample size as discussed above often masks the effect of modest risk alleles (251). To overcome this problem, we used our large sporadic cohort as discussed in chapter 3.1 to chapter 3.3. Power calculations showed that our sample has sufficient power (95%) to detect modest risk alleles with effect size ranging from 1.2 to 2.0.

Lack of consistencies among different studies could also be due to genotyping errors (257). This may give rise to spurious association. This is indeed in agreement with the study as described in chapter 3.2. We and others found no evidence of association with SNP rs6265 (G196A) in white Caucasian population suggesting that rs6265 is unlikely to play a major role in the pathogenesis of sporadic PD. On the contrary a study by Momose et al reported an involvement of rs6265 in the BDNF gene with PD in Japanese population. However, controls included in that study were not in HWE (189). The departure from HWE indicates a false positive signal either due to genotyping error or due to population admixture. Genotyping error can greatly reduce the power of the study. Recently, a study by Salanti et al evaluated the performance of HWE in published studies (258). They concluded that the majority of the studies do not report or test the HWE to assess the genotype quality in controls. It is

remarkable that this simple analysis is often not used in genetic case control studies to evaluate the control ascertainment and genotype quality (257, 259).

The effects reported originally in two studies in the iNOS gene were based on small sample sizes (182, 183). It is known that small sample size can provide imprecise or incorrect estimates of the magnitude of the observed effects (260). Moreover, it can result in insufficient power to detect the minor contributions of one or two alleles (260). Apart from genotyping the previously reported SNPs in our sample, we adequately enriched the iNOS gene to detect the effect of variants. Therefore, it is unlikely that we missed an association even if the reported SNP were in LD with a true causal variant (261). It is thus unlikely that variability in the iNOS gene plays a major role in the sporadic form of PD at least in our population (261).

Our failure to replicate the association signals could also be the effect reported by study is population specific. The study by Van der Walt was performed on familial data set. It is possible that familial background in their study as compared to our sporadic population contributes to the difference. Our LD data as discussed in chapter 3.3 are in agreement with the original study. It is therefore, unlikely that variability in the LD contributes to the differences. A recently published study analysed the same SNPs in the Greek as well as in the Finnish population (161). Their results are in agreement with our study suggesting that variations in FGF20 gene are unlikely to play an important role in the sporadic form of PD at least in the European population.

Careful selection of cases as well as controls, assessing the genotype quality by performing HWE test on controls and adequate sample sizes improve the quality of association studies. Moreover, the pooling of raw data should be encouraged if small effects are expected. Recent literature shows that more and more investigators are sharing their genotype data to dissect the pathologies of complex diseases (98, 249). By carefully considering the above discussed parameters, candidate gene studies offer an elegant approach to dissect the pathology of disease like PD in near future.

6.1 Clinical relevance

The discovery of PD related genes (aplha-synuclein, Parkin, PINK, DJ1, LRRK2 and ATP13A2) have revolutionized the PD genetic research (262). In the light of recent genetic data, the clinical classification of parkinsonism and PD should be revised (263). It is now clear that PD is not a homogenous entity as previously thought. Many cases of this seemingly sporadic disease have a family history, so in turn a positive family history should not be considered as an exclusion criteria for the diagnosis of PD (79). Longitudinal evaluation of mutation carriers might provide fundamental insights into the natural history of PD (79).

The genetic findings discussed above (chapter 2.1) provide the rationale for developing and testing novel pharmaceutical approaches to halt disease progression. Several possibilities are currently being explored in model systems including the use of immunological strategies to reduce alpha synuclein burden in transgenic mice and invertebrates (264). Recent data emerging from various studies link oxidative and mitochondrial damage to disease pathogenesis. For example, a study by Bender et al demonstrated that SN neurons have a high amount of mitochondrial DNA deletions in post-mortem PD patients when compared with SN neurons from healthy and age matched controls (265).

It is clear by now that the discovery of new genes has a major impact on understanding the pathophysiology of the disease. There is still a long way to fully understand the pathological mechanism of the disease. However; it is remarkable that a disease, once considered to be a "non -genetic“disorder might be the first to be prevented through a genetic understanding of its molecular causes.

Summary

Parkinson disease is the second most common neurodegenerative disorder. The main clinical phenotype of PD is parkinsonism, a movement disorder, that is characterized by tremor, rigidity and bradykinesia. Recent discoveries of PD related genes underline the importance of the genetic components of the disease. The goal of this thesis was to identify novel genetic susceptibility factors for PD (chapter 1).

Chapter 2 provides a comprehensive review on the genetic epidemiology of PD describing the current status on the genetic research of PD. In chapter 3, three candidate gene studies are described. Chapter 3.1 describes the findings on the iNOS gene. The study population consisted of 340 cases and 680 controls. We genotyped ten SNPs to cover the whole gene. Genotype and haplotype analysis did not show significant association with PD. Taken together, these data indicate that iNOS does not play a major role in the pathogenesis of PD. In chapter 3.2, we studied the role of the BDNF gene in PD. Recent studies provided conflicting results regarding the involvement of BDNF gene with PD. Lack of consistencies can be due to small sample sizes. To increase the sample size, we performed a meta analysis by pooling together all previously published studies. No significant association was observed between BDNF gene polymorphism with PD. Our result suggests that there is no major effect of BDNF gene polymorphisms with PD in the European population. Finally, in chapter 3.3 our findings on FGF20 are presented. A study by Van der Walt et al reported two SNPs to be significantly associated with PD. We could not replicate the findings in our population. It is likely that the effect reported by Van der Walt is population specific. The study by Van der Walt is performed with a familial data set. It is possible that different study design might contribute to the difference. A recently published study in the Greek as well as in the Finnish population found no evidence for this association. Their results are in agreement with our study suggesting that variations in the FGF20 gene are unlikely to play an important role in the sporadic form of PD at least in the European population.

In chapter 4, family based studies are described. In chapter 4.1, linkage and association analysis was performed. Different studies highlighted the SPR gene as a potential candidate gene for the PARK3 locus. A recent study reported SNP rs1876487 to be significantly associated with age of onset in the North American population. In our study, we thoroughly assessed the SPR gene. We genotyped 5 STR markers to cover the SPR gene. We obtained significant LOD scores with PD susceptibility at the two microsatellite markers D2S2110 and D2S1394, which encompass the SPR gene, in an independent large sample of multiplex families. Further refinement of this region showed that DNA polymorphisms in one large inter-correlated block appear to alter the susceptibility in sporadic as well as in the familial forms of PD. We found one haplotype (A-G-G) significantly associated with affection status in our population. Taken together results from our study as well as from previous published studies suggest that variability in SPR gene might modulate the progression of the disease. Chapter 4.2, describes our study on the European sib pair cohort to replicate the linkage on PARK11 locus. No evidence of linkage was observed in our sib pair population. Furthermore, the sib –transmission disequlibrium test (s-TDT) did not show any association with markers, suggesting that the PARK11 is not a major player at least in the European population.

In chapter 5, a meta analysis of genome wide linkage scans of PD is described. Pooling of linkage results across different studies will increase the sample size and hence help to find loci with small effects. In this meta analysis, we addressed the situation where there is no availability of raw genotype data from independent investigators. We solved this problem by reconstructing the figures and tables from previously published findings. By using our methodology, we found evidence for linkage to chromosome 1,5,9 and 14. Our findings on chromosome 1,5 and 9 confirms the signal of previous genome scans. The evidence of a linkage signal on chromosome 14 is noteworthy. The linkage signal on chromosome 14 requires further confirmation in a different population. Finally in chapter 6, our main findings are discussed. We conclude by

discussing the methodological issues of genetic case control studies, linkage studies as well as whole genome scan association studies.

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