A Clinical and Molecular Genetic Study Into Familial and Sporadic Parkinson's Disease

by

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease which causes tremor, muscular rigidity and bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive actions). Although the underlying causes remain unknown, there is evidence that genetic factors play an important role in the disease process. In this thesis I investigated the role of a recently identified hereditary PD gene *leucine rich repeat kinase 2* (*LRRK2*) in PD in the United Kingdom (UK).

In this thesis I have confirmed the importance of pathogenic *LRRK2* mutations in UK familial PD (fPD). In addition I identified three novel frameshift mutations. I investigated the functional effects of two of these mutations and provide evidence that nonsense mediated decay (NMD) is occurring in *LRRK2*-PD.

In this thesis I also present data from an extensive screen of *LRRK2* in UK subjects with sporadic PD (sPD). This confirms the importance of pathogenic *LRRK2* mutations in UK sPD.

In addition I report a novel missense mutation in the *GTP cyclohydrolase I* gene *(GCH1)* in a kindred with phenotypes ranging from PD to Dopa-responsive dystonia. The association of this novel *GCH1* mutation with late onset parkinsonism suggests a potential role for *GCH1* in PD.

This thesis is dedicated to the memory of my mother

Judith Constance Lewthwaite

who passed away in November 2008.

For all the love and support that she always gave me.

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List of Abbreviations

ABN	Association of British Neurologists
AD	Autosomal dominant
AD PD	Autosomal dominant Parkinson's disease
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APS	Ammonium persulfate
AR	Autosomal recessive
ARE	Antioxidant response element
AR PD	Autosomal recessive Parkinson's disease
ATP	Adenosine-5'-triphosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
ºC	Degrees Celsius
CBD	Corticobasal degeneration
CHX	Cycloheximide
CI	Confidence interval
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
COR	Carboxy-terminal of Roc
CO_2	Carbon dioxide
CRMP-2	Collapsin response mediator protein 2

CSF	Cerebrospinal fluid
CT	Computed tomography
CVA	Cerebrovascular accident
DAT	Dopamine Transporter
DBS	Deep brain stimulation
DGGE	Denaturing gradient gel electrophoresis
DHPLC	Denaturing high performance liquid chromatography
D-MEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxyribonucleotide triphosphate
ddNTP	Dideoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
dsDNA	Double stranded DNA
mtDNA	Mitrochondrial DNA
DLB	Dementia with Lewy bodies
DMSO	Dimethyl sulfoxide
DMV	Dorsal motor nucleus of the vagus
DRD	Dopa-responsive dystonia
DTT	Dithiothreitol
DZ	Dizygotic
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EJC	Exon-junction complex
ENS	Enteric nervous system
ERM	Ezrin, Radixin and Moesin
6-FAM	6-Carboxyfluorescein
F-dopa	6-[^{18} F]-fluorodopa
fPD	Familial Parkinson's disease
xg	Times gravity
GBA	Glucocerebrosidase
GPl	Lateral globus pallidus
GPm	Medial globus pallidus
GTP	Guanosine 5'-triphospate
GTPCH1	Guanosine 5'-triphospate cyclohydrolase 1
GWAS	Genome-wide association studies
HPD	Hereditary progressive dystonia
HRP	Horseradish peroxide
Hrs	Hours
HSP	Heat shock protein
HWE	Hardy-Weinberg equilibrium
Hz	Hertz
IBR	In-between RING motif
ION	Institute of Neurology
IPD	Idiopathic Parkinson's disease
kb	Kilobase pairs

kDa	Kilodalton
KRD	Kufor-Rakeb disease
LB	Lewy body
L-Dopa	Levodopa
LNs	Lewy neurites
LOD	Logarithim of the odds
LPS	Lipopolysaccharide
LREC	Local research ethics committee
LRR	leucine rich repeat
LRRK2	leucine rich repeat kinase 2
m	Milli (10^{-3})
M	Molar (moles per litre)
μ	Micro (10^{-6})
Mb	Megabase
MAO-B	Monoamine oxidase-B
MAOIs	Monoamine oxidase inhibitors
MAPT	Mictrotubule-associated protein tau
mg	Milligram
$MgCl_2$	Magnesium chloride
mins	Minutes
ml	Millilitre
mM	Millimolar
μg	Microgram

µl	Microlitre
µM	Micromolar
MAF	Minor allele frequency
MLPA	Multiple ligation-dependent probe amplification
MMSE	Mini Mental State Examination
MPP+	1-methyl-4- phenylpyridinium
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MREC	Multi centre research ethics committee
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSA	Multiple system atrophy
MZ	Monozygotic
ng	Nanogram
NHS	National Health Service
nm	Nanometre
NMD	Nonsense-mediated decay
NSAIDs	Non steroidal anti inflammatory drugs
OR	Odds ratio
p-value	Probability value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDD	Parkinson's disease with dementia

PET	Positron emission tomography
PGC-1a	PPARgamma coactivator 1 alpha
PINK1	Phosphatase and tensin homologue deleted on chromosome 10-induced kinase-1
PSP	Progressive supranuclear palsy
PTC	Premature termination codon
REM	Rapid eye movement
RFLP	Restriction fragment length polymorphism
RING	Really interesting new gene box domain
Rpm	Revolutions per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
Roc	Ras-like GTPase domain
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
s	Seconds
SDS	Sodium dodecyl sulfate
SNc	Substantia nigra pars compacta
SNCA	α-synuclein
SNr	Substantia nigra pars reticulata
SNP	Single nucleotide polymorphism
SPECT	Single-photon emission CT
SPR	Sepiapterian reductase

SPSS	Statistics package for social science
SSCP	Single-strand conformation polymorphism
STN	Subthalamic nucleus
SWEDDs	Scans Without Evidence of Dopaminergic Deficit
TBE buffer	Tris Borate-EDTA buffer
TE buffer	Tris EDTA buffer
TeMed	N,N,N,N –Tetramethyl-Ethylenediamine
TH	Tyrosine hydroxylase
UBL	Ubiquitin-like domain
UPD	Unique Parkin domain
UCH-L1	Ubiquitin carboxy-terminal hydrolyase L1
UK	United Kingdom
UKPDS	United Kingdom Parkinson's Disease Society
UPDRS	Unified Parkinson's disease rating scale
UPS	Ubiquitin-proteasome system
UPSIT	University of Pennsylvania Smell Identification Test
UV	Ultra violet
V	Volts
$\chi 2$	Chi-squared
YOPD	Young onset Parkinson's disease
Yrs	Years

1 General Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease, with a cumulative life-time incidence estimated at 2% [1]. The economic burden in the UK alone is said to be approximately £6 billion per annum [2]. There has been much progress over the last few decades in devising more accurate diagnostic criteria for PD and in development of treatments for the condition. However despite this progress, the aetiology of PD remains poorly understood, with the vast majority of cases considered to be idiopathic. It has been proposed that a complex interplay between genetic and environmental factors contributes to an individual's risk of developing PD.

In the introduction to this thesis I will cover a number of topics related to PD, including some historical aspects of the condition, the clinical features and differential diagnosis of the condition, and our current understanding of the aetiology of the condition, with particular emphasis on the genetic hypothesis.

1.1 Historical aspects of Parkinson's disease

The year 2005 marked the 250th anniversary of the birth of James Parkinson (1755-1824). His publication in 1817 *An essay on the shaking palsy* [3] gave a clear description of the condition that would eventually bear his name. The first few sentences of the essay evoke the main clinical features of PD, or *paralysis agitans*, perhaps better than any of the modern descriptions of the condition: *'muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.'*

This leaves an unanswered question, as to whether PD existed prior to the 19th century [28]. If it did not, then PD may be due to exposure to ubiquitous toxin or toxins which coincided with increasing industrialisation [29], [30]. Although their authors may not have been able to put together all aspects of the phenotype of the disease as Parkinson did, there do however appear to be several descriptions of PD in earlier literature. These include a description by Galen (Anno Domini 138-201) of a disorder compatible with the festinant gait of PD [28], and the description by Leonardo da Vinci (1452-1519) compatible with the involuntary rest tremor of PD [31]. This suggests that the disease entity is in fact much more ancient.

The term 'Parkinson's disease' was first used by Charcot in Paris [32] and Meynert was the first to suggest that PD was due to defective functioning of the basal ganglia [33]. Then in 1913 Lewy reported spherical inclusions in the nucleus substantiae innominatae and the dorsal nucleus of the vagus [34], although he did not examine the substantia nigra. These so called 'Lewy bodies' are a highly sensitive, but not entirely specific marker for PD [35]. However, it was another five years before the full importance of the substantia nigra became apparent after the work of Trétiakoff [36].

The biochemical abnormalities in PD only became apparent several decades later, when in 1960 PD was shown to be associated with depleted dopamine production in the caudate nucleus and putamen [37]. Indeed these findings led to the successful use of levo-dopa (L-Dopa) as a dopamine replacement therapy in PD [38]. From this time L-Dopa became the 'gold standard' by which other anti-parkinsonian drugs would be compared.

1.2 Clinical features of PD

Clinically PD is characterized by slowing of emotional and voluntary movement, muscular rigidity and tremor. Using the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria for PD [14] the diagnosis can be further refined as consisting of bradykinesia (i.e. slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive actions) along with at least one of the following: (i) muscular rigidity, (ii) 4-6Hz rest tremor, and (iii) postural instability that is not due to any other neurological cause. The full diagnostic criteria with exclusion criteria can be found below in table 1.1. PD is a progressive condition which will tend to evolve from mild unilateral symptoms through to end-stage nonambulatory state. The milestones in the illness are accurately outlined in the Hoehn and Yahr staging system [13] (see table 1.2).

Inclusion Criteria	**Exclusion Criteria**	**Supportive Criteria**
Bradykinesia ***And at least one of:*** Muscular rigidity 4-6 Hz rest tremor Postural instability	History of repeated strokes with stepwise progression of parkinsonian features History of repeated head injury History of definite encephalitis Oculogyric crises Neuroleptic treatment at onset of symptoms More than 1 affected relative Sustained remission Strictly unilateral features after 3 yrs Supranuclear gaze palsy Cerebellar signs Early severe autonomic involvement Early severe dementia with disturbances of memory, language and praxis Babinski sign Presence of cerebral tumour or communicating hydrocephalus on CT scan Negative response to large doses of levodopa (if malabsorption excluded) MPTP exposure	***Three or more required for diagnosis of definite PD*** Unilateral onset Rest tremor present Progressive disorder Persistent asymmetry affecting side of onset most Excellent response (70-100%) to levodopa Severe levodopa-induced chorea Levodopa response for 5 yr or more Clinical course of 10 yr or more

Table 1.1 UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria for PD. Adapted from Gibb and Lees 1988 [14]

Stage	Criteria
Stage 0	No signs of disease
Stage I	Unilateral disease
Stage I.5	Unilateral disease plus axial involvement
Stage II	Bilateral disease, without impairment of balance
Stage II.5	Mild bilateral disease, with recovery on pull test
Stage III	Mild to moderate bilateral disease; some postural instability; physically independent
Stage IV	Severe disability; still able to walk or stand unassisted
Stage V	Wheel chair bound or bedridden unless aided

Table 1.2 Hoehn and Yahr staging of PD. Adapted from Hoehn and Yahr 1967 [13].

There are a number of studies which have looked at the initial clinical features in PD, and there is now preliminary evidence that some clinical features may in fact influence disease progression [39], [40]. A recent study found that the most common initial motor symptom in PD was tremor (49%). In 21% of subjects bradykinesia was the initial motor symptom, followed by gait difficulty (10%), micrographia (8%), rigidity (5%) and other (7%) [17]. Indeed tremor (59%) and bradykinesia (16%) were also the most common initial motor symptoms reported in the DATATOP series [41]. The upper extremities were the most common location for initial symptoms (70%) [17]. Overall a slower rate of progression for tremor as compared to gait difficulties has been found in several studies [17], [42], [13], [43].

1.2.1 Nonmotor features of PD

Almost all patients with PD have nonmotor symptoms, which contribute prominently to reduced quality of life [5]. Autonomic symptoms include reduced gastrointestinal transit time, urinary frequency and orthostatic hypotension. Anxiety and mood disorders are each found in up to 40% of PD patients [44]. Sleep disturbance and fatigue are also almost universal in PD [5].

Cognitive changes are also common in Parkinson's disease and cause substantial disability [45], [5]. They can occur early in the disease and tend to involve impairments in executive functioning (ability to plan, organise, and regulate goal-directed behaviour). Over time PD with mild cognitive impairment can develop into a subcortical dementia, called PD with dementia (PDD). Furthermore, cognitive symptoms in PD patients are associated with more rapid disease progression [46].

The diagnosis of dementia in PD can be complicated by another disease entity called dementia with Lewy bodies (DLB). Indeed, debate still exists whether PDD and DLB are distinct disease entities or represent a spectrum of motor, cognitive and behavioural impairment [47], [20], [48]. The current clinical guidelines are that a diagnosis of DLB is made when cognitive symptoms appear within 1 year of the onset of motor symptoms, whereas a diagnosis of PDD is made when cognitive symptoms begin more than 1 year after onset of motor symptoms [49], [50]. The cognitive and psychiatric symptoms of DLB and PDD are essentially the same, with both having fluctuating abnormalities of cognition (confusion, disorientation, delusions) and well formed visual illusions and hallucinations [20], [49], [51]. The clinical profiles of PDD and DLB can be found below in table 1.3.

Neuropsychological performance almost identical
Psychiatric symptoms the same
Both have REM behavior disorder
Parkinsonism more symmetrical and tremor less in DLB
Neuroleptic sensitivity less in PDD
Levodopa response not as robust in DLB
Similar response to cholinergic treatment

Table 1.3 Clinical features of PDD and DLB. Adapted from Lippa et al. (2007) [20].

Depending on the population and criteria applied, reported rates of dementia in PD have been variable, ranging from 3 to 80% [52], [53], [54], [55], [56], [57]. It seems clear that the prevalence of PDD increases as PD progresses, for example one study showed a 26% prevalence at baseline, increasing to 51% at 4 years and to 78% at 8 years [58]. DLB is the second most common type of neurodegenerative dementia in older people, after Alzheimer's disease. Estimates of prevalence of DLB vary between 3% [59], [60] and 26.3% [61] of all demented cases over the age of 65. Autopsy series reveal similar estimates ranging between 15 and 25% [60], [62].

1.2.2 Heterogeneity of the PD phenotype

It is now well recognised that PD can present in a variety of ways and perhaps as such is best regarded as syndrome rather than a single disease. There are also conditions which can mimic

PD, including the parkinsonian plus syndromes multiple system atrophy (MSA) and progressive supranuclear palsy (PSP), drug induced parkinsonism, and vascular parkinsonism. However, the reported difficulties in clinical diagnosis of PD could also be explained by this heterogeneity in disease phenotype. Some series have shown a diagnostic accuracy of less than 80% [63]. However, a clinicopathological study has shown that strict implementation of diagnostic criteria, such as the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria for PD [14], can improve diagnostic accuracy to over 95%, in the hands of movement disorders specilaists [64].

Many attempts have been made to investigate and define this heterogeneity in PD. Most have used classifications based on pre-determined patient attributes such as age of disease onset [65]; cognitive performance [66], [67]; motor phenotype [13]; presence of depression [68], [69]; disease severity [13], [70]; or motor symptom laterality, [71], [72].

Some more recent studies have used cluster analysis to allow variables to be assessed in conjunction, rather than independently. Four cohorts of patients were identified: younger onset, tremor dominant, non-tremor dominant (with cognitive impairment and mild depression), and rapid disease progression (without cognitive impairment) [73], [74], [75].

These findings do require careful consideration as cluster analysis is dependent on the variables that are entered into it. However, the finding of a group of patients with young onset disease has been consistent amongst many studies [76], [77], [78]. Generally patients in this subgroup show a slower rate of disease progression [65], [43], [79], [41]; less cognitive impairment [43], [41], [80]; and a greater potential to develop motor fluctuations [63], [81]. Further clinicopathological study of these subgroups is required, but it does seem likely that patients with different clinical phenotypes may have different pathological processes and foci

[82], [83]. Therefore, further investigation of these subgroups, including genetic analyses, may prove to be important for our understanding of the aetiology of PD.

1.2.3 Differential diagnosis of PD

There are a wide range of conditions which can mimic PD clinically, as listed below in table 1.4.

Multiple system atrophies Striatonigral degeneration Olivopontocerebellar atrophy Shy-Drager syndrome	*Infectious disorders* Postencephalitis lethargica Postviral encephalitis
Hereditary disorders Wilson's disease Huntington's disease (rigid variant) Hakkervorden-Spatz disease Dopa responsive dystonia	*Other CNS disorders* Hydrocephalus Intracranial neoplasms Cerebral anoxia Repetitive head trauma Multiple cerebral infarcts Calcification of the basal ganglia
Other degenerative diseases Progressive supranuclear palsy Corticobasal degeneration Diffuse Lewy body disease Parkinsonism-dementia complex of Guam	Drug- induced parkinsonism *Toxins* N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Table 1.4 Causes of an akinetic-rigid syndrome. Adapted from Koller 1992 and Harding 1993 [4], [10].

With modern diagnostic criteria it is usually possible to differentiate between these conditions, although this is not always the case. The commonest causes of mis-diagnosis by non-specialists are probably essential tremor, artheriosclerotic pseudoparkinsonism [84] and drug-induced parkinsonism. However the commonest causes of misdiagnosis in prospective brain bank series are MSA and PSP [63], [64], [85]. These conditions are much rarer and largely escaped recognition as clinico-pathological entities until the 1960s.

There are a number of 'red flags' in the clinical features that may suggest alternative diagnoses (see table 1.5). Cognitive impairment within the first year should raise the possibility of DLB, corticobasal degeneration (CBD) or PSP. Symmetrical or prominent midline or bulbar signs, early gait disorder with falls, dependence on a wheelchair within 5 years of diagnosis, orthostatic hypotension, or urinary incontinence all suggest a 'parkinsonian plus' syndrome (MSA or PSP). An early gait disorder can also suggest vascular parkinsonism, whilst apraxia, alien limb, or cortical sensory loss suggest CBD.

Early or prominent dementia	Early autonomic failure
Symmetrical signs	Sleep apnoea
Bulbar dysfunction	Gasping respirations
Early gait disorder	Apraxia
Falls within the first year	Alien limb
Wheelchair dependence within 5 years	Cortical sensory loss

Table 1.5 Red flags in the diagnosis of PD. Taken from Shannon 2004 [5].

1.3 Pathology of PD

1.3.1 Functional anatomy of the basal ganglia

The structure of the basal ganglia is well understood, and it is known to be made up of the striatum (caudate nucleus and putamen), globus pallidus (medial and lateral segments), nuclei of the diencephalon (thalamus and subthalamic nucleus) and the mesencephalon (substantia nigra and pedunculopontine nucleus).

The striatum is regarded as the input area of the basal ganglia because it receives afferents from the cerebral cortex, thalamus and substantia nigra pars compacta (SNc). The medial globus pallidus (GPm) and substantia nigra pars reticulata (SNr) are the output area of the basal ganglia, with projections to a number of areas such as the thalamus and pedunculopontine nucleus (PPN). The GPm is known to give rise to inhibitory GABAergic output [86], [5], [11]. These structures are represented in figure 1.1.

The striatum gives rise to two inhibitory projections to the globus pallidus, the 'direct' pathway which projects to the GPm, and the 'indirect' pathway projects to the lateral globus pallidus (GPl). The direct pathway allows direct inhibitory control of the GPm whilst the indirect pathway controls the GPm via the subthalamic nucleus (STN). The STN also receives inhibitory projections from the GPl and projects excitatory fibres to the pallidum. Activation of the direct pathway therefore inhibits basal ganglia output, whilst activation of the indirect pathway will result in activation of the output neurons. These two pathways are known to be under differential dopaminergic control from the nigrostriatal pathway, with the direct pathway stimulated by D1 dopamine receptor activation, whilst the indirect pathway is inhibited by D2 dopamine receptor activation. There is in addition a GABAergic connection from the GPl to the basal ganglia output regions [86], [11].

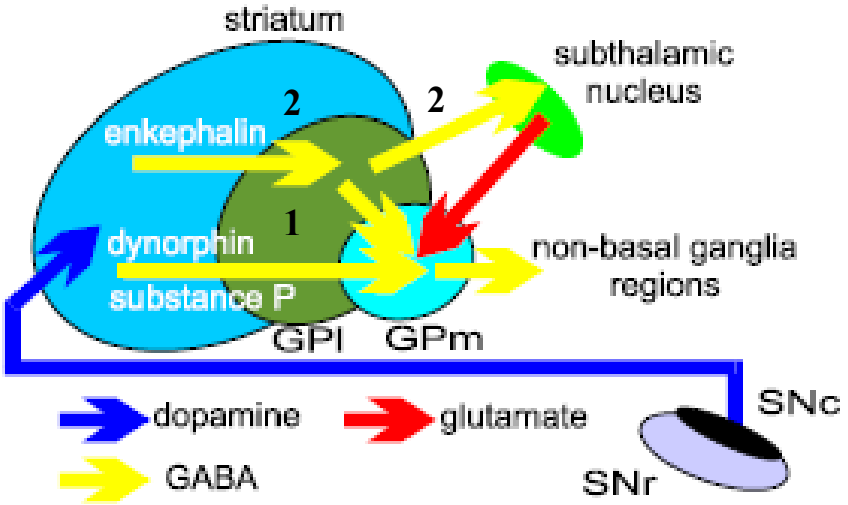

Figure 1.1 Schematic representation of the functional anatomy of the basal ganglia in health. The basal ganglia are made up of the striatum (caudate nucleus and putamen), globus pallidus (medial and lateral segments), nuclei of the diencephalon (thalamus and subthalamic nucleus) and the mesencephalon (substantia nigra and pedunculopontine nucleus). The striatum receives dopaminergic afferent input from the SNc and gives rise to two inhibitory (GABergic) projections to the globus pallidus. The 'direct' pathway (1) which projects to the GPm, and the 'indirect' pathway (2) which projects to the GPl. The direct pathway allows direct inhibitory control of the GPm whilst the indirect pathway controls the GPm via the STN. The STN also receives inhibitory projections from the GPl and projects excitatory (glutamatergic) fibres to the pallidum. Activation of the direct pathway therefore inhibits basal ganglia output, whilst activation of the indirect pathway will result in activation of the output neurons. There is in addition a GABAergic connection from the GPl to the basal ganglia output regions. Abbreviations: GPl= Globus pallidus lateral; GPm= Globus pallidus medial; SNc= Substantia nigra pars compacta; SNr= Substantia nigra pars reticulata; GABA= Gamma-aminobutyric acid. Taken from Crossman 2004 [11].

In subjects with PD there is known to be a loss of dopaminergic neurons in the SNc, which leads to dopamine loss in the striatum. This will lead to an under-activity of the direct pathway

and an over-activity of the indirect pathway from the striatum to the GPl. This over-activity will in turn inhibit the pallidostriatal neurons, causing a disinhibition of the STN. The resulting overactivity of the STN, and hence of the GPm, results in inhibition of the motor thalamus, cerebral cortical mechanisms, and brain stem locomotor region (including pedunculopontine nucleus) [87], [88], [11]. This disease state is represented in figure 1.2 below.

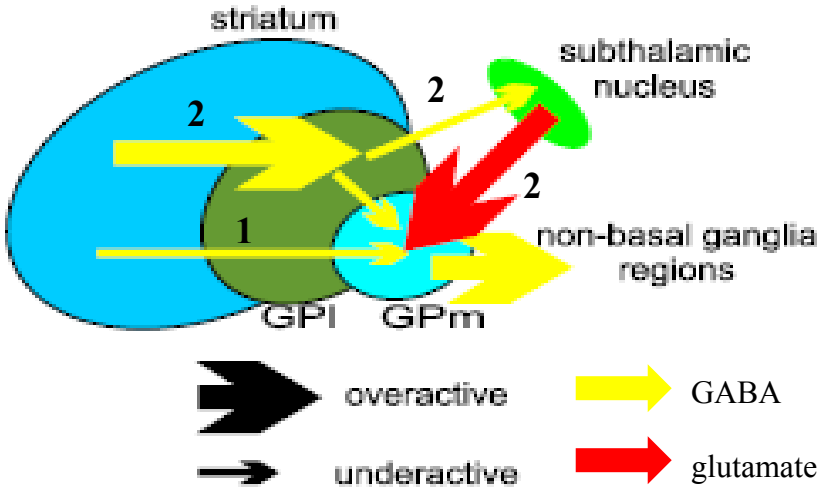

Figure 1.2 Schematic representation of the pathophysiology of parkinsonism in the basal ganglia. In subjects with PD there is known to be a loss of dopaminergic neurons in the SNc, which leads to dopamine loss in the striatum. This will lead to an under-activity of the direct pathway (1) and an over-activity of the indirect pathway (2) from the striatum to the GPl. This over-activity will inhibit the pallidostriatal neurons, causing a disinhibition of the STN. The resulting overactivity of the STN, and hence of the GPm, results in inhibition of the motor thalamus, cerebral cortical mechanisms, and brain stem locomotor region (including pedunculopontine nucleus). Abbreaviations: GPI= Globus pallidus lateral; GPm= Globus pallidus medial. Taken from Crossman 2004 [11].

Dyskinesias and dystonia are features that can be seen in PD, in the case of dyskinesias as side effects of medication. These symptoms appear to be mediated by the opposite changes to those seen in PD, with an overactivity of the direct pathway and an underactivity of the STN and GPm [11].

1.3.2 Cellular pathology of PD

The neuropathological hallmark of PD is depigmentation of the substantia nigra pars compacta (SNc), representing loss of neuromelanin containing dopaminergic neurons. Up to 80% of dopaminergic neurons are lost before clinical signs and symptoms of PD develop [89], [90], [91]. There is also an associated neuronal loss in the ventral tegmental area and locus coeruleus [91], [92].

A prerequisite for the postmortem diagnosis of PD is evidence of abnormal proteinaceous cytoplasmic inclusions in nigrostriatal neurons, which develop as spindle or thread-like Lewy neurites (LNs) in dendrites and axons, and in the form of globular Lewy bodies (LBs) in the somata of involved nerve cells [93], [94], [95], [96] (see figure 1.3). LBs are intracellular aggregations of proteins and lipids that were first identified by eosin staining. They are now known to be enriched in α-synuclein and other proteins that are often highly ubiquitinated [97], [98], [99].

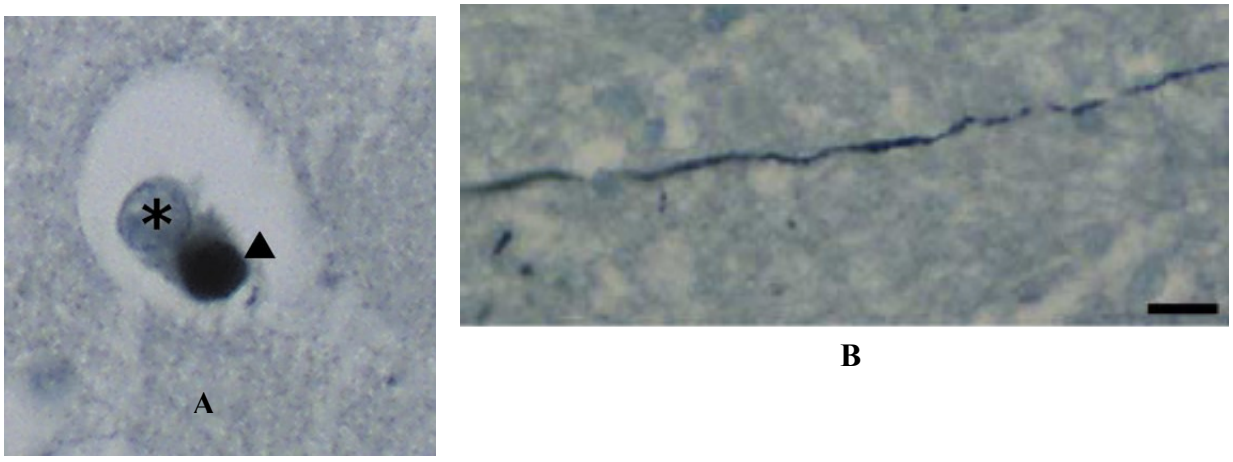

Figure 1.3 Immunolabelling for alpha-synuclein in human brain tissue from a subject with dementia demonstrating Lewy body pathology. Panel A= High-magnification photomicrograph of a Lewy body (arrowhead) in a pyramidal neuron of the orbital frontal cortex. Toluidine blue counterstaining allows the visualization of Alzheimer-type plaques (centre, labelled with asterisk), into which Lewy neurites have developed. Panel B= A Lewy neurite in horizontal section. It is notable that Lewy neurites can extend for long distances. The neurite visible in B is from a region of the anterior olfactory nucleus and is approximately 220 μm in length. Scale bar = 25 μm. The primary antibody used for the detection of alpha-synuclein was rabbit anti-alpha-synuclein antibody (Sigma). Taken from Hubbard et al. 2007 [24].

Whilst the LB is a sensitive marker for PD, it is not disease-specific and can be found in a number of other conditions such as Lewy body dementia, MSA and Hallervorden-Spatz Disease [100], [101], [102], [103]. Indeed LBs have also been detected in up to 10% of subjects over the age of 60 without clinical evidence of PD and it has been proposed that this may represent a pre-clinical phase of the disease [14], [104], [105], [106].

Whilst neuronal loss in the SNc is pronounced in PD, there is also widespread neurodegeneration in other areas of the central nervous system (CNS) and it is thought that the

SNc becomes involved in the middle stages of the disease [98], [107]. A pathological staging system for PD based upon the topographical extent of lesions has been proposed [107].

Braak et al. suggest that rather than evolving simultaneously at all the nigral and extra-nigral sites the pathological process may follow a pre-determined sequence [107]. It appears to target specific induction sites, with lesions initially occurring in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and anterior olfactory nucleus, progressing to involve the substantia nigra and eventually cortical areas become affected. The disease process in the brain stem progresses in an ascending course with little variation observed between individuals. Cortical involvement begins with the anteromedial temporal mesocortex, the neocortex then becomes involved, starting with the high order sensory association and prefrontal areas, followed by the first order sensory association/premotor areas and primary sensory/motor fields [107].

The sequential pathological process in PD proposed by Braak et al. may give important insights into which areas to focus on when we consider the aetiology of PD, and be important as we attempt to develop pre-symptomatic markers of the condition.

1.4 Investigation and treatment of PD

PD remains a clinical diagnosis and routine laboratory investigations are unhelpful in diagnosing PD. Furthermore, computed tomography (CT) and magnetic resonance imaging (MRI) are also not helpful in making a diagnosis of PD, although they may suggest alternative diagnoses such as PSP or MSA.

Olfactory testing and functional neuro-imaging in PD can be helpful for diagnosis, and even for pre-symptomatic screening, and are discussed below. The treatment of PD is also covered in section 1.4.3.

1.4.1 Olfaction and PD

Olfactory dysfunction (hyposmia) is found in 70-100% of PD patients, rendering it as common a clinical sign as pill rolling tremor [108], [109], [110], [111]. It is associated with neuronal loss and Lewy body deposition in the olfactory pathway [112]. Olfactory dysfunction is markedly reduced early on in the course of PD, as assessed by the University of Pennsylvania smell identification test (UPSIT) a well validated method of quantification of olfactory dysfunction [113], [108], [114], [115] [116], [117]. Marked olfactory dysfunction is also common in DLB, but it is not found in vascular parkinsonism, PSP and CBD, and only relatively mild olfactory dysfunction has been found in MSA.

The early or 'pre-clinical' detection of Parkinson's disease is an area in which olfactory testing may be of value in the future [118], [110], [117], [119], [120], [121], [122].

1.4.2 Functional imaging of the dopaminergic system

The pathological changes in the basal ganglia discussed above can now be detected by functional imaging. The radiopharmaceutical 6-[^{18} F]-fluorodopa (F-dopa) is taken up by dopaminergic neurons in the SN and metabolised to 6-[^{18}F]-fluorodopamine. Positron emission tomography (PET) scans using this radiopharmaceutical agent show reduced F-dopa uptake in dopaminergic nerve terminals in the putamen and caudate proportional to the severity of degeneration in the ipsilateral SN and symptoms in the contralateral hemibody [5].

Dopamine Transporter (DAT) imaging with single-photon emission CT (SPECT) enables in vivo demonstration of striatal dopamine activity, and yields results in keeping with other measures of the dopamine system such as autopsy studies [123], [124], [125]. DAT imaging is increasingly used in clinical practice. Although it is abnormal in PD, MSA and PSP it helps to distinguish theses conditions from essential tremor, vascular parkinsonism (unless there is focal basal ganglia infarction), drug-induced parkinsonism, or psychogenic parkinsonism in which DAT scanning is normal [126].

DAT is a sodium chloride-dependent protein on the pre-synaptic dopaminergic nerve terminal which controls dopamine levels by active re-uptake of dopamine after its interaction with the postsynaptic receptor [127]. The DAT ligands for SPECT, including [^{123}I]FP-CIT (DaTSCAN) [128] all show significantly reduced striatal uptake in PD [129], even in early cases [130]. The abnormal uptake progresses from the putamen to caudate, and matches contralaterally to the side of the body more affected clinically. DAT imaging is reproducible with test/retest variability in healthy controls and PD of 7% for DaTSCAN [131]. However, as discussed further in section 4.1.2, in some large studies of PD 11-15% of subjects have been found to have normal nigrostriatal uptake of presynaptic ligands [132], [133], [134], [135], [136]. The diagnosis in these patients remained unclear, and they have since been referred to as SWEDDs (Scans Without Evidence of Dopaminergic Deficit) [137].

1.4.3 Treatment of PD

The treatment of PD is a complex and ever developing topic. However, as this is not a major focus of the work in this thesis, only a brief summary is given here.

There have been two main strategies for the treatment of PD: (i) a disease modifying approach to slow or delay the progression of disease, and (ii) symptomatic treatment for the motor and non-motor features of the disease. The prolonged preclinical phase in PD makes it an excellent candidate for neuro-protective agents. However perhaps mainly because of our poor understanding of the aetiology of PD, there is to date no agent which has been proven in clinical trials to be neuroprotective [5], and the treatment of PD remains symptomatic.

The available symptomatic treatments for PD currently include both medical and surgical modalities. Since its discovery in the 1960s as a dopamine replacement therapy, L-Dopa has remained the 'gold standard' symptomatic medical therapy [38], [5]. Other medications which are available include anticholinergics, Amantadine, monoamine oxidase inhibitors (MAOIs), catechol-O-methyltransferase (COMT) inhibitors and dopamine agonists. The question as to which medication to use in an individual patient remains unanswered [5], although the ongoing 'PD MED' trial (www.pdmed.bham.ac.uk) aims to answer this question.

As the patient's symptoms progress the medical management of their PD becomes more complex as the medications become less effective, with patients often experiencing increasingly prolonged wearing off periods and dyskinesias [5]. In some patients, particularly with advanced disease, or where medical therapy has failed, surgical therapies are considered. Surgical techniques currently used include stereotactic destruction of physiologically defined overactive brain nuclei (e.g. pallidotomy) or more commonly deep brain stimulation (DBS) of the STN using implanted pulse generators [5].

1.5 Aetiology of PD

Despite much research over the last few decades, the aetiology of PD remains poorly understood. Without a better understanding of what causes PD it may prove difficult to develop more effective symptomatic and neuro-protective treatments for the condition, thus there is a strong and continuing stimulus for further research into this area.

Although it has been proposed for many years that PD may develop due to interplay between genetic and environmental factors, over the last 10 years the emphasis has shifted towards the genetic hypothesis of the aetiology of PD, mainly through the identification of a number of 'PD genes'. In the following sections I will summarise the evidence in support of the environmental and genetic hypotheses as well as outlining some of the current models for the aetiopathogenesis of PD.

1.5.1 Environmental hypothesis

A number of environmental risk factors have been proposed on the basis of presumed pathogenic mechanisms [138], [139]. The important discovery that 1-methyl-4-phenyl tetrahydropyridine (MPTP), a contaminant of a synthetic opiate, can cause parkinsonism, through its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+), stimulated intense interest in environmental chemical exposures as risk factors for PD [140], [141]. This form of parkinsonism was similar in its phenotype to idiopathic PD, apart from the young age of onset and the associated pathology showed evidence of nigral cell loss without Lewy bodies [142], [143]. A similar parkinsonian syndrome can also be induced in MPTP-treated primates and rodents, with nigral inclusion bodies similar to Lewy bodies seen in some aged primate models [144], [145], but not rodent models [146]. MPP+ irreversibly inhibits NADH CoQ1

reductase (complex I) activity of the mitochondrial respiratory chain, thus leading to increased free radical formation and subsequently cell death [147]. MAO-B and various members of the cytochrome P-450 family are important in the activation of MPTP and similar compounds, for example by CYP2D6, CYP1A2 [148], [149], [150] and CYP1A1 [151].

There have been numerous subsequent epidemiological studies designed to investigate environmental risk factors and putative MPTP-like compounds. However, many of the studies have been retrospective, and thus prone to recall bias, and have had other methodological flaws, such as small sample size and use of inappropriate controls. Furthermore 'reverse causality' needs to be considered. For example dopamine shortage can affect food preferences [152], and thus result in altered intake of certain nutrients in PD patients, rather than the nutrients themselves having any aetiological role.

Only over the last 5-10 years have larger prospective studies been performed. However even in these studies reversed causality can play a part, as can bias in exposure assessment before onset of PD and apparently asymptomatic participants may already have pre-symptomatic dopaminergic degeneration. Furthermore, the time period in which patients are at risk of developing PD is unknown and therefore it is not clear whether early, late, cumulative, or lifetime exposures should be studied [138], [153].

1.5.1.1 Toxin exposure and PD

The discovery of MPTP stimulated interest in finding environmental toxins in PD, and in particular finding a more common neurotoxin that might be implicated in the pathogenesis of PD. The association between PD and pesticide exposure was extensively investigated, given the similarity between paraquat and MPTP [154]. There has also been interest in associations

between rural living, farming and well water consumption (all of which may be surrogate markers for pesticide exposure) and PD (see section 3.1.5.4).

There have been a number of epidemiological studies into the relationship between pesticide exposure and incidence of PD. Most studies have used 'pesticide' or 'pesticide and herbicide' as contamination categories, and as in most environmental studies, have been retrospective case-control in design. A statistically significant link between pesticide exposure and PD has been found in approximately half of the studies. A meta-analysis of 19 of these studies calculated a pooled odds ratio of 1.94 (95% CI 1.49-2.53) [155], which fits with some of the more recent case-control studies [156], [157]. One large recent prospective study, with a 30 year follow-up, found a significantly increased risk of PD for men working for more than ten years on a plantation and a non-significant association for men exposed to pesticides [158].

The mechanism of pesticide toxicity in PD remains unknown. Pesticides are known to induce mitochondrial dysfunction through inhibition of complex I [159], [160], [161], [162], [163], [164], [165] and also modulate the xenobiotic metabolizing enzymes such as cytochrome P 450, CYP2D6 and glutathione transferase [166], [167], [168], [169], [170]. Two *in vivo* animal studies have shown dopamine depletion after infusion with the pesticide rotenone [165], [171]. The relevance of this is highlighted by the finding of complex I defects in the substantia nigra of patients with sporadic PD [172].

In addition to pesticides, much work has been done to investigate whether there may be links between other toxins, for example heavy metals, and the risk of developing PD. It has been proposed that these metals may accumulate in the substantia nigra and increase oxidative stress [173]. Case reports and case-control studies have linked long-term exposure to some specific metals such as manganese, copper, mercury, lead, iron, zinic, aluminium, amalgam,

and indeed combinations of these substances, with PD. However, the epidemiological evidence in support of a definite link remains inconclusive, as reviewed in Lai et al. 2002 and Jankovic et al. 2005 [173], [174].

The high incidence of a neurodegenerative disorder characterized by motor neuron disease, parkinsonism or dementia ('Lytico-Bodig') on the island of Guam in the western Pacific provides further supportive evidence of an environmental neurotoxin causing parkinsonism. Although there are inconsistencies between either a purely genetic or environmental hypothesis, the condition is thought to be related to the use of cycad seeds in the indigenous diet [175], [176].

There have also been a number of studies into potential associations between dietary factors and risk of developing PD. Some studies have shown a positive association between fat consumption and PD [177], [173]. Other studies show a negative association between dietary items such as niacin, nuts and vegetables and PD [178], [179], [180], [181], [173]. Overall a link between dietary factors and risk of PD remains unproven [182], [173]. The results for antioxidants are even more complex to interpret, with numerous studies into the effects of vitamins A, C and E on risk of PD, as reviewed in Lai et al. 2002. Overall there is as yet no consistent evidence to show that antioxidant vitamins have a protective role in the aetiology of PD [178], [179], [180], [182], [173].

It remains unclear if there is a definite link between environmental toxins and risk of PD. Given the prevalence of PD, a common low-level exposure to a putative neurotoxin, such as pesticides, experienced by the majority of the population, will be difficult to detect and will require much larger sample sizes [183]. How environmental toxins may interact with genetic

variations found in certain populations, or indeed individuals, is also an area of research which could prove to be crucially important for understanding the pathogenesis of PD.

1.5.1.2 Infections and PD

A link between cerebral infection and parkinsonism was first considered during the influenza pandemic of the late 1910s when it was observed that encephalitis lethargica often preceded parkinsonism, suggesting a possible infectious cause [184]. There is strong evidence to support the link between the pandemics of influenza and encephalitis lethargica, namely their close time link, global distribution and single aetiological agent (swine influenza virus) [185]. Furthermore the average age of onset of PD based on data from three London hospitals during the period of 1921-1930 was 36.8 years, compared to 54.7 years during the first 20 years of the 1900s, while the average age of diagnosis of postencephalitic parkinsonism from 1921 to 1942 was 27.4 compared to 55.6 for paralysis agitans from 1900 to 1942 [186].

Further evidence suggesting an infectious cause for Parkinsonism comes from reports suggesting that other infections such as AIDS [187], and Japanese B encephalitis [188], [189], [190] and encephalitis induced by cocksakie B [191], influenza A [192], herpes simplex [193], measles [194], or mumps [195] can be associated with temporary parkinsonism, either acutely, or as long term complications. These findings are also supported by *in vivo* work on mice inoculated with neurovirulent influenza A viruses, showing that the substantia nigra is a major target for these viruses [196]. There have also been some reports suggesting a link between intra-uterine and childhood infections and risk of PD [173].

There is, however, no evidence from serological analysis to support any specific infection being more common in PD [197], [198], and indeed pathological examination has also failed

to detect specific viral particles, inclusions or antigens in PD brain autopsy material [199], [173].

In summary, whilst there is enough evidence in support of a post-infective cause for parkinsonism to justify this being considered a potential mechanism, the infective hypothesis of PD remains far from proven.

1.5.1.3 Smoking and PD

Smoking is one of the most studied risk factors for PD, with a reduced risk of PD in smokers consistently found. Many of the earlier studies were retrospective case-control designs, but more recent cohort studies have also confirmed these results [200], [201], [202]. A large meta-analysis of case control (44) and cohort (4) studies reports a relative risk of PD, compared with never smokers, of 0.59 for ever smokers, 0.80 for past smokers, and 0.39 for current smokers [203]. The relative risk per 10 additional pack-years was 0.84 in case-control studies and 0.78 in cohort studies [203].

The reason for this association remains unclear. It may be due to bias from selective mortality of smokers among people without PD, or other unknown confounding variables. However, theories for true biological protection as a result of smoking have been proposed, along with laboratory evidence to back them up. The most likely proposed mechanisms involve nicotine's actions to inhibit MAO-B, and stimulate nicotinic acetylcholine receptors leading to dopamine release and neuroprotection of dopaminergic neurons in the nigrostriatal tract [204], [205], [206], [207]. Another explanation involves the role of dopaminergic pathways in reward mechanisms. Patients with PD might be less prone to addictive behaviours because of dopamine depletion or a pre-morbid personality characterised by a lower frequency of

behaviours associated with novelty seeking [208], [209]. A final theory is that genetic polymorphisms that reduce tolerance to tobacco smoke may also increase the risk of PD and account for this association [138].

1.5.2 Genetic hypothesis

The last decade has seen a revival of the genetic hypothesis of PD, mainly through the identification of a number of 'PD genes'. It is important to emphasise that after increasing age [210], [211] a family history of PD remains the strongest risk factor for PD. Genetic influence on PD has been recognized as far back as the nineteenth century, when Gowers noted that 15% of his patients had affected relatives [212].

There have been many recent studies of genetic influence on PD, for example the DATATOP study in which 19% of patients reported a positive family history of PD [41]. In a separate population-based study, the reported frequency of a positive family history of PD was 10.3% in subjects with PD and 3.5% in age and sex-matched controls [213]. Furthermore, Bonifati et al. had previously reported that a family history of PD was positive in 24% of 100 consecutive PD cases and 6% of spouse controls ($p<0.001$) [214]. The risk of developing PD does vary significantly according to the population examined, with the relative risk to first-degree relatives 2.7 in the United States [215], 2.9 in Finland [216], 6.7 in Iceland [217] and 7.7 in France [218]. This suggests the influence of different genetic polymorphisms, or indeed environmental exposures within these various populations.

1.5.2.1 Twin studies

Assessing the difference in concordance rates between identical and non-identical twins 'twin studies' has been the classic method of establishing whether a disease has a significant genetic aetiology. The initial twin studies in PD from the 1980s demonstrated similar concordance rates for monozygotic and dizygotic twins [219], [220]. However the results from more recent studies have been less clear cut. For example, in the largest PD twin study to date no difference in the concordance rate between monozygotic & dizygotic (MZ and DZ) twins was identified, for typical late onset PD. However, in the group of subjects whose age of disease onset was less than 50 years, genetic factors were demonstrated to be important [221].

One potential explanation for these findings is related to a fundamental problem associated with most studies of PD, that although the diagnosis of PD is entirely clinical there is a well recognised presymptomatic phase [14]. Thus those twin studies which have relied purely on clinical diagnosis alone will miss those subjects in the presymptomatic phase of disease. An attempt to counter this problem was made in a prospective study using fluorodopa PET to identify presymptomatic individuals. In this study the concordance rate for dopaminergic dysfunction and/or PD was 75% for the MZ twins compared with 12% in the DZ twins. Thus using this more sophisticated twin study design an important role for genetic factors in PD was demonstrated [222].

1.5.2.2 Familial studies

Another approach to establishing the genetic aetiology of PD has been familial studies. Although large kindreds with classical Mendelian inheritance of PD are rare, in the last decade family-based linkage approaches and positional cloning have identified a number of

monogenic forms of PD, which I will discuss below. The process of linkage analysis considers co-segregation of genetic markers (alleles) with a defined phenotype (disease state) within pedigrees with multiple affected family members in several generations. The likelihood of linkage is described as the logarithim of the odds (LOD) score (ratio of the chance of the marker locus and disease being linked versus the chance that they are not linked), with a score greater than 3.0 representing significant evidence for linkage, for autosomal loci [223], [224].

The findings from familial studies have generated a lot of interest, mainly because the study of these rare inherited forms of PD may enable us to obtain important pointers towards the pathogenesis of idiopathic PD.

1.5.2.3 Nomenclature

In this thesis I have used a nomenclature system for the description of changes (mutations and polymorphisms) in DNA and protein sequences based on that recommended by Antonarakis and the Nomenclature Working Group (http://www.dmd.nl/mutnomen.html) [225], [226].

I have used *sequence variation* or *variant* to describe sequence changes of unknown or unconfirmed functionality. This includes the term *polymorphism,* also referred to as *single nucleotide polymorphism (SNP),* which specifically refers to changes found at a frequency of 1% or higher in the population. I have used the term *mutation* to describe sequence changes with known medical significance or phenotypic consequence. I have used the term *pathogenic mutation* when there is strong evidence in support of a pathogenic role for a mutation, such as evidence of segregation with disease in large pedigrees, and/or laboratory evidence in support of a functional effect on the protein.

1.5.2.4 Autosomal Dominant and Recessive PD

Over the last 10 years through various population analyses a number of monogenic forms of PD, both dominant and recessive, have been identified. There is now robust evidence for five hereditary PD genes: *alpha-synuclein*, *DJ-1*, *LRRK2*, *Parkin* and *PINK1* (see table 1.6). There is less conclusive evidence implicating other possible PD genes, including: *NURR1*, *synphilin-1* and *UCH-L1* [227], [228], [229], [230], [25], [27], [231], [232].

Generally autosomal dominant PD (AD PD) is thought to present with a clinical and pathological phenotype similar to that of idiopathic PD (IPD) [233]. The AD loci identified to date are PARK1, PARK3, PARK8 and PARK11. Autosomal recessive forms of PD (AR PD) resemble IPD, but tend to present with an earlier age of onset and often demonstrate more slowly progressive disease [234], [235], [236]. The AR loci identified to date are PARK2, PARK6, PARK7 and PARK9. Importantly for our understanding of IPD, whilst both AD and AR PD resemble IPD clinically, they often lack specific pathological features. LBs, in particular, are an essential brain bank diagnostic criterion, but are inconsistently found in most forms of genetically mediated parkinsonism [237].

1.5.2.5 Dominant loci

1.5.2.5.1 Alpha-synuclein (PARK1)

The study of a large Italian pedigree of at least 60 members first led to the linkage of their form of AD PD to chromosome 4q. Subsequently an A53T (G209A) mutation in the *α-synuclein* gene (*SNCA*) was identified in this family, and five unrelated Greek families [238], [228]. Apart from a relative paucity of tremor, young onset, and long disease course, there were no clinical features to differentiate between PD families with the A53T mutation and

subjects with sporadic disease [239]. Subsequently other *SNCA* mutations in unrelated German (A30P) and Spanish (E46K) families have been identified [240], [241]. However, extensive studies have now conclusively demonstrated that *SNCA* mutations are a rare cause of fPD [242], [243], [244].

Additional mechanisms by which α-synuclein function can be altered have subsequently been identified. The level of protein expression may be altered by polymorphisms in the promoter or upstream regulatory regions, or by duplication or triplication of the gene. This latter phenomenon was initially erroneously allocated to the PARK4 locus [245], [246], [247], [248], [249], [250], [251], [252]. The most recent, and largest, analysis of *SNCA* promoter variability has indicated that allele length variability in the dinucleotide repeat sequence is associated with an increased risk of PD [253].

The importance of the genetic studies in *SNCA* was reinforced by the identification of α-synuclein as the major component of LB [97]. This finding created a new field of research focusing on diseases associated with the pathological aggregation and deposition of the α-synuclein protein, the so called 'alpha-synucleinopathies', which in addition to PD also includes diffuse LB disease, and MSA. Numerous histological studies have shown that α-synuclein forms an important component of LB and the oligodendroglial inclusions characteristic of MSA [103]. Furhermore, transgenic animal models expressing human α-synuclein, mutant A53T or A30P α-synuclein, or knock-out phenotypes have been developed showing a variety of phenotypic and pathological features with some similarities to IPD [254]. α-synuclein is now known to have lipid-binding properties [255], leading to speculation that the protein plays a role in stabilising lipid bilayers. Other studies suggest that the protein has a cellular housekeeping function, linking up with other synaptic vesicle proteins such as the

soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor 'SNARE' complex [256], altering proteasomal structure to modify protein synthesis and degradation, resulting in altered vulnerability to cellular stressors [257]. Despite this extensive and ongoing investigation, the normal physiological function of α-synuclein remains to be determined.

1.5.2.5.2 PARK3

The PARK3 locus was identified in 1998 in a genome-wide genetic linkage study using a group of families of European ancestry [258], with linkage to chromosome 2p13 found. Further linkage studies have subsequently confirmed linkage to the PARK3 locus in other families [259], [260], [261]. However, to date no relevant pathogenic mutations have been found in coding sequences of 14 candidate genes [262]. The associated clinical and pathological phenotype appears to be similar to IPD, with late-onset PD (average age of onset of 59 years) and associated LB pathology [259], [260], [261], [262]. A recent study showed linkage to the *sepiapterin reductase* (*SPR*) gene region, which is located in the PARK3 linkage region, in 122 families of European ancestry [263]. Interestingly *SPR* is implicated in dopamine synthesis, but this linkage needs to be confirmed.

1.5.2.5.3 UCH-L1 (PARK5)

In 1998 as part of a candidate gene approach, the study of two German siblings with apparently AD PD identified a missense mutation (I93M) in the ubiquitin carboxy-terminal hydrolyase L1 (*UCH-L1*) gene located at chromosome 4p14 [264]. The significance of this finding remains controversial, with no mutations identified in any other kindreds to date and a lack of pathological reports. A subsequent study suggested that another *UCH-L1* variant

(S18Y) reduces PD susceptibility [265], and this finding was confirmed in a pooled analysis from 11 studies involving 1,970 patients [266].

Intriguingly the *UCH-L1* gene codes for a neuron-specific protein that catalyses the hydrolysis of ubiquitin from the C-terminal end of substrates, and is a component of the ubiquitin-proteasome system [267], [268]. Although the functional relevance of mutations in this protein in PD are still being investigated, the findings to date suggest another potentially important link between protein processing, breakdown and fPD.

1.5.2.5.4 LRRK2/dardarin (PARK8)

This PD locus is the focus of the majority of the work presented in this thesis. In this section I will summarise in detail what was known about the PARK8 locus up until April 2005, when I started work on my project. More recent developments are covered in chapters 5, 6 and 7, in which I present my data.

The PARK8 locus for AD PD was first identified in 2002 in a large Japanese pedigree named 'the Sagamihara' kindred, after the region of origin in Japan, and was linked to chromosome 12p11.2-q13.1 (see figure 1.4) [269]. These findings were subsequently confirmed in non-Japanese families [270]. The clinical features of affected individuals were noted to be typical for idiopathic late-onset PD, including a good levodopa response [269], [270]. Pathological examination of patients found the expected nigral dopaminergic neuron degeneration, but a variable range of other features, including alpha-synuclein positive Lewy bodies (LBs) in some, but not all, cortical LB pathology, tau-positive axonal inclusions, or other uncharacterised neuronal cytoplasmic inclusions [271], [27].

In 2004 the gene linked to the PARK8 locus was identified simultaneously by two groups as *leucine rich repeat kinase 2* (*LRRK2*) [25], [27]. This large 51 exon gene encodes for a protein of 2527 amino acids (286kDa) called LRRK2, or dardarin, derived from the Basque word dardara, meaning tremor [25], [27].

LRRK2 is known to be part of the Roco protein family. Members of the Roco protein family are characterized by having a conserved supradomain that contains as Ras-like GTPase domain, called Roc, and a characteristic COR (Carboxy-terminal of Roc) domain. In addition, a kinase domain and diverse regulatory and protein-protein interaction domains are also often found in these proteins [272]. Sequence analysis indicates that the protein contains several discrete regions: an ankyrin repeat domain, a LRR (leucine rich repeat) domain consisting of 12 strands of a 22–28 amino acid motif, present in a tandem array; a Rho/Ras-like GTPase domain; a COR domain; a protein kinase domain related to the MLK (mixed lineage kinase) family; and a WD40-repeat domain consisting of approximately 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide (see figure 1.4).

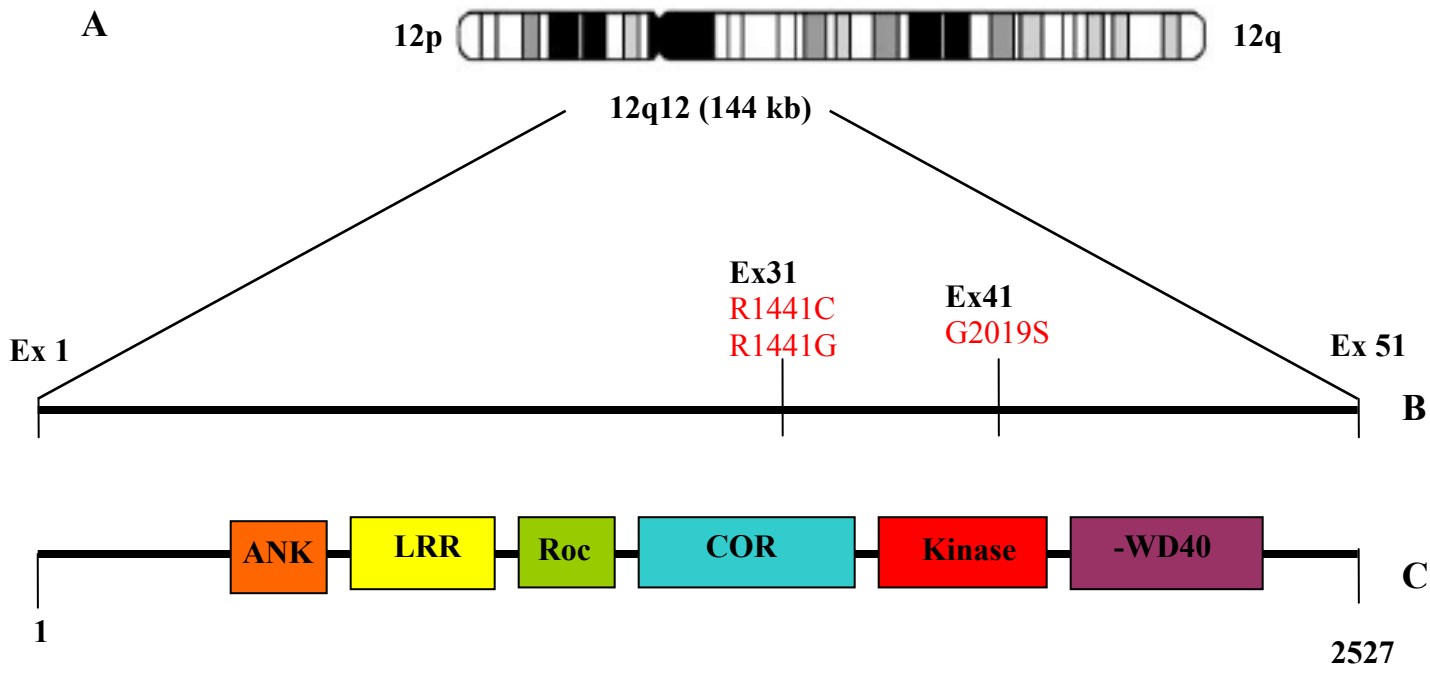

Figure 1.4 Schematic representation of the *LRRK2* region of chromosome 12, the LRRK2 protein domain structure and three pathogenic *LRRK2* mutations. Panel A= Ideogram of chromosome 12, *LRRK2* spans a genomic region of 144 kb. Panel B= Schematic representation of *LRRK2* exons 1-51 with three pathogenic amino acid variants. Corresponding exon numbers are shown in black. Panel C= Schematic representation of LRRK2 protein structure from residue 1-2527. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide; kb= kilobase pairs. Adapted from Mata et al. 2006, Paisán-Ruız et al. 2004 and Zimprich et al. 2004 [25], [27], [18].

Through what was already known about the types of functional domains identified in LRRK2, it was possible early on to speculate about what process the protein may be involved in. Ankyrin domains are known to be involved in protein-protein interactions [273]. The group of proteins which contain LRRs have been implicated in diverse cellular functions, ranging from

hormone receptor interactions, enzyme inhibition, cell adhesion, cellular trafficking, splicing, to substrate binding for ubiquitination. However an important and common property of these proteins again involves protein-protein interaction [274]. The Ras/GTPase domains are known to play a part in reorganisation of the actin cytoskeleton in response to external stimuli, with additional roles in cell transformation by Ras, cytokinesis, focal adhesion formation, and the stimulation of stress-activated kinases [275]. Enzymes which contain tyrosine kinase domains belong to a large family of proteins that share a conserved catalytic core common to both serine/threonine and tyrosine protein kinases. They are known to function by catalysing the transfer of the γ-phosphate of ATP to tyrosine residues on protein substrates [276]. Finally, WD40 domains have previously been found to be involved in signal transduction, pre-mRNA processing, and cytoskeleton assembly [277].

Following the original descriptions of mutations in LRRK2, a number of other putative pathological mutations were rapidly identified, including: R1441C, R1441G, and G2019S [18], [278], (see figure 1.3). These mutations were noted to be clustered around the kinase and GTPase domains. It soon became clear that *LRRK2* mutations appear to account for up to 10% of familial PD cases with AD inheritance [279], [280]. Of the mutations identified, the G2019S mutation, (c.6055G>A) in exon 41, appeared to be particularly important, as it alone seems to be responsible for up to 5%-6% of hereditary and, importantly, 1%-2% of apparently sporadic PD cases [281], [282], [283], [284], [285]. Indeed the G2019S common mutation was found at even higher frequencies in certain populations, including Portugese (6%) [286], and North African Arab patients (41%) [287].

The clinical phenotype of most *LRRK2* positive PD patients appears indistinguishable from that of typical IPD, including good response to Levodopa [27], [288], [283], [289], [290],

[285]. However, the age at onset is variable, ranging from 28 to 86 years in subjects with the G2019S mutation [285], [282], [284], [291].

One of the criticisms of the early work performed on LRRK2-PD was the relative dearth of studies in which a comprehensive analysis of the whole coding region of the gene had been performed on a large cohort of subjects with familial PD. By the end of 2005 there were only five such studies [27], [292], [279], [293], [294], compared to over 20 studies in which only a limited number of known *LRRK2* mutations were investigated. The major reason for this is likely to be the large size of the gene, such that comprehensive analysis of the entire gene is time-consuming and expensive. The majority of studies in sporadic disease had focused on screening for previously identified mutations in different populations. I recognised the main drawbacks of such a piecemeal screening approach, namely important pathogenic *LRRK2* mutations may not be identified and potentially important insights into the pathogenesis of PD missed, and also that estimates of mutation frequency may be inaccurate. This is discussed further in chapters 5 and 6.

When I commenced my study of LRRK2-PD in 2005 the putative functional domains of the protein had been proposed and various mutations identified. As the most common G2019S mutation is found in the kinase domain of the protein there was speculation that the pathogenic mechanism of this mutation may be via kinase over-activity. However, the physiological function of LRRK2 was unknown, as were the effects of the presumed pathogenic mutations. This is discussed further in chapter 7.

In summary, when I started this thesis the evidence suggested that LRRK2-associated PD was quite common, had an age of onset and phenotype that is closer to sporadic PD than most other forms of familial PD, and it demonstrated a wide range of pathology. In addition, for the

first time, a specific PD gene mutation (G2019S), identified in familial cases, had been implicated in apparently sporadic disease. Overall these findings represented a potentially major breakthrough in our understanding of the aetiology of PD. Further comprehensive analyses of the *LRRK2* gene were still required both in familial and sporadic PD, as well as a better understanding of the function of the protein and its potential role in the disease pathogenesis.

1.5.2.5.5 PARK11

In 2003 the PARK11 locus was identified with AD linkage to chromosome 2q36-37 in a sample of sibling-affected PD pairs, and subsequently expanded using 65 pedigrees to obtain a LOD score of 5.1 [295]. The significance of these findings remained controversial for some time as replication was not achieved in several other linkage studies [296], [297], [298].

More recently Lautier et al. investigated the *GIGYF2* gene encoding GRB10-interacting GYF Protein 2, which had been found to contains the PARK11 microsatellite marker (D2S206) with the highest LOD score (5.14) [299]. On sequencing all 27 coding exons of *GIGYF2* gene in over 200 patients and controls, they identified seven different missense mutations (single amino acid substitutions) in 12 unrelated index patients (4.8%) with none in controls. In addition three amino acid insertions or deletions were identified in four other index patients and none in controls. In four families with multiple affected family members amino acid substitutions (Asn56Ser, Thr112Ala, and Asp606Glu) were found to segregate with disease. In one family two unaffected mutation carriers were identified, which suggests age-dependent or incomplete penetrance for this mutation [299]. Whilst still to be replicated, this data does appear to support *GIGYF2* as the PARK11 gene with a causal role in fPD.

Intriguingly the investigators also identified one index case with early onset PD, age 33, who had inherited a *GIGYF2* mutation (Ile278Val) from her affected father (disease onset age 66) and a *LRRK2* mutation (Ile1371Val) from her affected mother (disease onset age 61). They speculated that the earlier onset and more severe clinical course of disease in the index patient may suggest an additive effect of the these two mutations [299].

1.5.2.6 Recessive loci

1.5.2.6.1 Parkin (PARK2)

Following the original descriptions of early onset AR PD in a series of Japanese families, the PARK2 locus was mapped to the long arm of chromosome 6, eventually leading to the cloning of the *Parkin* gene [229]. The associated clinical phenotype showed early onset of disease (typically with onset before age of 45), with slow progression, good levodopa response, and levodopa-induced dyskinesias. Whilst these cases can be difficult to distinguish clinically from Parkin-negative disease, they appear pathologically distinct, with nigral neuronal loss and deposition of extraneuronal melanin, but crucially LBs are absent in all but one of the reported cases [234], [235], [236], [300], [301], [302], [303].

It has now been demonstrated that homozygous parkin mutations are found in approximately 10-20% of patients with early onset PD (before age 45), depending on series, with this frequency increasing to 50% in AR early onset fPD cases [304], making *Parkin* mutations the most common cause of ARPD. Over 100 *Parkin* mutations have now been identified in many different populations [305], [234], and mutation analysis remains technically demanding, particularly because of possible rearrangements in exons 3, 4 or both [306], [307].

Parkin consists of 12 exons, which span over 1 megabase (Mb), and encode for a 465 amino acid protein widely expressed in both neuronal and glial cells as well in several extracerebral tissues. The protein contains an N-terminal ubiquitin-like sequence and a C-terminal RING 'really interesting new gene' domain composed of two RING finger motifs (see figure 1.5).

Figure 1.5 Schematic representation of human Parkin, showing its functional domains. This is a 465 amino acid protein, estimated domain boundaries are indicated by residue numbers above. Parkin contains a UBL= ubiquitin-like domain; a UPD= unique Parkin domain; and a RING= really interesting new gene box domain which consists of two RING finger motifs R1 and R2 separated by an IBR= in-between RING motif. Taken from Hampe et al. 2006 [22].

As with other RING finger proteins, parkin has E2-dependent E3 ubiquitin-ligase (E3) activity (E2 is the ubiquitin-conjugating enzyme UBCH7) [308]. Importantly E3 acts as a substrate-recognition molecule during ubiquitination by conjugation via a lysine residue to ubiquitin to form polyubiquitin chains, targeting proteins for degradation via the 26S proteasome. Thus Parkin would appear to provide a crucial link to proteasomal degradation [309].

However the true function of Parkin may be much more complex, with neuroprotective properties demonstrated in a variety of model systems [310]. For example in a *Drosophila* model of neuronal overexpression of mutant α-synuclein, Parkin was demonstrated to reduce dopaminergic neuronal loss [311]. In addition other reports have suggested that Parkin interacts with other proteins implicated in fPD, including PINK1, LRRK2 [312] and DJ-1 [313]. The pathogenesis of *Parkin*-related PD may involve several cellular processes,

including protein quality control, mitochondrial dysfunction, oxidative stress, and apoptosis. The extensive potential interactions of the Parkin protein may also suggest it has a role in the pathogenesis of other forms of fPD.

1.5.2.6.2 PINK1 (PARK6)

In 2001 the PARK6 locus was identified in a large consanguineous family from Sicily, with linkage to chromosome 1p35-p36 [314], and subsequently these findings were replicated in other European families [315]. After further mapping and candidate gene analysis, two homozygous mutations affecting the PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced kinase-1 (*PINK1)* gene on chromosome 1, in three consanguineous PARK6 families, were identified: a truncating nonsense mutation (W437X) and a missense mutation (G309D) [227]. Subsequently a variety of point mutations, splice mutations and large deletions of *PINK1* have been identified. Estimates of prevalence from European and Asian populations, suggest that *PINK1* mutations are the second most frequent cause of AR PD, after *Parkin* mutations, with reported frequencies ranging from 1% to 7% [316], [317], [318], [319]. The clinical phenotype associated with *PINK1* mutations demonstrates a wider age spectrum than *Parkin* related ARPD. The age of onset is usually in the third or fourth decade with similar features to *Parkin*-related disease, including slow progression, good response to L-dopa and frequent treatment-induced dyskinesias. Rare associated features also include rest dystonia, sleep benefit and psychiatric disturbances [227], [320], [321].

More recently several reports have suggested that heterozygous *PINK1* mutations may represent a susceptibility factor for PD, due to the increased frequency of *PINK1* heterozygous mutations in apparently sPD populations, as compared to matched controls [316], [322], [323],

[324]. Supportive data for this theory comes from a small study of three subjects using ^{18}F-dopa PET imaging, in which a 20 to 30% mean reduction in the ^{18}F-dopa uptake levels in the caudate and putamen was found in the heterozygote state [325].

PINK1 contains eight exons spanning 1.8 kb and encodes a 581 amino-acid, 63kDa, protein predicted to be a serine/threonine kinase of the Ca^{2+}/calmodulin family with a 8kDa amino-terminal, mitochondrial targeting sequence [227]. PINK1 is ubiquitously expressed throughout the human brain, and is found in both neurons and glial cells [326]. The complete protein is thought to be transcribed in the nucleus, translated in the cytoplasm and imported intact into the mitochondria, with subsequent processing and intra-mitochondrial sorting. There is also now evidence that PINK1 is localised to the inner mitochondrial membrane [327], [328].

Several *in vitro* and *in vivo* studies have provided evidence that loss of PINK1 function leads to a complex cellular phenotype including defects in mitochondrial morphology, increased sensitivity to cellular stressors and reduction in subsets of dopaminergic neurons [329], [330], [331], [332], [333], [334], [335], [336]. PINK1 inhibition has also been shown to lead to reduced Parkin expression, with an overexpression of Parkin rescuing some of the cellular defects in PINK1 mutants. This data suggests that the PINK1 and Parkin pathways interact, with Parkin appearing to function downstream of PINK1 [337], [338], [339].

1.5.2.6.3 DJ-1 (PARK7)

In 2001 the PARK7 locus was identified, following the study of a large Dutch consanguineous pedigree displaying AR PD, with linkage to chromosome 1p36 [340]. This linkage was subsequently confirmed in an Italian consanguineous pedigree [341]. Mutations in the *DJ-1* gene were eventually identified in these two families, a deletion in the Dutch family and a

missense mutation (L166P) in the Italian family [230]. Subsequently missense, truncating, and splice site mutations, as well as deletions have been described in several ethnic groups [342], [343], [344]. Much like for *Parkin*-related PD, the clinical phenotype associated with *DJ-1* mutations is of early-onset PD, with slow progression and good levodopa response. However, the frequency of *DJ-1* mutations in early onset PD appears lower than that for *Parkin* mutations, ranging from 1-2% [342], [344], [235], [345].

DJ-1 contains 8 exons, is 24 kb in length and encodes a 189 amino acid protein DJ-1, which belongs to the ThiJ/PfpI superfamily. DJ-1 is ubiquitously expressed throughout the brain and peripheral tissues, and is largely cytoplasmic with some localization to the mitochondria [230], [346], [347]. Whilst the exact role of DJ-1 in PD is not known, the protein appears to have several neural and non-neural functions, including cellular transformation, RNA binding, androgen-receptor signaling, spermatogenesis, and fertilization. [348]. *In vivo* and *in vitro* studies, including *Drosophila* and mouse knock-out experiments, indicate that DJ-1 may play a role in protecting dopaminergic neurons from oxidative stress [349], [350], [351], [352], [353], [354], [355]. DJ-1 is itself inactivated by extensive oxidation [352], and the observation that DJ-1 is oxidatively damaged in the brains of subjects with IPD, suggests that protein aggregation may be mediated by high levels of oxidative stress [353], [356]. Interestingly, DJ-1 has also been shown to associate with Parkin during oxidative stress [313].

1.5.2.6.4 PARK9 (Kufor-Rakeb disease)

This AR form of parkinsonism was described in a single consanguineous family from Kufor-Rakeb in the north of Jordan, with linkage to chromosome 1p36, and allocated as the PARK9 locus [357], [358]. The condition is known as Kufor-Rakeb disease (KRD), and is quite

distinct from other forms of fPD, with the additional features of pyramidal degeneration, cognitive dysfunction, supranuclear gaze palsy, oculogyric dystonic spasms, facial- faucial-finger mini-myoclonus and visual hallucinations described [359]. No other families with a similar disease phenotype have been described.

A recent study has determined that KRD is caused by loss-of-function mutations in a predominantly neuronal P-type ATPase gene, *ATP13A2*, leading to protein retention in the endoplasmic reticulum and subsequent enhanced proteasomal degradation [360]. Given its rarity, and distinct phenotype, the relevance of these findings to other forms of fPD remains unclear.

1.5.2.7 Loci of uncertain inheritance- PARK10, PARK12 & PARK13

The PARK10 locus was identified in 2002, following a genome-wide linkage analysis in Icelandic families, in which a susceptibility locus for late onset PD was discovered on chromosome 1p32 [361]. Subsequent studies have suggested that the PARK10 locus may control age of PD onset, and candidate genes including the gamma subunit of the translation initiation factor EIF2B gene (EIF2B3) and the ubiquitin-specific protease 24 gene (USP24) have been identified [362], [363]. However, to date the gene has not been identified, and there has been no reported confirmation of linkage in other population groups.

Also in 2002 linkage analysis in 160 families without *Parkin* mutations identified an additional locus, PARK12, on the X chromosome [364]. Again no PD genes have been identified to date.

The targeted disruption of the serine protease Omi/HtrA2 has previously been found to cause neurodegeneration and a parkinsonian phenotype in mice [365]. Therefore in 2005 a candidate

gene approach was used to screen a group of German PD patients for mutations in the *HtrA2* gene found on chromosome 2p13 (PARK13 locus). A novel heterozygous mutation (G399S) was found in four patients, and in addition a polymorphism (A141S) associated with disease. The associated patient phenotypes were typical for IPD [366]. Omi/HtrA2 is a nuclear encoded protein, with a mitochondrial targeting sequence and is proteolytically active during apoptosis. Intriguingly the G399S mutation led to mitochondrial dysfunction associated with altered mitochondrial morphology, and cells over-expressing the GS399S mutant protein were more susceptible to stress-induced cell death [366]. Omi/HtrA2 appears to interact with PINK1 and both are components of the same stress-sensing pathway, with Omi/HtrA2 phosphorylation decreased in brains of patients with PD carrying mutations in PINK1 [367]. Recently it has also been shown that HtrA2/Omi accumulates in the neuronal and glial inclusions found in brains of patients with alpha-synucleinopathies [368].

1.5.2.8 Candidate gene studies in PD

A wide range of genes and proteins have been investigated for an association with both sPD and fPD. A common theme for many of these studies has been difficulty in replication of results, which is most likely to be because of small sample sizes, inappropriate controls and flawed statistical analysis [369]. In this section I will outline the evidence for several of the candidate genes which have been most recently, and extensively, investigated.

The *NURR1* (*NR4A2*) gene located on chromosome 2q22-q23 encodes for the transcription factor Nurr1, which belongs to the orphan nuclear receptor superfamily. The protein is highly expressed in both developing and adult brain dopaminergic neurons, and appears to be critical in the development and maintenance of the dopaminergic system, via its regulation of the

expression of a number of proteins key to dopamine synthesis and storage [370]. From studies performed in Nurr1 knock-out mice, Nurr1 deficiency results in impaired dopaminergic function and increased vulnerability of the midbrain dopaminergic neurons that degenerate in PD [370]. Heterozygous mutations in the non coding region of exon 1 of *NURR1* were found in 10 out of 107 fPD patients [231]. However, numerous follow-up studies in larger patient populations have not identified *NURR1* mutations, and it is now considered unlikely that these are a significant contributor to the development of PD [371], [372].

The *Synphilin-1* gene encodes a protein which links α-synuclein, Parkin and other components of the ubiquitin-proteasome system. Synphilin-1 has been found in LB, interacts with α-synuclein and is a substrate for the ubiqutin E3 ligase function of Parkin [373], [374]. Furthermore a mutation (R621C) was described in two patients, with *in vivo* analysis of the mutant protein function demonstrating increased susceptibility to cell death [232]. However, a recent study of *Synphilin-1* mutations in sPD showed no significant association with disease [375]. Until more information is available, the significance of this gene in sPD and fPD remains unknown.

MAPT encodes the microtubule-associated protein tau. Meta-analyses have shown that the H1 haplotype (several *MAPT* polymorphisms which are in linkage disequilibrium) increases susceptibility to PD, with homozygosity for tau H1 giving a 1.57 times increased risk of PD [376], [377], [378], [379]. These genetic findings are of particular interest, as MAPT is known to co-aggregate with α-synuclein in LB [380], [381].

Finally, there have been several reports suggesting an association between Gaucher disease, a recessively inherited lysosomal storage disorder, resulting from mutations in the glucocerebrosidase gene (*GBA*) on chromosome 1q21, and parkinsonism [382]. An early

survey found that 31 out of 99 Asheknazi patients with apparently IPD had one or two mutant *GBA* alleles [383], and a subsequent post-mortem study found that 23% of cases of LB dementia had *GBA* mutations [384]. Thus a possible biochemical interaction between glucocerebrosidase and α-synuclein was proposed [385], [384]. Other populations have since been screened for *GBA* mutations and the findings suggest that *GBA* is a susceptibility gene in PD [386], [387], [388], [389], [390]. A recent interesting finding is that four subjects out of a study group of 420 demonstrated digenic inheritance of both a *GBA* mutation and a G2019S *LRRK2* mutation [388]. In summary, these findings may be of great importance for our understanding of the pathogenesis of PD, and point us in the direction of the lysosome, as does the recent identification of mutations in the *ATP13A2* gene responsible for the PARK9 locus [360].

1.5.2.9 Summary

As we have discovered more about the fPD genes over the last decade this knowledge has provided us with a better understanding of the potential underlying pathogenic mechanisms of both sPD and fPD. As outlined above, the known fPD genes have implicated a number of potentially pathogenic mechanisms, such as *PINK* being linked to mitochondrial dysfunction and *Parkin* being linked to dysfunction of the ubiquitin proteasome system. These mechanisms are discussed in section 1.5.3. Further expanding our knowledge of the known genes, and continuing our efforts to identify new PD genes, may therefore be of crucial importance to developing our understanding of the pathogenesis of PD.

Locus (gene,Location)	Inheritance	Population Affected	Clinical Description						Pathology
			Disease Type	Dementia	Asym.	Rest Tremor	L-dopa Resp.	Other	
PARK1 (*alphasynuclein*; 4q21)	AD	Italian-Greek (A53T), German (A30P), Spanish (E46K), US, French, Italian (gene triplication, duplication)	PD plus DLB	+	+++	+++	+++	Young onset (< 45 y)	PD plus DLB for A53T and E46K
PARK2 (*parkin*; 6q25)	AR, ?pseudo dominant	Global	PD, DRD, dystonia	No	+/no	+/no	+	Foot dystonia, sleep benefit, insidious course, dyskinesias	Nigral cell loss, no Lewy bodies except in 1 case
PARK3 (2p13)	AD, reduced penetrance	Northern European kindred	PD	+/no	+++	+++	+++	May resemble typical PD	PD, NFTs
PARK5 (*UCLH1*; 4p14)	?AD, only 1 family reported	German	PD	No	+++	+++	+++		No pathology reports
PARK6 (*PINK1*; 1p35-36)	AR	Italian, European, Asian	PD	No	+++	++	+++	Insidious progression, onset < 40 y	No pathology reports
PARK7 (*DJ-1*; 1p36)	AR	Italian, Dutch, British, Turkish, German, Serbian	PD	No	+++	+++	+++	Insidious progression, onset < 40 y	No pathology reports
PARK8 (*LRRK2*; 12p11-q13)	AD	Japanese, US, Britain, Spanish, Norwegian, Irish, Polish, Italian, Portuguese, Brazilian	PD	No	+++	+	+++	Adult onset, L-dopa responsive	Nigral cell loss; Lewy bodies, NFTs
PARK9 (1p36)	AR	Jordanian	Atypical PD	++		No	+	Dementia, spasticity, supranuclear palsy	No pathology reports
PARK10 (1p32)	Unclear	Icelandic	PD	No	+++	+++	+++	Resembles "typical" PD	No pathology reports
PARK11 (2q36-37)	AD	US	PD	No	++	++	++	Typical PD	No pathology reports
PARK12 (Xq21-q25)	unclear	North American	PD	No	++	++	++		No pathology reports
PARK13 (*HtrA2*; 2p13)	unclear	German	PD	No	++	++	++	Typical PD	No pathology reports

AD—autosomal dominant; AR—autosomal recessive; DLB—diffuse Lewy body disease; L-dopa—L-dopamine; DRD—dopa-responsive dystonia; NFT—neurofibrillary tangles; PD—Parkinson's disease; Asym.—-asymmetry; Resp. —response.

Table 1.6 Genetic causes of parkinsonism. Adapted from Lewthwaite & Nicholl 2005 [12].

1.5.3 Potential pathogenic mechanisms in PD

A number of potential pathogenic mechanisms involved in neurodegeneration in PD have been identified, often through *in vitro* and *in vivo* studies into the role of individual PD genes. These mechanisms include: mitochondrial and ubiquitin-proteasome dysfunction, abnormal protein aggregation and phosphorylation, oxidative stress, and inflammation [1], [391], [392], [393], [99], [394], [395], [396], [328] (see figure 1.6). There is also evidence that the final steps of cell death in PD may occur by apoptosis [397], [398], with several studies demonstrating increased expression of cell signals associated with this process, including p53 [397], [398], [399]. It has also been demonstrated in model systems that each of the putative pathogenic mechanisms in PD covered in this section is able to induce apoptosis [400].

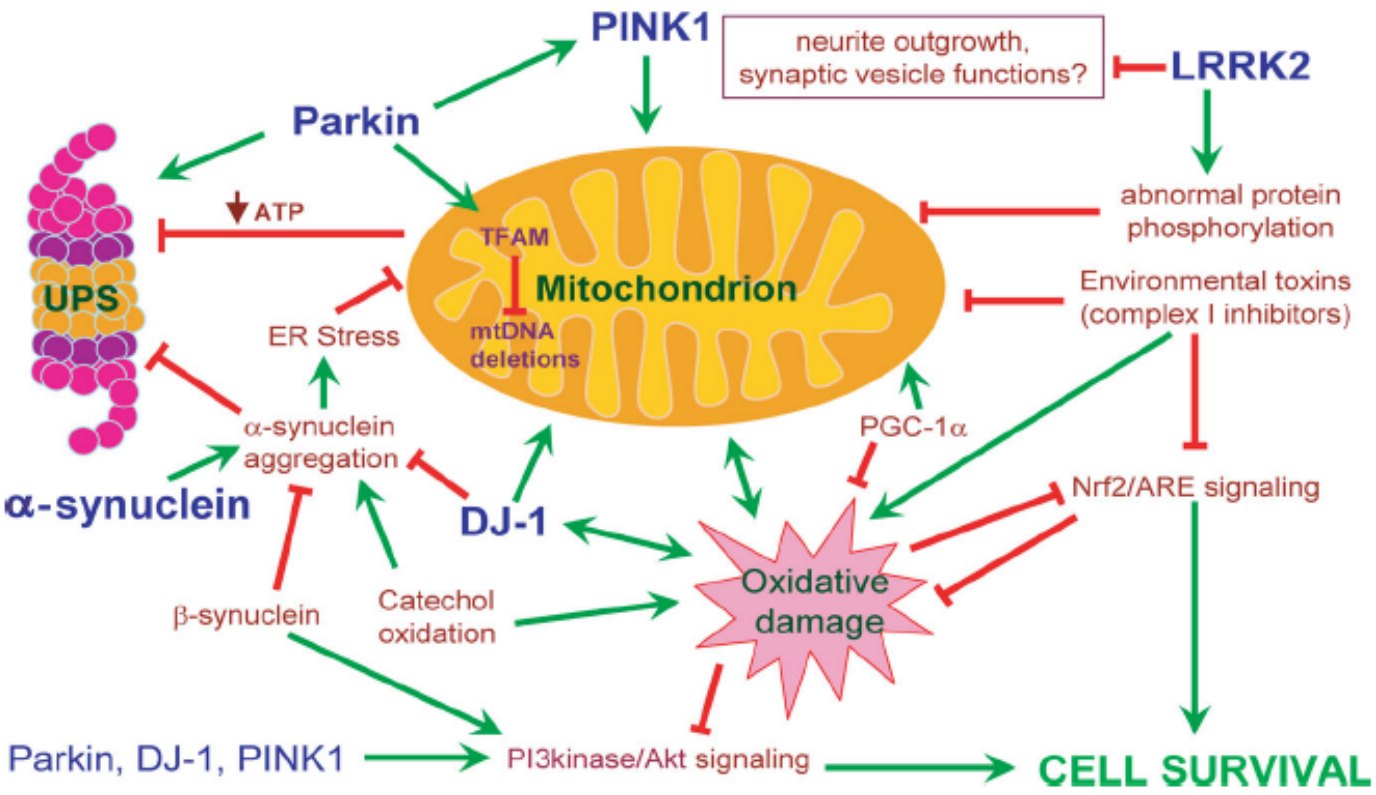

Figure 1.6 Putative interacting pathways underlying the pathogenesis of PD. Both mutations in fPD genes encoding α-synuclein, parkin, DJ-1, PINK1 and LRRK2, as well as environmental factors, are associated with PD pathogenesis, through mitochondrial dysfunction, oxidative damage, abnormal protein aggregation and protein phosphorylationl. β-Synuclein is known to prevent α-synuclein aggregation through activation of Akt signaling. UPS is ubiquitib-proteasome system; ATP is Adenosine-5'-triphosphate; TFAM is mitochondrial transcription factor A; PI3kinase-Akt is phosphatidylinositol 3-kinase/Akt; ER is endoplasmic reticulum; mtDNA is mitochondrial DNA. Green arrows indicate promoting or activating effects whilst red lines with blunt ends indicate inhibitory effects. Taken from Thomas & Beal 2007 [1].

1.5.3.1 Oxidative stress and mitochondrial dysfunction

Pathogenic roles for oxidative stress and mitochondrial dysfunction in PD have long been proposed. It has been recognised for many years that nigrostriatal dopaminergic neurons are under significant oxidative stress, probably as a result of redox cycling of catechols, leading to increased generation of reactive oxygen species (ROS) which are harmful to the cell [1].

Decrements in reduced glutathione levels in SNc of subjects with pre-symptomatic PD suggest that oxidative damage occurs earlier than the observed neuronal loss [401]. Recently a prominent role for Nrf2/ARE signaling in the pathogenesis of PD has been proposed [402]. The leucine-zipper transcription factor Nrf2 regulates the coordinated induction of the antioxidant response element (ARE)-driven cascade of cytoprotective genes, which includes numerous antioxidant and anti-inflammatory proteins [403]. In Nrf2 knockout mice neuronal tissues were shown to be more susceptible to oxidative stress and mitochondrial dysfunction [404], [405], [406].

The phosphatidylinositol 3-kinase/Akt pathway has also come to prominence as it may be of importance for dopaminergic neuronal survival. Recent animal studies have demonstrated that overexpression of the oncoprotein Akt protects against 6-hydroxydopamine (6-OHDA)-induced dopaminergic toxicity and confers neurotrophic effects on dopamine neurons [407]. Parkin [408], DJ-1 [409] and PINK1 [227] are also all known to mediate cell survival through the Akt pathway.

Some of the recent evidence of a pathogenic role for mitochondrial dysfunction includes the identification of deficits in the subunits and activity of complex I, and a high amount of mitochondrial DNA (mtDNA) deletions in the SNc of subjects with PD [410], [411], [412], [413]. Animal studies also provide supportive evidence, with a low mitochondrial mass observed in mouse SNc [414], and with a targeted deletion of mitochondrial transcription factor A in mouse midbrain dopaminergic neurons leading to Parkinsonism [415].

The evidence for interaction between oxidative stress and mitochondrial dysfunction comes from a recent *in vitro* study which showed an impairment of mitochondrial complex I due to chronic depletion of antioxidant glutathione [416]. Furthermore, PPARgamma coactivator

1alpha (PGC-1a), which is involved in mitochondrial biogenesis and respiration, is known to be a modulator of ROS generation during oxidative stress [417]. Nigrostriatal dopaminergic neurons in PGC-1a knockout mice are more vulnerable to MPTP, whilst overexpression of PGC-1a protected neural cells from oxidative stress-induced death [418].

Several 'PD genes' also provide evidence for the link between mitochondria and oxidative damage. The *α-Synuclein* gene appears to link oxidative stress and mitochondrial dysfunction to abnormal protein degradation. Mice overexpressing human mutant A53T α-synuclein induce mitochondrial damage due to aberrant α-synuclein accumulation [419]. It has also been shown that mice lacking α-synuclein are resistant to mitochondrial toxins such as MPTP, 3-nitropropionic acid and malonate, while overexpression of human α-synuclein in mice enhances their vulnerability to MPTP [420], [421].

The *Parkin* gene appears to link oxidative stress and mitochondrial dysfunction to the ubiquitin proteasome system, with apparently neuroprotective properties demonstrated in several model systems [310]. It has been shown to prevent mitochondrial swelling, cytochrome c release and caspase activation, whilst these functions are impaired when parkin mutations are present [422]. Furthermore Parkin knockouts in mice and *Drosophila* show increased oxidative stress and mitochondrial dysfunction [423], [424]. Oxidative modifications of the protein, which impair its ubiquitin E3 ligase activity, have been shown to compromise its protective function [425], [426]. Finally, in proliferating cells, Parkin has been shown to localise to the mitochondria, associate with mitochondrial transcription factor A (TFAM), and enhance mitochondrial biogenesis [427].

The association between PINK1 and the mitochondrion, as well as the role of the antioxidant DJ-1, have already been reviewed (sections 1.5.2.5.2. and 1.5.2.5.3.). It is also of interest that

there is recent evidence to suggest that oxidative damage due to dysfunction of PINK1 results in recruitment of DJ-1, thus maintaining steady-state levels of PINK1 through a physical interaction and overexpression of DJ-1 [332]. Although little is known about the function of LRRK2, recent evidence suggests that up to 10% of the protein may be associated with mitochondrial membranes [428], [312], [429].

1.5.3.2 Protein aggregation, phosphorylation and the Ubiquitin-proteasome system

The Lewy body (LB) is one of the neuropathological hallmarks of PD, and therefore it is unsurprising that 'proteolytic stress' has gained early support as one of the key underlying pathogenic mechanisms in PD [394]. There is evidence that LBs might represent aggresome-like inclusions which form as a protective response to high levels of misfolded/ubiquitylated proteins [430]. Another concept which provides support for this theory is that with PD being an age-related disorder, there is a good correlation with the aging process itself which is associated with a gradual increase in levels of damaged proteins that need to be cleared [431], [432], [433]. Furthermore the dopamine neurons in the SNc appear particularly vulnerable in PD, with relatively low levels of proteasomal enzyme activity [434], and a high level of dopamine metabolism which leads to the formation of oxidized proteins [435].

The ubiquitin-proteasome system (UPS) is the mechanism by which eukaryocytic cells remove mutant, damaged, misfolded or unwanted intracytoplasmic proteins [9], [436], [437], [438], [394], [395]. The proteins which are to be removed are tagged with ubiquitin molecules, signaling for their transport to the proteasome, where they are degraded. The peptides and amino acids which are generated by this process are then recycled, with the resulting ubiquitin monomers also re-used (see figure 1.7).

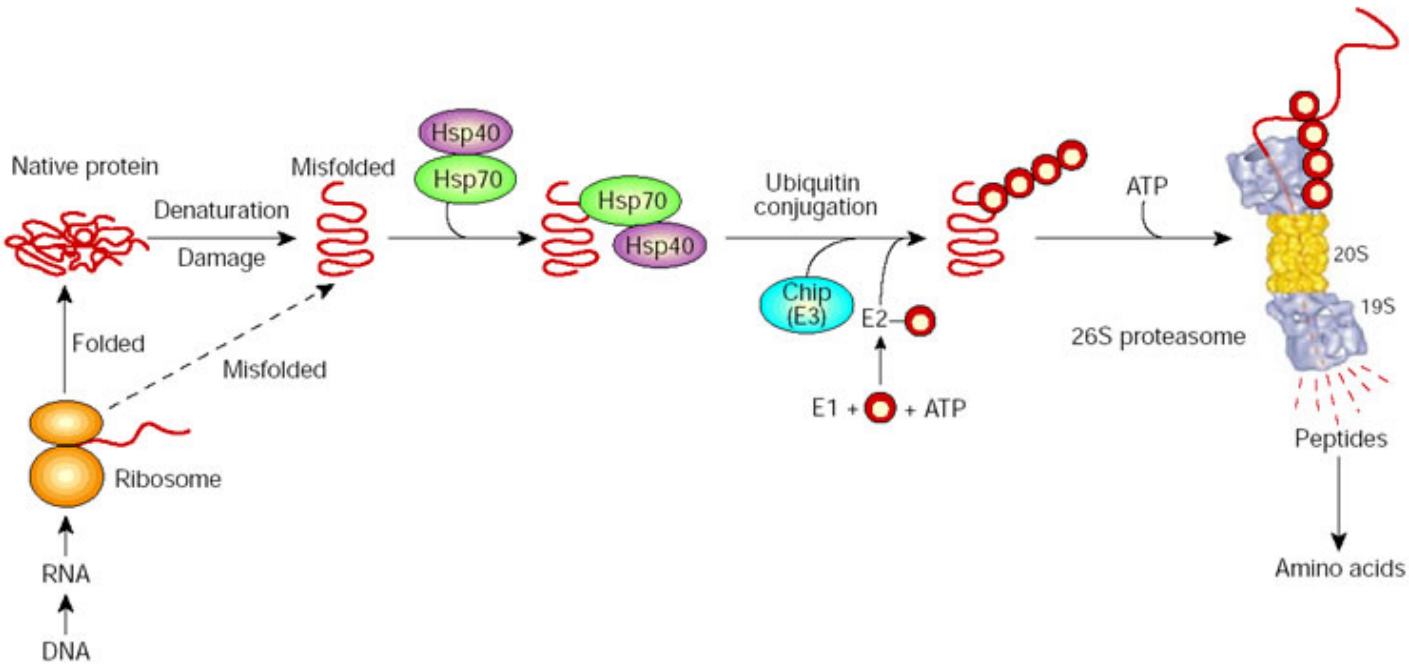

Figure 1.7 The Ubiquitin-proteasome system (UPS). The steps by which the UPS clears unwanted proteins: ATP-dependent activation of ubiquitin monomers; labelling of unwanted/damaged proteins with chains of activated ubiquitin molecules by CHIP; transport of ubiquitinated proteins to the proteasome by chaperone molecules such as heat shock proteins (HSPs); recognition and unfolding of ubiquitinated proteins by proteasome regulators; and ATP-dependent degradation of unwanted proteins by the proteasome. The red circles represent ubiquitin. Taken from Goldberg 2003 [9].

The process of 'proteolytic stress' will occur if the capacity of the UPS to degrade unwanted protein is exceeded, for example if excess misfolded protein is produced. If this occurs an accumulation and aggregation of abnormal proteins will develop, which will have an effect on the ability of the cell to perform various cellular functions, including apoptosis [394], [395].

In fPD mutations in *α-synuclein*, *Parkin*, and *UCH-L1* have all been linked to proteolytic stress. α-Synuclein is a naturally unfolded protein that is prone to misfold and to aggregate; this tendency is enhanced when the protein is in a mutant form or present in excess levels,

possibly exceeding the capacity of the proteasome to clear them. Indeed, overproduction of α-synuclein has been shown to lead to neuronal degeneration with aggregate formation [439], [440]. *Parkin* mutations could hinder ubiquitylation, and hence impede the functioning of the UPS, leading to cell death. Investigators have demonstrated that in subjects with *Parkin* mutations, excess levels of substrate proteins, in a non-ubiquitiylated state, can be detected [441]. *UCH-L1* mutations could also disrupt the UPS, and it has now been demonstrated in experimental models that treatment with inhibitors of this protein leads to dopamine neuronal degeneration with inclusion bodies [442].

More evidence for the importance of the UPS comes from the observation, in postmortem studies, of proteasomal impairment in the SNc of subjects with sPD [434]. Furthermore, in model systems proteasome inhibitors can induce selective degeneration of cultured dopamine neurons with the formation of LB-like inclusions [442], [443], [444], [445], [446], although it is important to point out that these results have not been replicated by several other investigators [447], [448], [449].

Therefore from the evidence available to date an intervention that prevents or reverses proteolytic stress might be neuro-protective in PD [450]. One such strategy is to upregulate heat shock proteins (HSPs) and make use their ability to promote protein refolding and facilitate the degradation of severely misfolded proteins. In model systems upregulated HSPs have been shown to protect dopamine cells from toxicity induced by proteasome inhibition or excess levels of misfolded proteins [451], [452] and also to prevent aggregate formation and degeneration of dopaminergic neurons induced by over expression of α-synuclein in *Drosophila* [453], [454], [455], [456].

The physiological function of LRRK2 is as yet unknown, and hence so are the underlying pathogenic mechanisms of *LRRK2*-related PD. However, its structure suggests that LRRK2 functions as a kinase, and there is now evidence that its kinase activity is regulated by GTP, and that any alterations in the protein which reduce its kinase activity cause a corresponding reduction in neuronal toxicity [457]. Therefore it has been proposed that for *LRRK2*-related PD abnormal protein phosphorylation may be the key step in the pathogenic mechanism.

1.5.3.3 Inflammation

There is a long-standing debate in the literature regarding the role that inflammation may play in the pathogenesis of PD. The well documented association between encephalitis and parkinsonism has already been mentioned here (see section 1.5.1.2). More recently it has been proposed that pathogenic process in PD could be due to activation of microglia and upregulation of inflammatory cytokines such as TNF-α in the striatum of affected subjects [458], [392], [459]. Both *in vitro* and *in vivo* studies have suggested that inducing cytokine formation leads to dopamine neuronal cell death, with anti-inflammatory compounds being shown to be attenuate MPTP and 6-OHDA induced toxicity [460] [461], [462], [463].

The intriguing report of a case of parkinsonism induced by accidental exposure to lipopolysaccharide (LPS) from *Salmonella minnesota* [464] has also provided additional support for the role of inflammation in PD. There is evidence that the presence of LPS in the basal ganglia can induce microglial activation, increasing cytotoxic molecules and leading to dopamine cell death [465], [466], [467], [468], [469], [470], [471], [396].

A recent study in which both celecoxib and pioglitazone were demonstrated to reduce mitochondrial dysfunction suggests that the mitochondrial impairment seen in PD may be caused by inflammation [472].

The final piece of evidence to support a major role for inflammation in PD comes from several cohort studies in which subjects who took non steroidal anti inflammatory drugs (NSAIDs) were found to be less likely to develop PD than were controls, suggesting a possible neuroprotective role in PD [473], [474], [475], [476]. However other more recent studies have failed to show a protective effect of NSAIDs for PD [477], [478], [479], [480], [481].

Whilst a role for inflammation in the pathogenesis of PD appears attractive, there is clearly more work to be done in order to determine its true importance in this process.

1.5.3.4 Summary

The identification of the 'PD genes' over the last decade has provided the major impetus for us to gain a better understanding of the pathogenic mechanisms involved in PD. Despite this progress, none of the mechanisms discussed above has been proven to be the primary cause of cell death for either fPD or sPD, although there is now increasing evidence that these various mechanisms are in fact interactive, promoting cell death through a complex network (see figure 1.5). Other processes not covered here, for which there is little evidence at present in PD, such as excitoxicity, may also be important. The discovery of mutations in *LRRK2* in both fPD and sPD raises hope of further progress in this field. The role of *LRRK2* in the pathogenesis of PD is still poorly understood and is one of the major questions for ongoing research. This project aims to contribute to this area by investigating the contribution of *LRRK2* to UK fPD and sPD both at the level of genetic variation and functional effect.

1.6 Aims of the work presented in this thesis

The overall aims of this study are:

1. To recruit a large well characterised cohort of subjects with autosomal dominantly inherited PD from around the UK.
2. To elucidate an accurate diagnosis in various affected members of a UK family with an inherited autosomal dominant movement disorder using imaging, molecular genetic and pathological techniques.
3. To perform a comprehensive genetic analysis of the entire coding region of *LRRK2* in the cohort of familial PD patients.
4. To perform an analysis of key coding regions within *LRRK2* in a large cohort of UK sporadic PD patients.
5. To perform initial functional characterisation of novel *LRRK2* mutations identified in this study.

The specific aims of each section of the study are outlined in the introduction to each chapter.

2 Materials and Methods

2.1 Subjects

2.1.1 Familial PD

Subjects were recruited from around the UK with familial PD (fPD). The majority of these subjects had self reported to our group previously, in response to advertisements in the national press, or via our website (http://medweb.bham.ac.uk/clin_neuro/genetics/Patient_Information.html). Some subjects were new referrals from other clinicians around the UK. In addition a small number were recruited as part of a study of fPD undertaken in conjunction with colleagues at the Institute of Neurology (ION) and the Queen Square Brain Bank for Neurological Diseases in London. These subjects were recruited to the study between April 2005 and March 2008.

For the purpose of this thesis inclusion criteria to the study were: (i) the subject must have definite PD, according to UKPDS (United Kingdom Parkinson's Disease Society) Brain Bank clinical diagnostic criteria for PD (table 1.1) [14], family history was not used as an exclusion criteria. The diagnosis was determined on clinical examination by myself. (ii) the subject must report at least 2 other family members with PD. Where possible other living affected family members were examined to confirm the diagnosis, but if the diagnosis in deceased family members was by historical family report strenuous effort was made to obtain copies of death certificates on these individuals. (iii) segregation of PD in the subject's family had to be consistent with an autosomal dominant pattern, with three or more PD cases in at least two consecutive generations (see figure 2.1).

- Subject must have definite PD, according to UKPDS (United Kingdom Parkinson's Disease Society) Brain Bank clinical diagnostic criteria for PD.
- Subject must report at least 2 other family members with PD.
- Segregation of PD in the subject's family had to be consistent with an autosomal dominant pattern, with three or more PD cases in at least two consecutive generations.

Figure 2.1 Inclusion criteria for entry into the familial PD study.

All subjects gave their informed written consent to take part in the study. The study had appropriate ethical approval from South Birmingham LREC, Sandwell and West Birmingham LREC and London MREC. All data was kept under the provision of the Data Protection Act.

2.1.1.1 Familial PD methods

A search of the database for patients with Parkinson's disease and at least 2 other affected family members was performed. 190 subjects who had previously expressed an interest in taking part in our research and were registered on the database were contacted by letter or telephone to determine whether they were still willing or able to take part in the study. In addition those subjects who had recently been referred to our group and those subjects who had been identified via the ION collaborative study were also contacted by letter or telephone.

Subjects who wished to participate in the study were then offered a research appointment in clinic or at home.

At the time of recruitment to the study a full medical history and epidemiological questionnaire, based on the PD GEN questionnaire (http://www.pdgen.bham.ac.uk/docs/PG%20GEN%20Questionnaire.pdf) (figure A.3), were recorded and a neurological examination performed on each subject. The UPDRS III (Unified Parkinson's Disease Rating Scale, Part III) [6] and the Hoehn and Yahr [13] PD rating scales were used to obtain a disease severity score in each subject in their 'on state' (figure A.1 and table 1.2). In addition a Folstein MMSE (Mini Mental State Examination) [19] (figure A.2) was performed on each subject as an assessment of their cognitive function. A levodopa equivalence dose was calculated for all individuals who were taking anti-parkinsonian medication, based on a previously used scale: levodopa 100mg=133mg of controlled release levodopa=1mg Pergolide=6.67mg Bromocriptine=1mg Pramipexole=4mg Ropinirole [17]. In each case, where the subject gave their permission, a video was taken to visually record the clinical features of disease. Olfactory function was also tested in each subject using the University of Pennsylvania Smell Identification Test (UPSIT-40, Sensonics, Haddon Heights, NJ) and olfaction was categorised as normal or pathologic according to normative data in relation to gender and age [482], [483]. Furthermore, with permission of the subject, and where possible, I reviewed the medical records of the subject relating to their PD to confirm clinical details such as initial presenting symptoms and response to medication.

2.1.2 Sporadic PD

The largest number of subjects with PD came from the Parkinson's Disease DNA Bank (PD GEN study). The aim of this ongoing study is to obtain epidemiological data and DNA samples on a large cohort of subjects with PD from around the UK who had entered two other studies, a large randomised assessment of the relative cost-effectiveness of classes of drugs for Parkinson's disease (PD MED study) and a large randomised assessment of the relative cost-effectiveness of surgery for Parkinson's disease (PD SURG trial). These are pragmatic trials of medical and surgical therapy, details of which can be found at www.pdmed.bham.ac.uk; www.pdsurg.bham.ac.uk; and www.pdgen.bham.ac.uk. The DNA samples were collected between 2002 and 2007.

The second largest collection of subjects with PD came from an existing local study, the Birmingham PD study. The aim of this study was to obtain demographic data and DNA samples from subjects with unselected PD. These samples were collected by Dr David Nicholl, predominantly from neurology outpatient clinics in the West Midlands between 1994 and 1998.

Some subjects with PD came from our local ongoing study entitled the Birmingham Neurodegenerative Disorders DNA Bank (NDD). The aim of this study is to obtain demographic details and DNA samples on a cohort of subjects with unselected PD from neurology outpatient clinics at the University Hospitals Birmingham NHS Trust and Sandwell and West Birmingham Hospitals NHS Trust. Samples for this study were collected by myself or Dr Nicholl between April 2005 and March 2008. With permission of the participating subject, I reviewed the subject's medical records relating to their PD to confirm clinical details such as initial presenting symptoms and response to medication.

The final source of subjects with PD came from the PINE study. The aim of this ongoing clinic based study is to obtain demographic data and DNA samples on subjects with unselected PD from Aberdeen, Scotland. These samples were collected by Dr Carl Counsell between 2002 and 2005.

In each of the four studies covered above there is also a control cohort. This consists of DNA samples from the unaffected spouse or carer of a subject with PD who had themselves donated a DNA sample to the collection. Demographic details were also obtained from the control subject.

All subjects had given their informed written consent to take part in the studies listed above. The studies had appropriate ethical approval from London MREC, South Birmingham LREC, West Midlands MREC, and North of Scotland LREC. In addition appropriate ethical approval from Coventry LREC was obtained in order to perform molecular genetic analyses on samples from the PD GEN collection. All data was kept under the provision of the Data Protection Act.

2.2 Functional neuro-imaging

In order to evaluate basal ganglia function seven subjects underwent dopamine transporter SPECT scanning using [^{123}I]-FP-CIT SPECT (DaTSCAN, Amersham Health) at Sandwell and West Birmingham Hospital NHS Trust. This technique enables *in vivo* demonstration of striatal dopamine activity, and has been shown to demonstrate significantly reduced striatal uptake in PD [129].

Six subjects from family 6, the kindred investigated in detail in chapter 4 (subjects III:1-III:5 and IV:1) and the proband subject from family 12 underwent DaTSCANs. These images were reviewed by Dr Alp Nothgi and Dr Edward Rolfe, consultant radiologists at Sandwell and

West Birmingham Hospital and University Hospitals Birmingham NHS Trusts, respectively. They are experienced in the interpretation of DaTSCANs, and were blinded to the demographic and clinical data on these subjects.

2.3 Genomic DNA extraction from Venous blood

Following informed consent whole venous blood was collected from patient and control subjects in ethylenediaminetetraacetic acid (EDTA) vacuette tubes (Griener Bio-One Ltd). These samples were stored at 4°C or at -80°C (if extraction was delayed by more than 48 hours) until DNA extraction was performed using the Nucleon^{TM} BACC3 kit (GE Healthcare UK Ltd).

The principle steps of this technique are: cell lysis, followed by deproteinisation, DNA extraction, DNA recovery, and the final step of DNA washing. Lysis of red blood cells was performed by adding four volumes of lysis solution A (proprietary) to whole blood and mixing for 10mins at room temperature. Next, white blood cells were pelleted by centrifugation at 1600xg for 8 mins and then re-suspended in 2ml of re-suspension solution B (proprietary). After vigorous mixing and incubation at 37°C, the samples were deproteinised by adding 500µl of sodium percholrate. To extract the DNA 2ml of chloroform (Fisher Scientific) was added, followed by 300µl of Nucleon resin (proprietary) and the samples centrifuged at 1400xg for 4 mins. Next the upper aqueous layer, which contains the DNA, was harvested and 2 volumes of ice cold 100% ethanol (Sigma) added to precipitate the DNA. The precipitated DNA was desalted with 70% ethanol, air dried and re-suspended in Tris-EDTA (TE) buffer (10mM Tris pH 7.6, 1mM EDTA) (Sigma). Finally, in order to dissolve the DNA, the samples were left at room temperature for 24-48hrs on a rotatory mixer. After 24-48 on

this mixer the DNA concentration of each sample was measured on a spectrophotometer, before being stored at -80°C. These stock DNA samples were diluted to 100ng/µl in TE buffer to create the working solutions for PCR, these were also stored at -80°C until needed.

2.4 Polymerase chain reaction (PCR)

Since its development in the 1980s PCR has revolutionised molecular genetics as a technique for rapid analysis of DNA. It permits the exponential amplification of specific regions of interest in the DNA, with the use of pre-designed DNA 'primers' which flank the target sequence, and a heat stable DNA polymerase 'Taq' named after the thermophilic *Thermus aquaticus* in which it was discovered. The region of interest is amplified in a series of reactions, which are illustrated in figure 2.2.

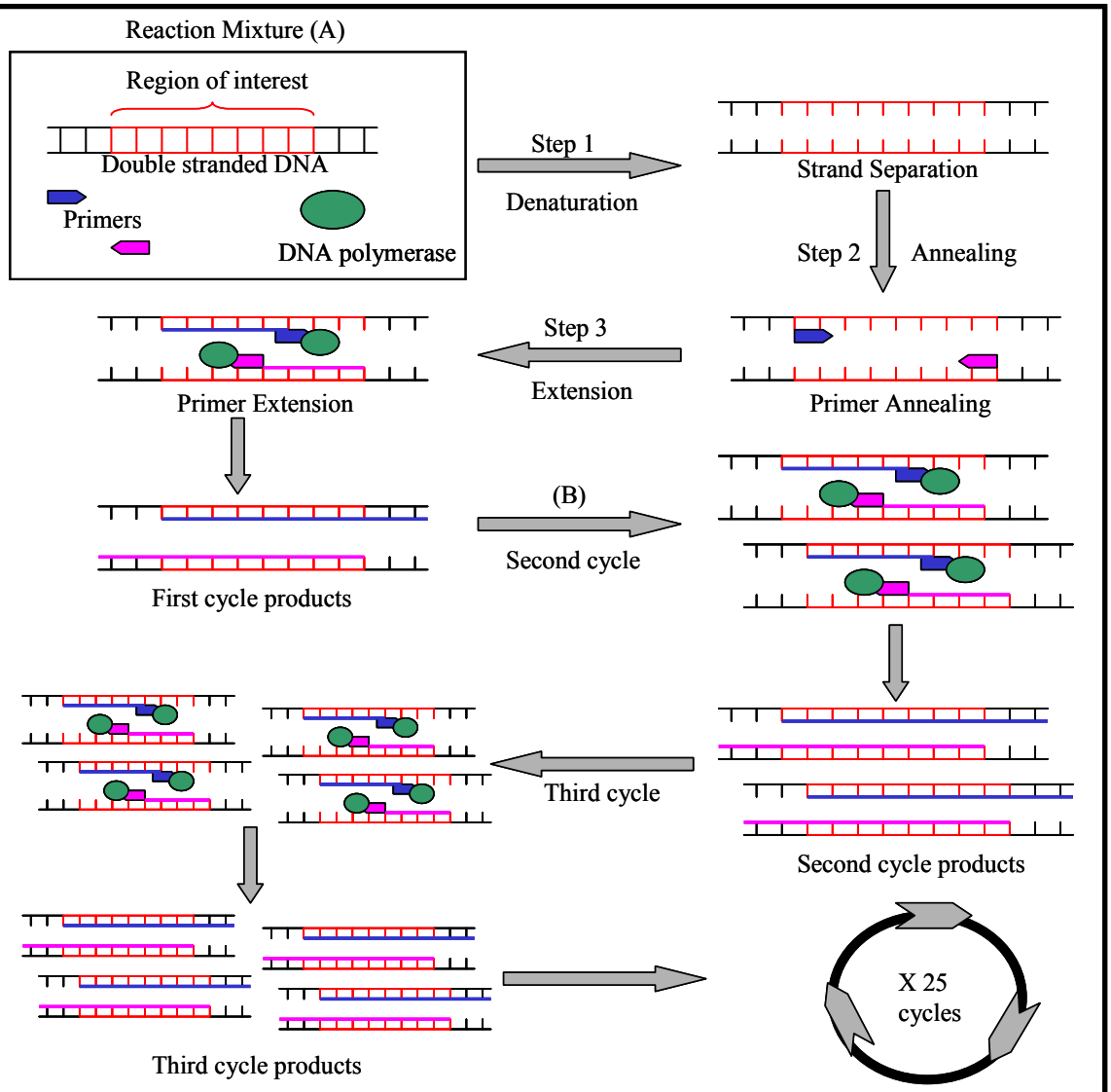

Figure 2.2 The principle of PCR. The reaction mixture contains double stranded DNA, Taq DNA polymerase and primers (A). A denaturation step (step 1) separates double stranded DNA to allow primers to anneal (step 2). Complimentary strands are then produced from the primers by the DNA polymerases (step 3) to result in first cycle products. During the second cycle, first strand products and genomic DNA act as template for primer annealing and extension (B). During the subsequent cycles the amount of DNA doubles, leading to an exponential increase typically over 25 cycles.

The reagents and cycling conditions used in a standard PCR reaction to amplify genomic DNA for genotyping are given below in table 2.1 and figure 2.3. However, for each PCR primer sequence, the annealing temperature, cycling times and component concentrations had to be optimized, details of which can be found in appendix C. Each annealing temperature was dependent upon the specific primer sequences, with extension times determined by PCR product length, with 1min per kb used as a guide.

Reagent	Stock concentration	Final concentration
Water (distilled and autoclaved)	N/A	N/A
10x Buffer (Bioline)	10x	1x
dNTP mix (Fermentas)	2mM	160µM
$MgCl_2$ (Bioline)	50mM	1.5mM
Forward primer (AltaBioscience) / (Eurogentec)	10pmole/µl	0.5pmole/µl
Reverse primer (Altabioscience) / (Eurogentec)	10pmole/µl	0.5pmole/µl
Taq (Bioline)	5u/µl	0.1u/µl
DNA	100ng/µl	5ng/µl

Table 2.1 Constituents of a standard polymerase chain reaction (PCR).

	95°C	5mins	
Denaturation:	95°C	30s	x30 cycles
Annealing:	50-60°C	30s	
Extension:	72°C	1mins	
	72°C	7mins	

Figure 2.3 Standard cycling conditions of a polymerase chain reaction (PCR).

2.4.1 Primer design

In order to achieve successful PCR reactions, the specific oligonucleotide primer sequences, and annealing temperatures, are crucial. Here I have listed some of the general principles which I followed when designing my primers: (i) primers were between 18 and 24bp in length; (ii) the melting temperatures of forward and reverse primers were approximately equal, and varied between 50-60°C; (iii) the GC content varied between 40-60%; (iv) primers contained no more than three consecutive bases of the same type; (v) the sequence of the primers were tested using BLAST to ensure that they were unique to the target resgion.

The primers and temperatures used to study each of the 51 exons and exon-intron boundaries of *LRRK2* by PCR were those described previously [283], [280]. In addition I designed specific primer pairs for the DNA sequence of *LRRK2* to screen for variations within exons 41, 42 and 49, and for the cDNA sequence of the gene in order to investigate the effect of novel mutations on RNA expression, all by the use of the Primer3 program

(http://fokker.wimit.edu/primer3/input.htm). These primers are all listed, with annealing temperatures and $MgCl_2$ concentrations, in appendix C.

2.4.2 PCR troubleshooting

The various primer pairs used in this study all have different optimal PCR conditions. The optimal annealing temperatures were available for the primer pairs used in previous studies, but not for the newly designed primer pairs. However, the other reaction conditions were not available for any of the primers, hence the standard PCR protocol, as listed above, was used as a starting point for optimisation. The different problems encountered during this optimisation process, and the methods used to overcome them, are outlined here.

2.4.2.1 Non-specific products

Non-specific amplification occurs as a result of primers annealing to DNA sequences other than that required. When this problem was encountered the annealing temperature of the PCR was increased by 2-5°C and/or the $MgCl_2$ concentration decreased from the standard 1.5mM to 1mM in order to make the reaction more specific.

2.4.2.2 Low product yield

If in contrast to non-specific amplification, when there was little or no product formed by the PCR, the melting temperature was reduced by 2-5°C and/or the $MgCl_2$ concentration increased up to 2.5 or 3mM. This had the effect of making the PCR less stringent.

2.4.2.3 Primer dimers

This occurs when primers interact with each together to form small products called 'primer dimers', which are seen at the bottom of the agarose gel. This can result in a reduction in product yield and may then interfere with genotyping processes such as sequencing. When this problem was encountered, several strategies were adopted to increase the likelihood of primers annealing to the DNA rather than other primers. These included adding 5-10% dimethylsulfoxide (DMSO) (Sigma) to the reaction mix, which can aid strand separation and reduce primer interactions, and/or increasing DNA concentrations whilst decreasing primer concentrations. If these steps were unsuccessful then the primers were re-designed.

2.5 Gel electrophoresis

Agarose gel electrophoresis is a standard method by which DNA fragments can be separated by size. Different concentrations of agarose were used to give maximal separation of differently sized fragments, such that higher concentrations were used for better resolution of smaller fragments. Typically a 1% gel was used to visualize PCR products. Agarose gels (Bioline) were made up in 1x Tris-Borate-EDTA (TBE) buffer (89mM Tris-borate, 2mM EDTA, pH8.3) (Sigma) containing ethidium bromide (Sigma). Ethidium bromide intercalates with DNA to allow visualisation with ultra violet (UV) light. During the course of my project the ethidium bromide was replaced by the newer and less toxic nucleic acid gel stain GelRed^{TM} (Biotium). Before loading onto the gel, PCR products were combined with 1µl of loading dye (Invitrogen) which added colour and weight to the sample. A DNA ladder (Helena Biosciences) of an appropriate size-range was loaded into one lane of each gel to allow sizing of DNA fragments. Then electrophoresis was carried out for 1-2 hours at 120V in horizontal

gel tanks. Finally DNA bands were visualised on a UV illuminator and pictures were taken using Genescan (SynGene) gel documentation system.

2.5.1 Mutation detection by gel electrophoresis

The principal of a higher percentage agarose giving better resolution was used to screen PCR products for a novel five-base deletion, see section 5.3.2.4. Specific primers were designed (see appendix C) to generate a small PCR product of 60bp in length, with a 55bp product produced if the deletion was present. A 3% high resolution RESponse^{TM} Research agarose (Geneflow) gel was used, with a low size-range ladder, Hyperladder^{TM} V (Bioline), and electrophoresis carried out at 120V for 3 hours in order to resolve this 5bp size difference (see figure 5.3).

2.6 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis is a commonly used technique for genotyping which exploits the ability of restriction endonucleases to recognise specific DNA sequences, and cleave at particular sites in this sequence. DNA polymorphisms can change the recognition sequence of a restriction endonuclease, altering whether the enzyme will cleave the DNA at this position or not. Hence, different DNA banding patterns will be produced on an agarose gel, depending on whether the polymorphism is present or not, allowing wild-type to be distinguished from mutant DNA.

In this study RFLP analysis was used to screen for the G2019S mutation (c.6055G>A) in exon 41, as described in section 6.3.2.2. A restriction endonuclease which recognised this polymorphic site, Sfc I from *Streptococcus faecium* (New England Biolabs), had been described previously in the literature [288], [484]. I used the online NEBcutter program

(http://tools.neb.com/NEBcutter2/index.php) to confirm that the different alleles of this polymorphism did indeed alter the recognition sequence of the enzyme. I then designed PCR primers to amplify this region of interest with the polymorphic site asymmetrically placed within it, in order to allow discrimination between homozygous and heterozygous individuals. The PCR primers and conditions can be found in appendix C.

The Sfc I recognition site was confirmed as:

- 5'…. C^TRYAG….3'; where Y= C or T and R= A or G.

The DNA target sequence in the PCR product is wild type CTACGG and mutant CTACAG. The PCR product size is 257bp, hence after restriction digest the products are 239bp and 18bp if the homozygous mutation is present, and 257bp, 239bp and 18bp if the heterozygous mutation is present.

Following the manufacturer's guidelines, the PCR products were incubated with Sfc I at 37°C for 2½ hours, 4µl of loading dye was then added and the samples were run on a 2.5% agarose gels for 2 hours to ensure clear band separation. The bands were visualised on a UV illuminator in order for the genotype to be determined, and pictures were taken using Genescan (SynGene) gel documentation system. Using control samples known to either heterozygous for the G2019S mutation or wild type, I was able to consistently show a band of 257bp for wild-type samples with bands of 257bp and 239bp for subjects heterozygous for the mutation (see figure 6.3).

2.7 Sequencing

I used the automated Sanger (dideoxy) DNA sequencing method to determine the nucleotide base sequence of my DNA and cDNA samples. This is a PCR based technique which uses a small concentration of fluorescently tagged dideoxynucleotides (ddNTPs) in the reaction mix,

along with the standard deoxynucleotides used in normal PCR reactions. The ddNTPs lack 3' hydroxyl groups such that phosphodiester bonds cannot be formed with other nucleotides, and hence if incorporated into the sequence will cause abrupt termination of DNA strand elongation. The random incorporation of these ddNTPs results in a collection of DNA strands of different lengths with tagged ddTNPs at the 3' end. Electrophoresis of these tagged products on a denaturing polyacrylamide gel separates the strands by size. The four types of ddNTPs (adenosine, cytosine, guanine and thymine) have different fluorescent tags, such that the 3' base of each strand can be determined by laser excitation of theses tags, and the sequence of the template DNA is obtained by compiling the nucleotide base information from the different length strands.

In preparation for sequencing, 15µl of each PCR product was 'cleaned-up' by the addition of 2µl of antarctic phosphatase (New England Biolabs) to catalyze the removal of 5′ phosphate groups, and 2µl of exonuclease 1 (New England Biolabs) to degrade single-stranded primer DNA. These enzymatic mixes were then incubated at 37°C for 15 mins, followed by 15mins at 85°C to inactivate the enzymes. The treated PCR product was then diluted with water to an appropriate concentration for sequencing, 5-20ng depending upon product length. The next step was to prepare these diluted samples for cycle sequencing using the BigDye terminator v3.1 kit (Applied Biosystems) as outlined in table 2.2. I prepared 10µl sequencing reactions and used the cycling conditions described in figure 2.4.

Reagent	Stock concentration	Final concentration
Water (distilled and autoclaved)	N/A	N/A
BigDye Buffer (Applied Biosystems)	5x	1x
BigDye (Applied Biosystems)	N/A	N/A (1µl per 10 µl reaction)
Forward or Reverse Primer (AltaBioscience) / (Eurogentec)	10pmole/µl	5pmole/µl
DNA	Variable	Variable

Table 2.2 Constituents of a standard cycle sequencing reaction.

	96°C	2mins	
Denaturation:	96°C	20s	x30 cycles
Annealing:	50°C	15s	
Extension:	60°C	4mins	

Figure 2.4 Standard cycling conditions of sequencing reaction.

The products from the cycle sequencing reaction were then purified using the EDTA/ethanol method. First DNA was precipitated by adding 2.5µl of 0.125M EDTA and 35µl of 95% ethanol to each sample, and incubating at room temperature for 30mins. The precipitated DNA was then pelleted by centrifugation at 2,250xg for 30mins at 4°C. These pellets were then washed twice with 95% ethanol and dried at 65°C for 20mins. Finally, the purified DNA pellets were re-suspended in 10µl of HiDi formamide, (Applied Biosystems), incubated at 95°C for 5mins and then loaded onto capillary sequencer ABI 3700 machines to read the sequence. The data obtained from the sequencer was analysed using Chromas version 2.13 (Technelysium, Australia) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw/).

When there were only a small number of samples to be sequenced I organised for the process to be performed by the Functional Genomics and Proteomics Unit (University of Birmingham) 'plasmid to profile' service. In this instance 200-500ng of PCR product was mixed with 10pmole of either forward or reverse primer, and the volume made up to 10µl with distilled water. These samples were sequenced using a similar protocol to that described above, and loaded onto an ABI 3700 capillary sequencer machine. The data was collected via the functional genomics website (http://www.genomics.bham.ac.uk/) and analysed using software as previously described.

2.8 PCR fragment analysis

Automated high-resolution PCR fragment analysis allows separation of PCR products differing in length by as little as 1 base pair [485]. Either the forward or reverse PCR primer is labelled with a fluorescent compound, so that after amplification the labeled PCR products can be separated with an automated sequencing system. With the use of a fluorescent-labeled

DNA length marker or 'size standard' and GeneScan® software (Applied Biosystems), the exact size of each peak can be displayed and analysed. This technique can also be used with cDNA for a semi-quantitative estimation of the relative abundance of wild-type and aberrant transcripts [485], [486], [487].

I used this fragment analysis technique to analyse post-PCR products for both DNA (section 5.3.2.6) and cDNA (section 7.5.2). In the PCR reactions either the forward or reverse primer was labeled with 6-Carboxyfluorescein (6-FAM), the primers and reaction conditions can be found in the relevant sections. I first prepared a 'HiDi master mix' by adding GeneScan™ 500 LIZ® internal 'size standard' (Applied Biosystems), which allows sizing of DNA fragments 35-550bp long, to HiDi formamide (Applied Biosystems). 4µl of the LIZ® size standard was added to every 500µl HiDi used for the reaction and then mixed by vortexing. 10µl of this 'HiDi master mix' was added to 1µl of each PCR product. The PCR products were used neat or diluted 1:10 with distilled water, depending on strength of PCR fragments visualised on agarose gel electrophoresis.

The reaction mixture was then incubated at 95°C for 5mins, before being loaded onto a capillary sequencer ABI 3700 machine. The data obtained was analysed with the ABI Prism Genemapper 3.5™ software (Applied Biosystems).

2.9 Denaturing high performance liquid chromatography (DHPLC)

DHPLC is a well validated high throughput technique used to identify DNA sequence variations via the detection of heteroduplexes in PCR products by HPLC [488], [489], [490], [491], [492], [493]. Before analysis by DHPLC, the PCR products were denatured and slowly

re-annealed to create homo and heteroduplexes (see section 6.1.2 for details) using the conditions described below (figure 2.5).

Denaturation:	95°C	6mins
Reannealing:	93.5°C	1min
	-1.5°C per cycle for 29 cycles	
	25°C	10mins

Figure 2.5 Standard cycling conditions for 'Duplexing' reaction

The DHPLC reactions were performed using the Transgenomic WAVE® system (Transgenomic Ltd). Column temperatures for analysis of each PCR product were determined by examination of PCR product melt profiles generated by WAVE® navigator software to cover the entire region (see figure 2.6). All PCR products required two column temperatures, some required four (see appendix F). Time shifts were also used for some temperatures to ensure that the entire peaks of the chromatogram could be visualised. 5µl of duplexed PCR product was loaded onto the DNASep column (Transgenomic Ltd) at the pre-selected analysis temperatures and eluted from the column by an acetonitrile gradient (see section 6.1.2 for details). Eluted DNA fragments were subsequently detected by UV absorption and chromatograms generated by the WAVE® navigator software. Chromatograms were analysed

automatically by the 'mutation detection' software and also manually to detect mutations or to confirm the presence of wild-type sequence.

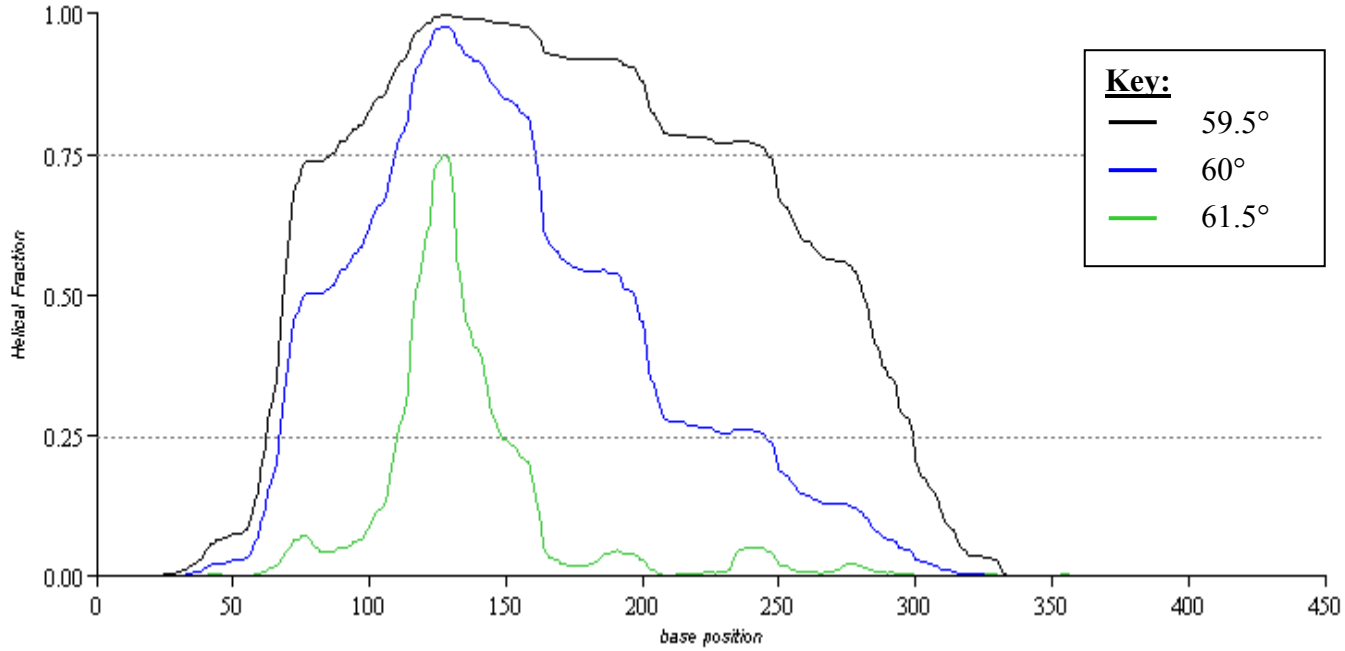

Figure 2.6 Example of PCR product melt profile created by the Transgenomic Navigator™ software. Base position is plotted against helical fraction and the melt profiles at three different temperatures are represented by different colours. Temperatures for analysis were chosen which corresponded to a helical fraction of between Melting temperatures are selected by trying to get the target sequence between the 0.60 and 0.99 helical fraction range. The helical fraction gives the proportion of DNA that is double stranded and therefore allows the DHPLC machine to detect homo- and hetero-duplices.

2.10 Primary cell lines

Two subjects carrying novel *LRRK2* mutations consented to having both a further blood sample taken, for the extraction of lymphocytes, and to having a skin biopsy performed. Lymphocytes were extracted from whole blood and transformed with Epstein-Barr Virus (EBV) into immortalized lymphoblast cell lines. Fibroblasts obtained from the skin biopsies were grown into fibroblast cell lines. Both of these cell lines were kindly generated for us by

Professor Malcolm Taylor in the Institute for Cancer Studies, at the University of Birmingham. Control lymphoblast and fibroblast cell lines were obtained as gifts from Professor Taylor and Dr Andrew Filer in the department of Rheumatology, University of Birmingham.

2.10.1 Lymphoblast lines

The media I used to maintain the lymphoblast cell lines was made up of Roswell Park Memorial Institute medium (RPMI) (Invitrogen) supplemented with L-glutamine, 10% bovine foetal calf serum (Sigma) and penicillin/streptomycin antibiotics (1000 units/ml penicillin and 0.1 mg/ml streptomycin) (Sigma). Lymphoblast cultures (10-15ml) were maintained in T75 flasks, and kept at 37°C in a humidified atmosphere containing 5% CO_2. These cells were grown in suspension, and were split every 4-5 days by removing 5-6ml of the media containing lymphoblasts from the flask. The removed cells were either then discarded or further processed, dependent upon the nature of their use in planned experiments, as covered in the relevant sections below. Finally, 5-6ml of fresh culture media, pre-warmed to 37°C, was added to the flask and the cells placed back into the incubator.

2.10.2 Fibroblast lines

The media I used to maintain the fibroblast cell lines was made up of Dulbecco's Modified Eagle Medium (D-MEM) (Invitrogen) supplemented with 4500mg glucose/l, L-glutamine, $NaCO_3$, 10% bovine foetal calf serum (Sigma) and penicillin/streptomycin antibiotics (1000 units/ml penicillin and 0.1 mg/ml streptomycin) (Sigma). Fibroblast cultures were maintained

in T75 flasks, covered with 10ml of growth media, and kept at 37°C in a humidified atmosphere containing 5% CO_2.

The cells were split every 5-7 days when they reached 80-90% confluency. Prior to splitting, complete growth media, phosphate buffered saline solution (PBS) (Gibco) and 1x trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA) were pre-warmed to 37°C. The media was removed from the flask and cells washed twice with PBS, to remove any of the bovine foetal calf serum which could have inhibited trypsin activity. 2ml of trypsin/EDTA solution was added to each flask and incubated at 37°C for 5 minutes in order to detach cells from the flask. Trypsin is a serine protease which cleaves adhesion proteins, whilst EDTA chelates the calcium required by some adhesion proteins. 5ml of pre-warmed complete growth media was then added to inactivate the trypsin and re-suspend the cells. The cells were transferred to a 15ml centrifuge tube, and pelleted by centrifugation at 1500rpm at 20°C for 5 minutes. The supernatant was then removed, cells were re-suspended in fresh growth media, plated at 20-25% confluency in T75 flasks and re-incubated. The method by which fibroblasts were harvested was dependent upon the nature of their use in planned experiments, as covered in the relevant sections below.

2.10.3 Harvesting of cells

Prior to DNA and RNA isolation, the cell density of lymphocytes was measured using a haemocytometer, before they were pelleted by centrifugation at 500 x g at 20°C for 5 minutes, and the supernatant removed. For Fibroblasts, after trypsinisation the cell density of the re-suspended cells was measured, using a haemacytometer. The cells were then re-pelleted by centrifugation at 500 x g at 20°C for 5 minutes and the supernatant removed.

2.10.3.1 DNA isolation

DNA was isolated using the DNeasy® blood and tissue kit (Qiagen) according to manufacturer's instructions for cultured cells. All steps of the DNA isolation process were carried out a room temperature. Using the maximum number of cells (5 x 10^6) permitted by the protocol, the cell pellet was re-suspended in 200µl PBS and 20µl of proteinase K was added to lyse the cells. I then added 200µl of Buffer AL (without added ethanol), the sample was mixed by vortexing and then incubated at 56°C for 10 min. Next, 200µl of 100% ethanol (Sigma) was added before vortexing again. This mixture was pipetted into a DNeasy® mini spin column which was placed in a 2 ml collection tube. This was centrifuged at 8000 rpm for 1 min. The flow-through and collection tube were discarded and the spin column placed into a new 2 ml collection tube. Then 500µl Buffer AW1 was added and the column was again centrifuged at 6000 x g for 1 min. The flow-through and collection tube were again discarded and the spin column placed into a new 2 ml collection tube. Next 500µl of Buffer AW2 was added and the column centrifuged at 20,000 x g for 3 min to dry the DNeasy® membrane. The flow-through and collection tube were once again discarded. This centrifugation step ensured that no residual ethanol was carried over this can interfere with subsequent reactions. The spin column was placed into a clean 2 ml microcentrifuge tube, and 200µl of Buffer AE was pipetted directly onto the DNeasy® membrane. After incubation for 1 min the tube was centrifuged at 20,000 x g for 1 min to elute. This elution step was repeated once to increase DNA yield.

A sample of the isolated DNA was then assessed in a nanodrop ND-100 spectrophotometer (Labtech International) to make ensure a high yield of DNA was obtained with no solvent contamination. DNA was stored at -80°C until use in PCR.

2.10.3.2 RNA isolation

RNA was isolated using the RNeasy® mini kit (Qiagen) according to manufacture's instructions for cultured cells. All steps of the RNA isolation process were carried out at room temperature, with RNase free reagents, tubes and pipette tips. Before isolating RNA, Buffer RPE had to be prepared from concentrate by adding 4 volumes of 100% ethanol (Sigma) to obtain a working solution. As the number of pelleted cells used was $<5 \times 10^6$, the cell pellet was dissolved in 350µl Buffer RLT, to disrupt the cells, and the mixture vortexed for 1 min. Then 350µl of 70% ethanol (Sigma) (diluted in RNase-free water (Qiagen)) was added and mixed well by pipetting. The sample was transferred to an RNeasy® spin column and placed in a 2 ml collection tube. This was centrifuged at 8000 x g for 15 seconds and the flow-through discarded. Next 700µl of Buffer RW1 was added to the spin column, which was centrifuged at 8,000 x g for 15 seconds to wash the spin column membrane, and the flow-through again discarded. 500µl of Buffer RPE was then added to the spin column, which was centrifuged at 8,000 x g for 15 seconds to wash the spin column membrane, and the flow-through discarded. A further 500µl of Buffer RPE was added to the spin column, which was centrifuged at 8,000 x g for 2 mins to again wash the spin column membrane, and the flow-through discarded. The spin column was then placed into a new 2 ml collection tube and centrifuged at 8,000 x g for 1 min to eliminate any possible contamination with Buffer RPE. Next the spin column was placed into a new 1.5 ml collection tube, 30µl RNase-free water (Qiagen) was added to the spin column membrane, which was then centrifuged at 16,000 x g for 1 min to elute the RNA. This elution process was then repeated with another 30µl RNase-free water (Qiagen) to obtain a higher yield of RNA.

A sample of the isolated RNA was then assessed in a nanodrop ND-100 spectrophotometer (Labtech International) to ensure a high yield of RNA was obtained with no solvent or DNA contamination. The RNA was stored at -80°C until further use.

I used the DNA-free™ (Applied Biosystems) kit according to manufacture's instructions to remove any contaminating DNA from the RNA samples before cDNA generation. This kit can remove trace to moderate amounts of contaminating DNA (up to 50µg DNA/ml RNA) from purified RNA. The routine DNase treatment protocol was used, as less than 200µg nucleic acid was present per ml of samples used. The manufacture's recommendations are to use 1µl (2U) of the recombinant DNase I (rDNase I) for up to 10µg of RNA, hence all reactions were performed with less than 10µg of RNA. The reagents, tubes and pipette tips were RNase free.

I used 35µl reactions in RNase free microtubes (Applied Biosytems), adding 1µl of rDNase I, 3.5µl (0.1 volume) of 10X DNase I reaction Buffer, and 2µl of RNase free water (Qiagen) to 28.5µl of RNA isolate. The reaction contents were mixed gently and then incubated at 37°C for 30 minutes. After 30 minutes 3.5µl (0.1 volume) of re-suspended DNase inactivation reagent was added, mixed well and then incubated for 2 minutes at room temperature, ensuring that the contents of the tube were mixed 3 times during the incubation period to redisperse the inactivation reagent. The samples were then centrifuged at 10,000 x g for 11/2 minutes and then the supernatant, which contains the RNA, was transferred into a fresh RNase free microtubes (Applied Biosytems). The RNA was then assessed in a nanodrop ND-100 spectrophotometer (Labtech International) and stored at -80°C until use in cDNA generation.

2.10.3.3 cDNA generation

cDNA was generated using the High capacity cDNA archive kit (Applied Biosystems) according to manufacture's instructions. Nuclease-free reagents, tubes and pipette tips were used. Approximately 0.9-1µg of total RNA was used in each Reverse Transcription (RT) reaction. For each reaction I prepared a 20µl master mix (see table 2.3 for constituents) which was added to 30µl of the isolated RNA (30ng/µl) and mixed well.

A negative control without the MultiScribe™ Reverse Transcriptase (Applied Biosystems) was also prepared for each reaction. These mixtures were incubated at 25°C for 10 minutes and then at 37°C for 120 minutes. The cDNA generated was stored at -20°C.

Component	**Volume (µl)/ reaction**
10X Reverse Transcription Buffer (Applied Biosystems)	5µl
25X dNTPs (Applied Biosystems)	2µl
10X random primers (Applied Biosystems)	5µl
MultiScribe™ Reverse Transcriptase 50U/µl (Applied Biosystems)	2.5µl
Nuclease-free water (Qiagen)	5.5 µl

Table 2.3 Constituents of a reverse transcription reaction.

2.11 Nonsense-mediated decay (NMD) assay

NMD is a cellular mechanism which reduces the abundance of potentially deleterious transcripts containing a premature termination codon [494], [495], [15], [496]. In order to study the effects of NMD in our patient and control fibroblast cell lines, I designed an assay using cycloheximide (CHX), a potent inhibitor of NMD [497]. This is a well recognised technique that has been used before in fibroblast cell lines, although numerous concentrations of CHX, numbers of cells and incubation times have been used [498], [499], [500], [501], [502], [503]. The protocol I used was based upon that of Baker et al. (2006) [498] and was also recommended by a collaborator at the Institute of Neurology in London, Dr Henry Holden.

Fibroblast cell lines from two subjects with PD and two control subjects were grown to confluency in T75 flasks as described previously (section 2.10.2). These cells were split and re-plated into T25 flasks, 15 flasks for each of the 4 subjects. These cells were subsequently grown to 80-90% confluency. Once confluent, the cells from 3 flasks for each subject were harvested for RNA extraction as previously described (section 2.10.3.2). Then 6 of the remaining 12 flasks for each subject were incubated with 500μM CHX (Sigma) and 6 without CHX. After 2 hours incubation the cells from 3 CHX-treated flasks and 3 untreated flasks for each subject were harvested for RNA extraction. This procedure was repeated again after 8 hours incubation. cDNA was subsequently generated from each RNA sample, as previously described (section 2.10.3.3), for further analysis.

2.12 Western blotting

Western blotting is a commonly used technique in which the specificity of antigen-antibody recognition is utilised in order to detect, and quantify, a particular protein of interest in various samples. In this technique, proteins are first size-fractionated on polyacrylamide gels, by electrophoresis. The samples are then transferred or 'blotted' onto a nitrocellulose membrane again via electrophoresis, and antibodies employed to bind to the protein of interest and allow detection. The protocol which I used to perform western blots is outlined below.

2.12.1 Sample preparation

Samples were prepared differently for the lymphoblast and fibroblast cells, but all protein extractions were performed on ice, with a protease inhibitor cocktail (Sigma) and Cytobuster^{TM} protein extraction reagent (Promega) chilled to 4°C, in order to minimise proteolysis.

Prior to protein isolation, the cell density of lymphocytes was measured using a haemocytometer, before they were pelleted by centrifugation at 500 x g at 20°C for 5 minutes, and the supernatant removed. The pellet was washed once with sterile PBS pre-warmed to 37°C to remove all trace of media that might be inhibitory to protein analysis. Pre-chilled CytoBuster^{TM} (Promega) (150µl per 10^6 cells) containing 1:100 dilution of protease inhibitor cocktail (Sigma) was then applied. This mixture was incubated on ice for 30 minutes, transferred into a fresh 1.5ml eppendorf tube, and then centrifuged at 16,000 x g at 4°C for 5 minutes. The supernatant containing the cell extract was subsequently removed and transferred into a clean tube for further analysis, the pellet was stored at -80°C.

In the case of the fibroblast lines, cells were harvested when they had reached 80-90% confluency. Cell culture flasks were washed twice with sterile PBS pre-warmed to 37°C to remove all trace of media. The relevant volume of pre-chilled CytoBuster™ (Promega) (500µl per T25 and 1.5ml per T75 flask) containing 1:100 dilution of protease inhibitor cocktail (Sigma) was applied. The flask was then incubated on ice for 30 minutes, before cells were detached using a cell scraper, the cell suspension aliquoted into a fresh 1.5ml eppendorf tube, and then centrifuged at 16,000 x g at 4°C for 5 minutes. The supernatant containing the cell extract was subsequently removed and transferred into a clean tube for further analysis, the pellet was stored at -80°C.

In order to quantify the total protein concentration in each sample, the Biorad *DC* protein assay (Biorad) was used according to manufacture's instructions for a microplate assay protocol. This is a colorimetric assay similar to the Lowry assay [504]: the assay is based on the reaction of protein with an alkaline copper tartrate solution, and the subsequent reduction of Folin reagent by the copper-treated protein [504]. Proteins cause a reduction of the Folin reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour, with a maximum absorbance at 750nm and minimum absorbance at 405nm [505].

Prior to each assay run I prepared an aliquot of 'working reagent A' by adding 20 µl of reagent S to each ml of reagent A needed for the run. In order to prepare a standard curve I prepared 9 dilutions of a protein standard, bovine serum albumin (BSA) (Sigma), ranging from 0 to 1mg/ml of protein. The BSA was diluted in the same buffer as the samples, CytoBuster™ (Promega). Then 5 µl of each of the standards and samples were pipetted into separate wells in a clean, dry micrplate. I added 25µl of 'working reagent A', followed by 200µl of reagent B,

into each well. The plate was gently agitated for 5 seconds, ensuring that any bubbles were popped with a clean, dry pipet tip. After 15 minutes at room temperature and in the dark, absorbances were read on a microplate spectrophotometer, VICTOR 3 ™ 1420 multilabel counter (PerkinElmer), at 750nm. The absorbance readings of the samples were then compared to the standard absorbance curve, to calculate protein concentrations.

The preparation of the lysate for western blotting was carried out on fresh samples, and was not performed on samples which had been pre-frozen. To prepare lysates for western blotting, proteins within the samples were denatured by the addition of Novex sodium dodecyl sulphate (SDS) sample buffer (Invitrogen) containing 20mM of the reducing agent dithiothreitol (DTT) (Sigma) and boiled for 5mins. This was to linearise the proteins, thus allowing them to be separated by size as well as ensuring that peptide antigen sequences were available for antibody binding. The sample buffer also contained bromophenol blue, which weighed down the samples so that they sank to the bottom of the wells, and allowed them to be visualised.

2.12.2 Polyacrylamide gel preparation

Polyacrylamide gels were prepared with two different components, an 8% resolving gel for separating the protein samples, and a 6% stacking gel containing wells into which protein samples were loaded. The constituents of each gel are listed in Table 2.4. This ‘discontinuous’ gel system was used to enhance band resolution. It works by creating an ion gradient in the stacking gel that causes the proteins to ‘stack’ into a focused band; once the proteins enter the separating gel the ion gradient is lost and the proteins are separated according to size.

Components		Volume for one gel
Resolving gel (8%)	Water	2.8ml
	Protogel (National Diagnostics)	1.6ml
	1.5M Tris/HCl buffer pH 8.8 (Sigma)	1.5ml
	SDS (10%) (Sigma)	60µl
	APS (10%) (Sigma)	21µl
	TeMed (Sigma)	9µl
Stacking gel (6%)	Water	1.85ml
	Protogel (National Diagnostics)	0.4ml
	0.5M Tris/HCl buffer pH 6.8 (Sigma)	0.75ml
	10% SDS (Sigma)	30µl
	10% APS (Sigma)	15µl
	TeMed (Sigma)	7.5µl

Table 2.4 Polyacrylamide gel recipe. Components were mixed in the order listed. SDS is sodium dodecyl sulphate. The ammonium persulfate (APS) and N,N,N,N - tetramethyl-ethylenediamine (TeMed) caused the acrylamide and bisacrylamide contained within the Protogel to polymerise, so were added immediately before gel pouring.

The resolving gel was prepared first and added to pre-made gel casting cassette (Invitrogen) using a Pasteur pipette, until it was three quarters full. A layer of 70% ethanol was then applied to remove any air bubbles and produce a level surface. This was left to set at room temperature for approximately 15mins. After removing the ethanol, the stacking gel layer

applied on top. A comb was inserted to create lanes for sample loading, and the gel left to set at room temperature for a further 15 minutes.

2.12.3 Gel loading and electrophoresis

Polyacrylamide gel cassettes were placed into the X-cell surelock mini gel system (Invitrogen), clamped tight and Novex tris-glycine running buffer (Invitrogen) (10x stock solution diluted 1:10 with distilled water) added to fill the central chamber, before the gel combs were removed. In order to allow sizing of protein bands 8µl of HiMark™ pre-stained protein standard (Invitrogen) consisting of 9 pre-stained protein bands in the range of 31-460 kDa was loaded to at least one lane of every gel. The prepared lysate samples were then loaded onto the gel lanes, using the known protein content of each sample to ensure that similar quantities of protein were loaded into each lane. Electrophoresis took place at 160V for 2hrs.

2.12.4 Protein Transfer

After electrophoresis the polyacrylamide gels were removed from the cassettes and the proteins transferred onto hybond nitrocellulose membrane (GE Healthcare UK Ltd) in the XCell II blot module (Invitrogen) according to manufacturer's instructions. Transfer buffer was prepared with 20ml Novex transfer buffer (Invitrogen), 100ml of methanol (Fisher Scientific) and made up to 500ml with distilled water. All components in the XCell II blot module (nitrocellulose transfer membrane, blotting paper and blotting pads) were soaked in this buffer prior to stacking, except the gel which was placed in distilled water prior to transfer. They were stacked as shown in figure 2.7, inserted into the gel tank and clamped tightly together. The transfer module was then topped up with transfer buffer to make sure all

the components were bathed in the electrolyte. I performed the protein transfer at 60V for 3 hrs at room temperature.

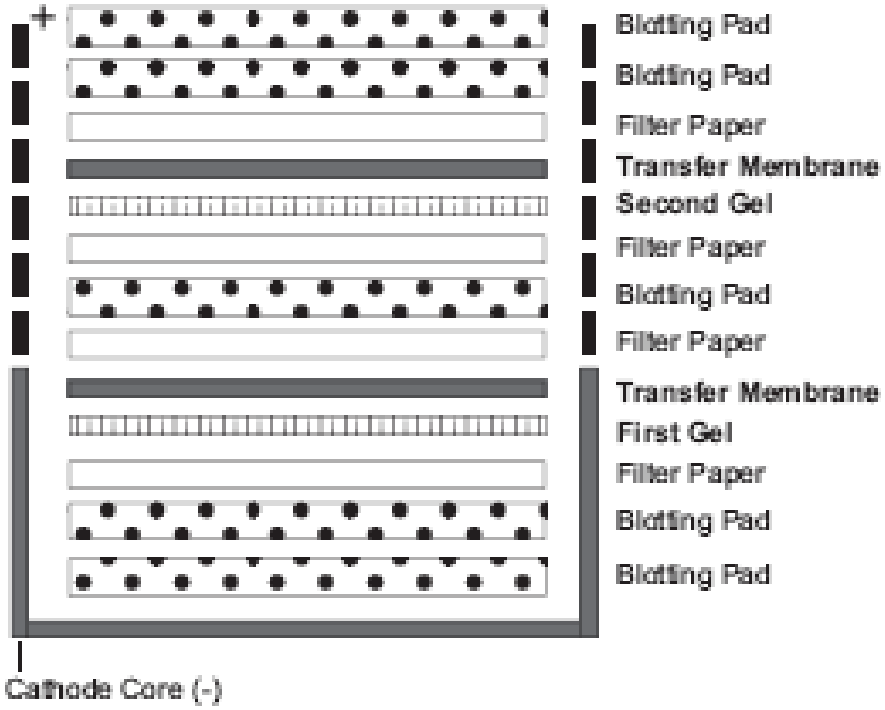

Figure 2.7 Diagrammatical representation of how the different components of the transfer module were stacked. Taken from Invitrogen website (http://www.invitrogen.com/site/us/en/home/Global/.html)

2.12.5 Antibody application

Once the proteins were transferred onto the nitrocellulose membrane, they were ready to be probed with antibodies. In order to prevent non-specific binding of the primary and secondary antibodies to background endogenous proteins, the membrane was first 'blocked' in 10ml of 5% non-fat dry milk (Marvel®, International Foods Ltd) diluted in 1x PBS containing 0.1%

Tween 20 (Sigma) and left to agitate on a rotatory mixer for 1hr at room temperature. The primary antibody was then applied to the membrane and left to incubate overnight at 4°C with agitation. These antibodies were diluted in the 5% Marvel® in PBS/Tween solution, dilution factors varied (see section 7.3.9). After primary antibody incubation, the membranes were rinsed for approximately 10s, and then washed three times for 10mins each in the PBS Tween to remove any of the primary antibody.

Horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako), specific to the species of animal in which the primary antibody was generated, were then diluted in the 5% Marvel® in PBS/Tween (see section 7.3.9 for dilution factors). The membranes were incubated with the secondary antibody solution for 1hr at room temperature, on the rotatory mixer. Finally excess secondary antibody was removed by washing in the PBS/Tween solution three times, as after the primary antibody incubation.

2.12.6 Signal detection

I used enhanced chemiluminescent solution plus (ECL plus) western blotting detection reagents (GE Healthcare UK Ltd) according to manufacturer's instructions to detect HRP signals on the conjugated secondary antibodies. The ECL system uses the oxidation of Lumigen PS-3 Acridan by the HRP enzyme to produce light (figure 2.8).

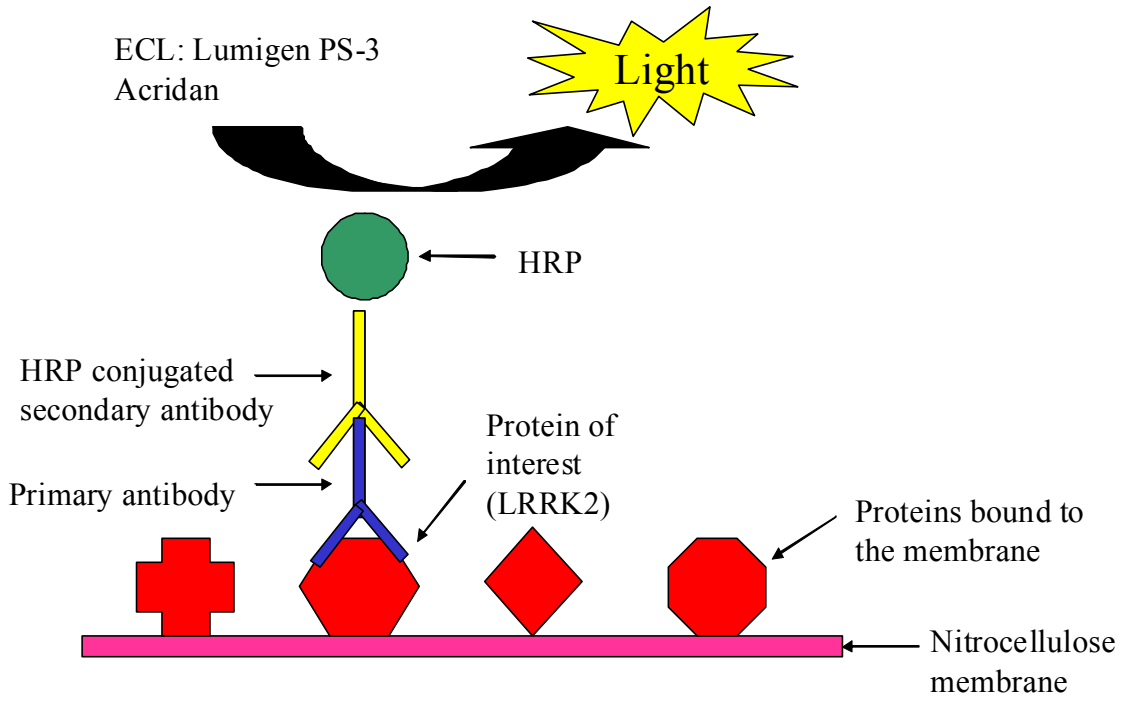

Figure 2.8 Western blot protein detection. Proteins bound to the nitrocellulose membrane have been separated by size on a polyacrylamide gel. Primary antibodies bind to the protein of interest and HRP-conjgated secondary antibodies bind to the primary. The HRP enzyme converts Lumigem PS-3 Acridan to ester intermediates, which react with peroxide to produce light that can be detected on X-ray film.

I added 50µl of solution B to 2ml of solution A, mixed well, applied it evenly to the nitrocellulose membrane with a Pasteur pipette, and then incubated the membrane for 5 minutes at room temperature. The membrane was then patted dry, wrapped in cling film and exposed to X-ray film (Thermoscientific) prior to development using an automated x-ray film processor (Xograph).

3 Clinical Studies of Familial and Sporadic Parkinson's Disease

3.1 Introduction

The variability of the presenting features and rate of progression of idiopathic Parkinson's disease (IPD) suggests multiple pathogenic mechanisms and thus implying complex gene-environment interactions. This would appear to suggest that we should regard PD more as a syndrome rather than a single disease.

When studying the role of genetic factors in the pathogenesis of PD it is therefore also crucial to consider a number of other potentially important factors such as age, presenting symptoms, co-morbid dementia and environmental exposures. Furthermore, when studying genetic factors in distinct cohorts of subjects with familial and sporadic PD it is vitally important to consider the demographics of the cohorts in order to put this data into the context of IPD in the community.

3.1.1 Demographics

The demographics of a PD cohort may have important implications for such crucial factors as disease phenotype and progression. Indeed, there are many important factors to consider, such as age, gender, ethnicity, handedness and family history.

Increasing age has been shown to be the strongest risk factor for PD [210], [211] and has also been shown to be associated with an accelerated rate of symptomatic progression [40], [42].

PD also appears to occur more commonly in men than women; indeed in most clinic based studies males outnumber females by approximately 2:1. Furthermore, recent meta-analyses of age-adjusted male to female incidence ratios for PD have shown a male to female ratio of 1.46

and 1.49 [506], [507]. Some differences in disease phenotype have been noted between the genders, but the reasons for this are unclear. It may be that male gender itself is a risk factor, or it may be a surrogate for other factors. Another explanation comes from *in vitro* evidence that oestrogen may be neuroprotective [508].

There is evidence to suggest that incidence of PD may also vary by ethnicity. Prevalence rates appear to be lower in Asians than in whites, with lower rates in blacks than those in non-Hispanic whites [509], [510], [511], [512], [513], [514], [515]. Indeed, in a recent study age- and gender-adjusted incidence rates were highest amongst Hispanics, followed by non-Hispanic whites, Asians and then blacks [515]. These potential ethnic variations may point to important differences in genetic factors or may be as a result of different environmental exposures.

Handedness and PD has also been investigated and in a large clinic-based study of patients with PD 92% were found to be right handed [516]. This fits with population figures which indicate that 8-15% of people are left-handed [517], [518]. Handedness was found to be significantly related to side of asymmetric disease, such that left-handed individuals tended to have more severe disease on the left hand side of the body. This may be due to a combination of anatomic vulnerability and/or hand usage [516].

Family history of neurodegenerative disorders is another potentially important factor and in a recent study 25% of PD patients reported a positive family history of neurodegenerative disorders [17]. In the DATATOP study of PD 19% of patients reported a positive family history of PD [41]. In a population-based study, the reported frequency of a positive family history of PD was 10.3% in subjects with PD and 3.5% in age- and sex-matched controls

[213]. Furthermore, Bonifati et al. reported that a family history of PD was positive in 24% of 100 consecutive PD cases and 6% of spouse controls ($p<0.001$) [214].

Generally fPD is thought to have a different phenotype to sPD, for example *Parkin* related PD typically presents at a younger age and shows slower disease progression [234], [235], [236]. Recently there have been some more comparative studies which have re-investigated this proposed difference. Carr et al. compared 26 fPD patients to 52 age-matched sPD patients [233]. Baba et al. compared 40 fPD patients to 1277 consecutive PD patients (1087 without a family history of PD and 190 with a family history of PD) [519]. Furthermore in a recent prospective study by Papapetropoulos et al. 50 fPD patients were compared to 50 age- and sex-matched sPD patients [520]. All three studies showed similar demographic and phenotypic characteristics between the two groups, suggesting a similar topography of neurodegenerative insult and shared aetiological factors between the two groups [233], [519], [520].

3.1.2 Initial motor symptoms

There has been relatively little work done into the nature of initial motor symptoms in PD and their predictive utility for disease progression. A recent study, however, did investigate the pattern and relationship between initial symptoms and clinical examination findings in 840 patients with PD [17]. The initial motor symptoms were: 49% tremor, 21% bradykinesia, 10% gait difficulty, 8% micrographia, 5% rigidity and 7% other. Indeed tremor (59%) and bradykinesia (16%) were also the most common initial motor symptoms reported in the DATATOP series [41]. In general there appeared to be a consistent relationship between initial motor symptoms and symptom rating at first clinic visit.

3.1.3 Cognitive dysfunction and PD

Cognitive changes are common in PD and cause substantial disability [45]. Indeed mild cognitive impairment can develop into a sub cortical dementia called PD with dementia (PDD). As outlined in the introduction, the diagnosis of dementia in PD is complicated by the other disease entity of dementia with Lewy bodies (DLB). The reported rates of dementia in PD are variable, ranging from 3 to 80% [521], [53], [54], [52]. A recent systematic review on the prevalence of PDD suggested that 24 to 31% of PD patients have dementia [55]. One of the most established risk factors for dementia in PD is advanced age, whilst other factors such as disease duration and later onset of PD have not been consistently associated with the development of dementia [56], [522], [523], [58], [524].

3.1.4 Olfaction and PD

Olfactory dysfunction is found in 70-100% of subjects with PD, making it is as common a clinical sign as resting tremor [108], [109], [525], [110]. It is known to be associated with neuronal loss and Lewy body deposition in the olfactory pathway [112], [525]. Olfactory identification is the most widely used assessment, with the University of Pennsylvania Smell Identification Test (UPSIT) validated as a method of quantification [116], [117]. Indeed, olfactory dysfunction has been shown to appear prior to the onset of motor symptoms [117]. Olfactory testing has also been performed in asymptomatic individuals, and in some studies olfactory dysfunction has been shown to be predictive of development of PD [119], [120], [121], [122].

3.1.5 Environmental exposures and PD

As outlined in the introduction, a number of environmental risk factors for PD have been proposed on the basis of presumed pathogenic mechanisms. However, many of the epidemiological studies designed to investigate these factors (such as smoking, toxin exposure and infections) were retrospective, with small sample sizes and in some cases used inappropriate controls. Furthermore, the time period in which patients are at risk of developing PD is unknown and therefore it is not clear whether early, late, cumulative or lifetime exposures should be studied. This section will briefly outline evidence for and against a pathogenic role in PD for some other environmental exposures, not covered in the introduction, which have been recorded on subjects studied in this project.

There have been numerous studies investigating a link between caffeine consumption and PD. An inverse association between PD and coffee consumption [201], [526], [527], [528], [529] and between PD and tea consumption has been identified by many of these studies [527], [530]. Indeed a meta-analysis of eight case-control and five cohort studies showed a significantly decreased PD risk for coffee drinkers (pooled relative risk 0.69) that was independent of smoking [203].

The role of occupation and risk of PD has also been investigated, and certain occupations do have an increased risk of exposure to potentially important environmental factors, such as head injury, pesticides or toxins. However, interpretation of data from the numerous studies of the effects of lifestyle is difficult due to the overlapping nature of the risk factors, and the variability of the results. Some studies have shown a positive association between PD and farming, rural living and ingestion of well water [531], [532], [533], [534]. This relationship could be due to chemical or pesticide exposure. Indeed one study found that associations with rural living were a function of exposure to pesticides [535]. Furthermore the association of

well water ingestion might be due to leaching of pesticides from soil into ground water [536]. Thus rural living and farming might simply be surrogate markers for pesticide exposure, which is discussed in the introduction.

Several recent studies into the long suspected link between head trauma and PD have been performed. Bower et al. counted only head traumas that required medical care, and found the frequency of head trauma to be higher in PD cases than controls (OR 4.3, 95% CI=1.2-15.2) [537]. In another study involving siblings with PD, history of head trauma was associated with a younger onset of PD by a mean of 3.3 years [538]. A case control study of 93 pairs of twins discordant for PD also found an association between PD and head trauma (OR= 3.8, 95% CI=1.3-11.0) [539]. Most recently the Geoparkinson study found that head injury showed an exposure-response relationship with PD (for being knocked unconscious more than once, (OR 2.56, 95% CI= 1.78-3.69) [153].

3.2 Aims of the chapter

The three major aims of the work in this chapter were firstly to recruit a large cohort of subjects with familial PD (fPD) from around the UK and to collect demographic, phenotypic and epidemiological data on this cohort. Secondly to analyse and discuss the demographic, phenotypic and epidemiological data collected. Thirdly to analyse and discuss the demographic data on several cohorts of patients recruited from around the UK with predominantly sporadic PD (sPD).

3.3 Subjects and methods

3.3.1 Familial and sporadic PD

I recruited subjects with familial PD (fPD) from around the UK between April 2005 and March 2008. Proband cases were selected where segregation of PD in their family was consistent with an autosomal dominant (AD) pattern, with three or more PD cases in at least two consecutive generations, as covered in section 2.1.1.

Subjects with sporadic PD (sPD) came from four sources, as covered in section 2.1.2. The largest number of subjects with sPD came from the Parkinson's Disease DNA Bank (PD GEN study) (www.pdgen.bham.ac.uk), DNA samples were collected between 2002 and 2007. Subjects with sPD also came from local studies, the Birmingham PD study in which samples were collected between 1994 and 1998 and the Neurodegenerative Disorders DNA Bank in which samples were collected between April 2005 and March 2008. The final source of subjects with sPD came from the PINE study, samples were collected in Aberdeen between 2002 and 2005.

All subjects had given their informed written consent to take part in the studies listed above and appropriate ethical approval had been obtained (see sections 2.1.1 and 2.1.2). All data was kept under the provision of the Data Protection Act.

3.3.2 Statistical methods

Statistical analysis was performed using the SPSS for Windows release 15.0 (SPSS Inc, Chicago, Ill., USA). Continuous variables were tested for normal distribution using Kolmogorov-Smirnov test and homogeneity of variance was tested using the Levene's statistic where appropriate. Categorical variables were compared using χ^2 analyses, whilst continuous

variables (age at evaluation, and age at disease onset) were investigated using analysis of variance (ANOVA), Hochberg's GT2 Post Hoc analysis and two-tailed independent *T*-tests. GraphPad software (http://graphpad.com/quickcalcs/) was used to compare my data to that from previously reported studies, with advice and assistance from a statistician, Dr Peter Nightingale, Wellcome Trust Clinical Research Facility, University of Birmingham. A p value <0.05 was considered significant.

3.4 Results of the fPD study

I recruited a total of 46 proband cases from families containing three or more members affected by PD, where segregation of PD in the proband's family was consistent with an AD pattern, to the study. From these 46 families seven other members affected by PD, two members affected by dopa responsive dystonia (DRD) and 16 unaffected family members were also recruited to the study.

In addition I recruited ten proband cases from families in which, after detailed examination of family history, only two affected family members could be confidently identified. From these ten families two other members affected by PD, one member affected by Alzheimer's disease and three unaffected family members were also recruited to the study. Data on the geographical location, demographic and clinical characteristics and epidemiological survey on this cohort is available but is not presented in this thesis.

3.4.1 Geographical location of families

As can be seen in figure 3.1 below 44 of the fPD probands were recruited from throughout England, with a bias towards the West Midlands (48%). Two of the fPD probands were recruited from Wales.

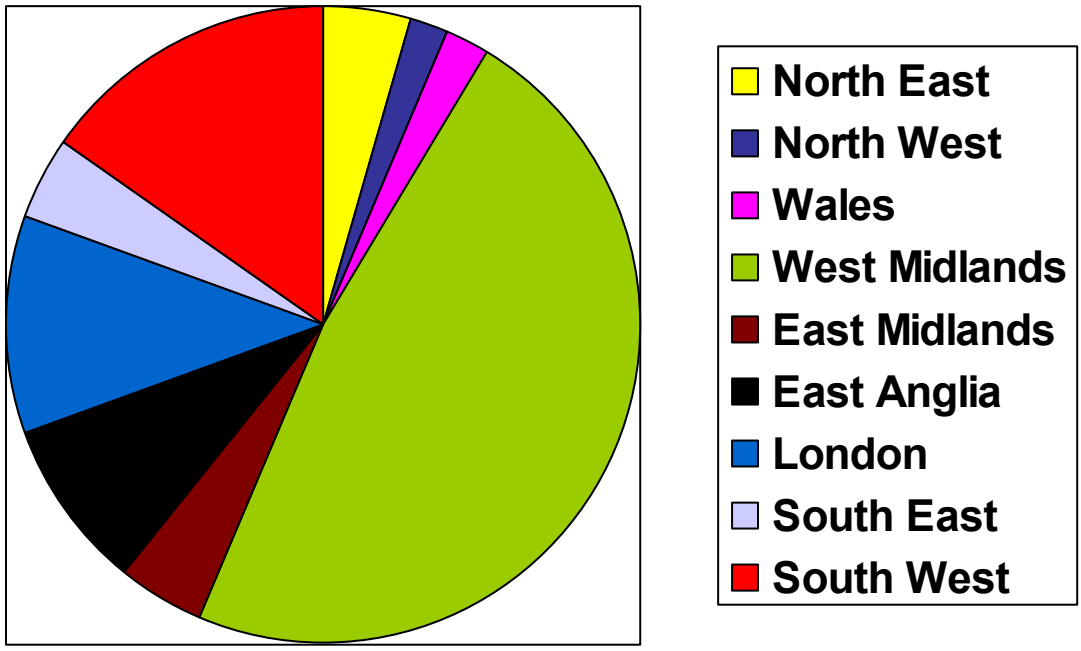

Figure 3.1 Pie chart representing geographical location of probands with familial PD recruited to this study.

3.4.2 Number of affected family members

As can be seen in figure 3.2, of the 46 families recruited to the study there were 35 families (76%) with three members affected by PD, eight families (17%) with four members affected by PD and three families (7%) with five members affected by PD.

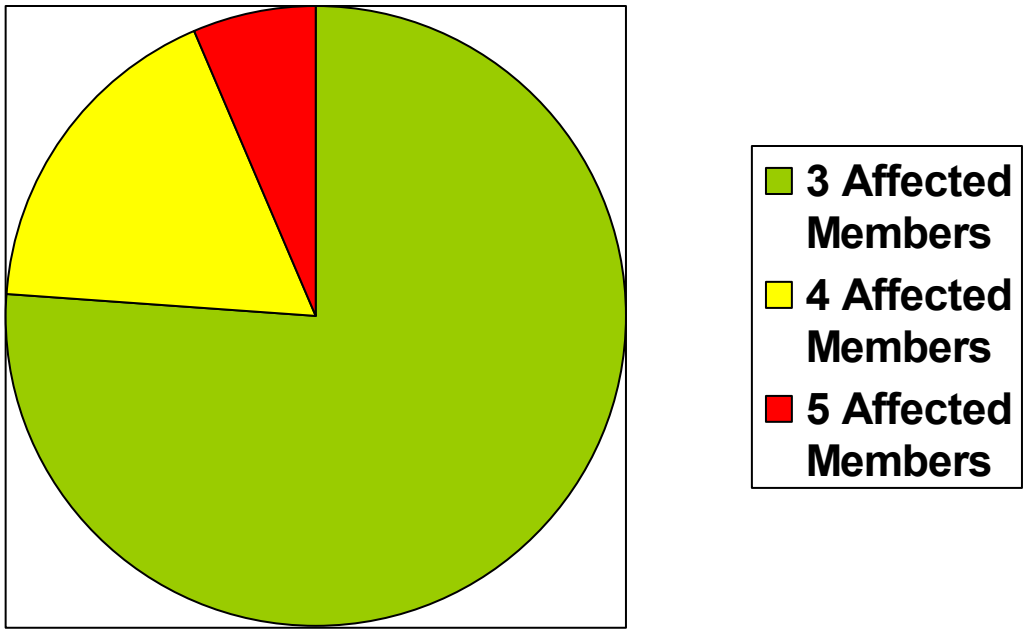

Figure 3.2 Pie chart representing number of family members affected by PD for the 46 proband subjects recruited to this study.

3.4.3 Pedigrees

Figure 3.3 gives examples of the pedigrees recorded for four of the families with three or more affected members, other pedigrees can be found in chapters 4 and 5 and appendix D.

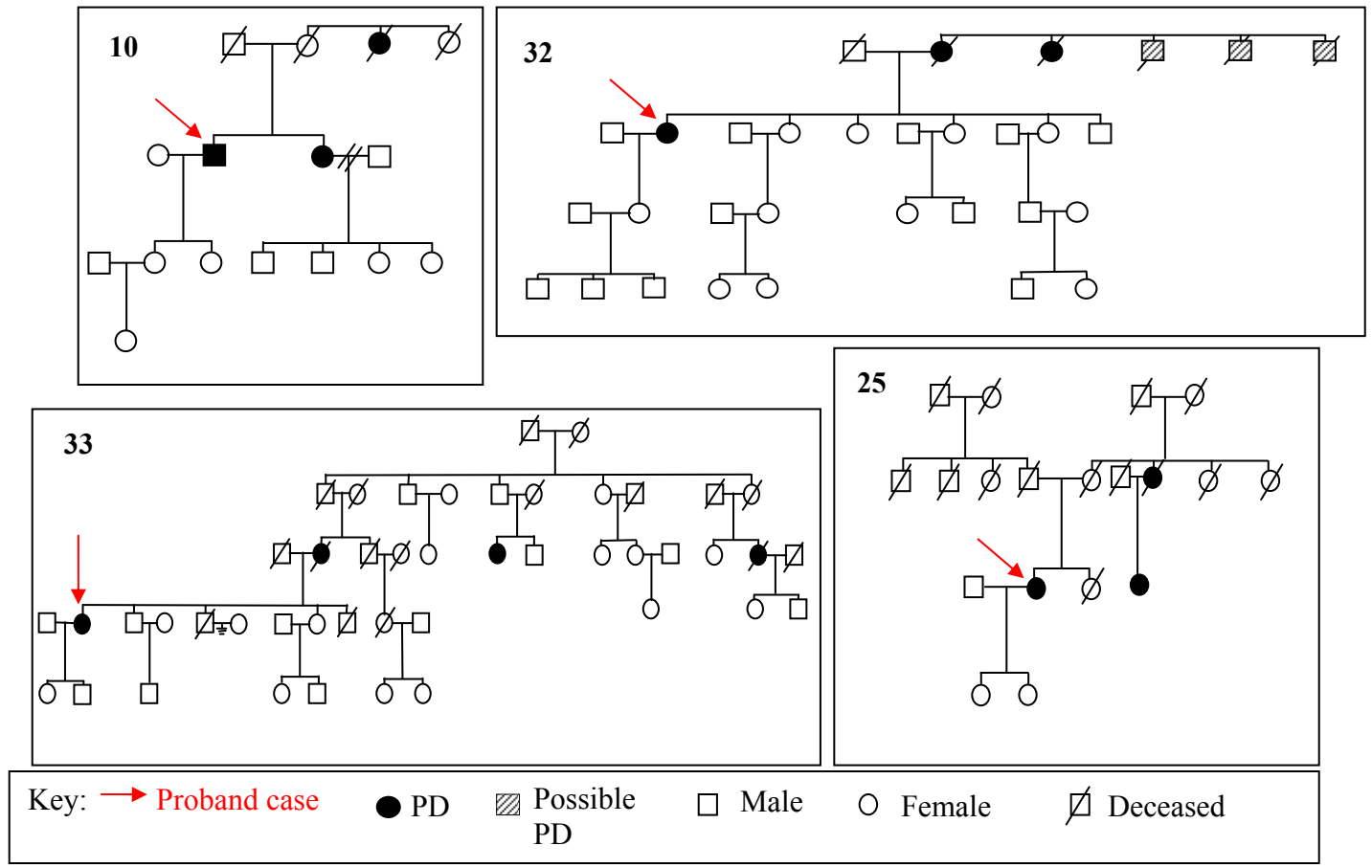

Figure 3.3 Example pedigrees of PD families with three or more affected members.

3.4.4 Demographics and clinical characteristics

The demographics and clinical characteristics of the 46 proband cases from families containing three or more members affected by PD are listed in tables 3.1 to 3.5, with the numbers of individuals affected by PD in each family presented in figure 3.2. The continuous variables (age at evaluation, and age at disease onset) were found to be normally distributed. I compared my data to that available from previous studies, and where possible performed statistical analysis. However, as discussed below, unless stated no statistically significant differences were identified, probably due to the small group size of my fPD cohort.

	3 or more affected family members
	Mean (SD)
Age at evaluation	68.0 (11.3)
Age range	39-89
Age at symptom onset	57.9 (12.6)
Age range	30-78
	Number (%)
Gender Male	27 (59)
Female	19 (41)
Ethnicity Caucasian	45 (98)
Other	1 (2)
Handedness Right	36 (86)
Left	6 (14)
F/H other Y	14 (30)
neuro.conditions N	32 (70)

Table 3.1 Demographics and clinical characteristics of fPD proband cases (a).

	3 or more affected family members
Initial motor symptom (s)	*Number* (%)
Tremor	27 (61)
Bradykinesia	8 (18)
Rigidity	9 (20)
Gait difficulty	3 (7)
Micrographia	7 (16)
Other	6 (14)
Initial motor symptom location	*Number* (%)
Right arm	17 (39)
Left arm	18 (41)
Both arms	0 (0)
Right leg	5 (11)
Left leg	2 (5)
Both legs	3 (7)
Other	8 (18)

Table 3.2 Demographics and clinical characteristics of fPD proband cases (b).

	3 or more affected family members
	Mean (SD)
UPDRS III score	31.4 (14.8)
Hoehn and Yahr stage	2.8 (1.1)
Hoehn and Yahr stage	*Number* (%)
Stage 1	3 (7)
Stage 1.5	1 (2)
Stage 2	12 (26)
Stage 2.5	7 (15)
Stage 3	8 (17)
Stage 4	5 (11)
Stage 5	5 (11)
Missing	5 (11)
Parkinsonian features on examination	*Number (%)*
Bradykinesia	44 (96)
Rigidity	45 (98)
Tremor	30 (65)
Postural instability	39 (85)
Missing	1 (2)

Table 3.3 Demographics and clinical characteristics of fPD proband cases (c).

		3 or more affected family members
Other clinical features		*Number (%)*
Response to anti-PD therapy	Yes	41 (89)
	No	1 (2)
	No Rx	3 (7)
	Not recorded	1 (2)
Mean L-dopa dose, mg/day (SD)		636.8 (382.9)
Levodopa		34 (74)
Dopamine agonists		22 (48)
COMT Inhibitor		13 (28)
Other anti-PD Rxs		16 (35)
Dystonia	Yes	26 (57)
	No	13 (28)
	Not recorded	7 (15)
Dyskinesias	Yes	22 (48)
	No	18 (39)
	Not recorded	6 (13)
Dementia	Yes	10 (22)
	No	36 (78)

Table 3.4 Demographics and clinical characteristics of fPD proband cases (d).

	3 or more affected family members
Other clinical features	*Mean (SD)*
MMSE	28.0 (2.8)
UPSIT Score	18.4 (6.8)
Range	9-32
Missing data	20
Male UPSIT Score	15.4 (5.4)
Male Range	9-25
Missing data	12
Female UPSIT Score	23.3 (6.0)
Female Range	14-32
Missing data	8

Table 3.5 Demographics and clinical characteristics of fPD proband cases (e).

3.4.5 Epidemiological survey

The epidemiological data obtained on the 46 proband cases from families containing three or more members affected by PD are listed in tables 3.6 and 3.7.

		3 or more affected family members
		Number (%)
Well water consumption	Yes	8 (17)
	No	31 (67)
	Not recorded	7 (15)
Head injury	Yes	3 (6)
	No	38 (83)
	Not recorded	5 (11)
Meningitis or encephalitis	Yes	0 (0)
	No	41 (89)
	Not recorded	5 (11)
Smoking	Never	24 (52)
	Yes or Ex	19 (41)
	Not recorded	3 (7)
		Mean (SD)
Pack years (smokers or ex smokers)		16.9 (13.2)

Table 3.6 Epidemiological data on fPD proband cases (a).

		3 or more affected family members
		Number (%)
Toxin exposure	Yes	11 (24)
	No	28 (61)
	Not recorded	7 (15)
Lived most of life	Town	27 (59)
	Country	10 (22)
	Both	5 (11)
	Not recorded	4 (9)
Occupation	Manual	13 (28)
	Non manual	27 (59)
	Farmer	3 (7)
	Not recorded	3 (7)
Tea or coffee drinker	Yes	16 (35)
	No	1 (2)
	Not recorded	29 (63)
		Mean (SD)
No. cups per day (tea or coffee drinkers)		4 .9 (1.2)

Table 3.7 Epidemiological data on fPD proband cases (b).

3.5 Results of the sPD study

A total of 697 unselected PD cases from four different collections were available for this part of the study. There were 357 subjects from the PD GEN study, 254 subjects from Birmingham PD study, 57 subjects from the Neurodegenerative Disorders study, and 29 subjects from the PINE study. The demographics of the 697 subjects from are given in table 3.8. Where available, data on family history of PD is given, as are the initial presenting symptoms for subjects from the PINE study.

	PD GEN study	B'ham PD study	NDD study	PINE study
	Mean (SD)	*Mean (SD)*	*Mean (SD)*	*Mean (SD)*
Age at evaluation	72.6 (9.1)	62.7 (11.5)	67.5 (10.3)	73.1 (11.8)
Age range	47-102	31-90	45-86	42-95
Age at symptom onset	67.2 (9.2)	57.2 (13.9)	55.6 (9.7)	71.9 (11.7)
Age range	40-99	18-85	39-72	42-95
	Number (%)	*Number (%)*	*Number* (%)	*Number* (%)
Gender Male	236 (66)	151 (59)	34 (60)	15 (52)
Female	121 (34)	103 (41)	23 (40)	14 (48)
Ethnicity Caucasian	346 (97)	251(99)	54 (95)	29 (100)
Other	11 (3)	3 (1)	3 (5)	0 (0)
Family Yes	52 (15)	79 (31)	13 (23)	7 (24)
history of PD No	233 (65)	77 (30)	25 (44)	22 (76)
Not recorded	72 (20)	98 (39)	19 (33)	0 (0)
Initial motor symptoms	*Number* (%)	*Number* (%)	*Number* (%)	*Number* (%)
Tremor				17 (59)
Bradykinesia				3 (10)
Rigidity				0 (0)
Gait difficulty				4(14)
Micrographia				0 (0)
Other				5 (17)

Table 3.8 Demographic & clinical characteristics of 4 sPD cohorts.

The continuous variables (age at evaluation, and age at disease onset) were found to be normally distributed and were investigated with ANOVA. This revealed significant differences between the four cohorts for both age at evaluation and age of disease onset (F-value 47.806, P-value <0.001, 3 degrees freedom and F-value 38.744, P-value <0.001, 3 degrees of freedom). Subsequent investigation was with Hochberg's GT2 Post Hoc analysis and independent *T*-tests and this is presented below in tables 3.9 and 3.10.

Cohort	PD GEN study	B'ham PD study	NDD study	PINE study
PD GEN study		$p<0.0001$*	$p<0.0001$*	$p=0.7814$
B'ham PD study	$p<0.0001$*		$p=0.0040$*	$p<0.0001$*
NDD study	$p<0.0001$*	$p=0.0040$*		$P=0.0259$*
PINE study	$p=0.7814$	$p<0.0001$*	$P=0.0259$*	

Table 3.9 Statistical analysis (independent *T*-tests) of the comparison in age at evaluation between four sPD cohorts. *= statistically significant difference.

Cohort	PD GEN study	B'ham PD study	NDD study	PINE study
PD GEN study		$p<0.0001$*	$p<0.0001$*	$P=0.010$*
B'ham PD study	$p<0.0001$*		$p=0.4099$	$p<0.0001$*
NDD study	$p<0.0001$*	$p=0.4099$		$p<0.0001$*
PINE study	$P=0.010$*	$p<0.0001$*	$p<0.0001$*	

Table 3.10 Statistical analysis (independent *T*-tests) of the comparison in age at disease onset between four sPD cohorts. *= statistically significant difference.

χ^2 analyses of the categorical variables showed no significant difference between gender or ethnicity in any of the four sPD cohorts (Pearson Chi-Square=4.626, p=0.201, 3 degrees of freedom and Pearson Chi-Square=4.943, p=0.176, 3 degrees of freedom). However, there was a smaller proportion of subjects from the PD GEN cohort (15%) who reported a family history of PD, compared to 24% of subjects in the PINE cohort, 31% of subjects in the Birmingham PD cohort and 23% of subjects in the NDD cohort. Despite missing data for the PD GEN, Birmingham PD and NDD studies (20%, 39% and 33% respctively) χ^2 analyses revealed there to be a significant difference in family history of PD between the four sPD cohorts (Pearson Chi-Square=85.643, p<0.0001, 3 degrees of freedom). Further analysis between individual cohorts revealed that the only statistically difference was between the PD GEN and Birmingham PD cohorts (Pearson Chi-Square=50.669, *p*<0.0001, 1 degree of freedom).

3.6 Discussion of demographic and clinical characteristics of the fPD cohort

I will first consider the demographics and clinical characteristics of my cohort of 46 proband subjects with fPD.

3.6.1 Demographics

I recruited 46 proband cases from UK families with three or more members affected by PD, with a geographical bias towards the West Midlands (see figure 3.1). This bias was because I was based in this region and received a larger number of referrals from colleagues within the region. This may be important when considering the generalisability of the results of this study to the UK as a whole.

3.6.1.1 Number of affected family members

With regard to the number of affected family members, there is data available from two previous studies of fPD (with AD inheritance) for comparison. In the first study 72% of the families had 2-5 known affected individuals, 20% had 6-10 affected individuals and 8% had more than 10 affected individuals [293]. In the second study 58 % of the families had 3 or more affected members and 42% of the families had 2 individuals with PD [280]. However, these data are not directly comparable as one of the original entry criteria to my study was for families to have 3 or more affected individuals with PD. The proportion of families with different numbers of affected individuals clearly varies between different studies, probably reflecting differing recruitment criteria.

In the majority of the families recruited to my study the proband had self-reported their family history of PD. This so called 'family history method' is the method used by most studies of

fPD. Where possible I examined the reportedly affected relatives of the proband for signs of PD, or other neurological disease. However, in most cases the other affected family members were deceased at the time of the study, also in some cases it was not possible to contact the affected relatives. In addition, copies of death certificates of deceased affected family members were obtained in ten cases, to help confirm the diagnosis of PD reported by the proband.

This raises for discussion the accuracy of the family history data which I obtained. Several studies have compared the validity of the 'family history method' in first-degree relatives to the study of each relative, the 'family study method'. The findings were generally similar, in that reliable and accurate family history information on PD in first degree relatives can be obtained. However, there was some over-reporting of PD in relatives who were not affected and the family history information was more reliable when a more conservative diagnostic algorithm was used [540], [541], [542]. Although in some cases in my study, information on relatives other than first degree relatives was given, this suggests that the reliability of the data I obtained should be good.

3.6.1.2 Age at symptom onset and gender

The demographics of a PD cohort will depend on a number of factors, but most notably in this study on the fact that the subjects were selected for study because of their strong family history of PD. This makes direct comparison between demographic data from my cohort and demographic data from the clinic-based cohorts listed in the introduction difficult. Nevertheless, the basic demographics of this PD cohort appear comparable to those from a number of different clinic based populations of PD presented in table 3.11.

Author	Number of subjects	Age (years)	Symptom onset (years)	% Male
Uitti et al. (2005)	840	70.0	61.0	68
Jankovic et al. (2001)	297	61.6	55.1	61
Goetz et al. (2000)	100	72.0	58.0	53
Scigliano et al. (1996)	335	59.2	54.8	52
Hely et al. (1995)	125	62.6	60.7	55
Starkstein et al. (1992)	105	66.0	56.3	60
Diamond et al. (1990)	371	60.5	55.8	67
Jankovic et al. (1990)	800	61.1	59.0	66
Current study	*46*	*68.0*	*57.9*	*59*

Table 3.11 Demographics of clinic-based PD populations. Adapted from Uitti et al. 2005 [17]

The data on ages at evaluation and symptom onset, and the slight predominance of males in this cohort is all in general agreement with the previously reported data (see table 3.11). The age of disease onset in the fPD cohort (57.9) does appear to be younger than that reported in the largest population based study (61.0), although this difference is not statistically significant (P=0.06). The difference in proportion of males versus females between these two cohorts is also not statistically significant (p=0.19). These differences may not have been statistically significant because of the small group size of my fPD cohort.

The general similarity in the age of symptom onset data from my fPD cohort to the population-based studies does suggest that in this aspect at least the cohort is more similar to sPD than some forms of fPD (such as *Parkin* related PD) which tend to have a younger age of onset [234], [235], [236]. One reason why fPD cases report an earlier age of disease onset may be that they are more aware of the symptoms of the condition, notice them earlier and self-refer. Interestingly, even though the majority of our fPD subjects self-referred themselves to my study, the age of symptom onset is still similar to that reported in sPD.

A more direct comparison of the demographics of my fPD cohort can perhaps be performed with the limited data available from three other recent studies of fPD in which cohorts of patients with fPD and apparently AD inheritance (in the case of the first two studies) have been recruited [293], [280], [26]. In addition a useful comparison can also be made with data from the two studies mentioned in section 3.1.1, which compared cohorts of patients with fPD and sPD [233], [520]. This data is presented below in table 3.12.

.

Author	Number of subjects	Symptom onset (years)	% Male
Mata et al. (2005)	100	53.0	65
Di Fonzo et al. (2005)	60	49.2	Not given
Nichols et al. (2007)	88	61.2	58
Carr et al. (2003)	26	60.1	69
Papapetropoulos et al. (2007)	50	61.1	58
Current study	*46*	*57.9*	*59*

Table 3.12 Comparative demographics of fPD cohorts.

Therefore, the gender distribution of 59% male and mean age of onset of 57.9 (12.6) years for my fPD cohort is generally comparable with the figures from previous studies of fPD. Where available data permitted comparison, differences were not statistically significant, although this may again be due to small group size.

3.6.1.3 Ethnicity

There was only one non-Caucasian patient recruited to my fPD cohort. Given the increasingly diverse ethnicity of the UK, with 7.9% of the population reporting as non-white at the 2001 census [543] there does appear to be an underrepresentation of non-Caucasians. This perhaps can be explained by two factors, first most patients self referred via advertisements in the media and internet. There may be a proportion of non-Caucasians whose first language is not English, especially amongst the age group associated with PD, and therefore these patients would be less likely to respond to advertisements in English. Second, different genetic variations and environmental exposures amongst different ethnic populations may give different risks of developing PD. Indeed there is evidence of ethnic variations in incidence of PD, recently Van Den Eeden et al. found that age and gender-adjusted incidence rates were highest amongst Hispanics, followed by non-Hispanic whites, Asians and then blacks [515].

3.6.1.4 Handedness

The data on handedness from this study shows that 86% of subjects were right handed. This fits with the general population figures in which 8-15% of people are found to be left-handed [517], [518]. Indeed in a single large study on a clinic-based sample of patients with PD 92% were found to be right handed [516].

3.6.1.5 Family history of other neurodegenerative diseases

As outlined in section 3.1.1, up to 25% of PD patients report a family history of neurodegenerative disorders [17]. In my study 30% of the patients reported a family history of neurodegenerative disorders, apart from PD, ranging from multiple sclerosis to motor neurone disease; the largest single condition reported being dementia. Given that all the subjects were selected because they had a family history of PD, and extensive family histories were taken, this finding is perhaps not surprising. Most of the probands were unable to confirm the type of dementia in their families, or describe the clinical features, however one explanation is that this dementia was associated with Lewy body pathology and hence this could be important to associate with subsequent genetic data from these families.

3.6.2 Clinical characteristics

A number of different phenotypic characteristics of this cohort of fPD patients have been presented in tables 3.2 to 3.5, which are discussed below.

3.6.2.1 Initial motor symptoms

In my study tremor was the most commonly reported initial motor symptom (61%). This was also the most commonly reported initial symptom in previous studies, including in fPD, at frequencies of 47%, 54% and 59% respectively [17], [41], [519]. In these studies bradykinesia was the next most common initial symptom, in my current study 18% reported their first symptom to be bradykinesia with 20% reporting rigidity. Furthermore, the upper extremities were the most frequently reported location for initial motor symptoms in my study (80%).

This tallies with data from the previous studies which also found upper extremities to be the most common site for initial motor symptoms (68% and 89% respectively) [17], [519].

It is important to point out that for a number of patients there may have been some recall bias as at time of recruitment to the study, as it was a number of years since their symptoms first began. This was minimised by referring to medical records to confirm the subject's reports in as many cases as possible. In addition a number of subjects reported more than one initial motor symptom and in this study these were all recorded, hence the total percentage is greater than 100%. The data from this part of the study provides further support that the PD phenotype of subjects in my fPD cohort is generally similar to the that which one would expect to find from a clinic based cohort of predominantly sporadic disease.

3.6.2.2 Severity of disease

In this study, the mean UPDRS III motor score was 31.4 and the mean modified Hoehn and Yahr stage was 2.8, on treatment. Although there is missing data on five patients, the data suggests that within this cohort there is an equal mixture of early stage (modified Hoehn and Yahr stage of 2.5 or less) and more advanced disease. In a recent study with a symptomatic disease duration of 7 years, compared to 10 in my study, the UPDRS III score on treatment was 30.9 and 73% of patients had a modified Hoehn and Yahr stage, on treatment, of 2.5 or less [17]. Furthermore in a study of fPD patients with a mean disease duration of 8.9 years, the mean UPDRS III score on treatment was 32.1 (24.0) and the mean modified Hoehn and Yahr stage, on treatment, was found to be 2.7 [520].

3.6.2.3 Clinical signs

The data on clinical features demonstrates that a very high proportion of probands have evidence of bradykinesia, rigidity and postural instability, whilst only 65% have tremor. In a previous study the proportion of patients with tremor was 66% for fPD patients, with 74% having rigidity, and 74% for sPD patients, with 82% having rigidity [520]. In another study only 4% of fPD patients had tremor, although in the same study only 8% of the sPD patients had tremor [233]. Whilst data on the other features of PD were not given in these two papers, there does appear to be a discrepancy between the higher percentage of patients with rigidity in my study, and the very small proportion of patients with tremor in the previous study.

There are several possible explanations for these apparent differences, first both the fPD and sPD cohorts in the Carr et al. study had very low frequencies of tremor and therefore it may be explained by methodological variations or the different populations from which the two studies recruited. The higher proportion of patients with rigidity in my study may be explained by the longer disease duration. Another explanation is that because the current study cohort is composed solely of patients with autosomal dominantly inherited PD these patients may have a different disease phenotype to the previously studied fPD patients.

3.6.2.4 Treatment

In my study, 89% of probands reported positive response to anti-PD therapy and the average daily L-dopa dosage was 636.8mg (SD 382.9). Comparison of this average daily dosage of L-Dopa to equivalent figures reported in previous studies is difficult as different methods have been used to calculate L-Dopa equivalencies. However in a recent study of a large cohort of PD patients (fPD and sPD) which used the same L-Dopa equivalency calculation, with an

average disease duration of 7 years, the average daily L-Dopa dose was 589.6mg [17]. The higher average daily dose seen in my study may be explained by the longer disease duration, although variations in prescribing patterns from country to country may also partly explain the difference. The relatively long disease duration of the patients in my study is also reflected by the data showing the proportions of patients receiving different PD medications, suggesting that a number of these patients have complex treatment regimens.

The proportion of patients in the current study who reported treatment-induced dyskinesias (48%) fits with the average daily dose of L-dopa given and the average disease duration of ten years. In two other recent studies 27% and 22% of fPD patients, compared to 37% and 24% of sPD patients respectivel, reported dyskinesias [233], [520]. The average disease durations in these studies were 5.9 years and 8.9 years for fPD and 8.5 years and 7.5 years for sPD respectively [233], [520]. Therefore the data on treatment and dyskinesias fits with a cohort of patients in which the average disease duration is 10 years and clinical features are typical of IPD.

3.6.2.5 Dementia

In this study 22% of probands were reported to have dementia. The dementia type was Parkinson's disease with dementia (PDD) as the onset in all cases had been more than one year after the onset of motor symptoms. The estimates of prevalence of dementia in PD from the literature are very variable, but 22% seems comparable to those of 24% to 31% quoted in a recent systematic review [55]. The mean MMSE score for proband cases in my study was 28.0, whilst in a recent study of fPD and sPD mean MMSE scores of 23.6 and 24.0 respectively were recorded [520]. Whilst the cognitive function of probands in the my study

may actually be better than expected, and could be explained by genuine differences in disease phenotype, and potentially disease mechanism, there is limited data available on similar cohorts of patients and any assumptions must be made with caution.

3.6.2.6 Olfactory dysfunction

In this study the fPD probands were found to have impaired olfactory function with a mean UPSIT-40 score of 18.4 (males 15.4 and females 23.3). These scores were similar to the recently reported mean score of 17.1 found in a cohort of British patients with IPD (mean age 70.6) and contrasts with the mean score of 27.6 found in normal age-matched controls [118]. The mean score is lower for the male subjects, which is as expected as normal values are known to be lower in men and decrease with age [483]. Wenning et al. also recorded UPSIT scores in 118 patients with IPD and 123 healthy control subjects. They found a marked impairment in olfactory function the IPD group compared to controls [544]. Some subjects in my study had scores within the normal range, this is to be expected as up to 30% of PD patients have normal smell despite Lewy body disease [110].

Numerous other studies of olfactory dysfunction in PD have been performed, including a study in which the UPSIT scores of subjects with 'malignant' PD were significantly worse than those of subjects with 'benign' PD [114]. In another study of fPD, olfactory dysfunction was found to be a phenotypic characteristic of familial parkinsonism [545]. In a recent comparison of tremor-dominant and standard PD no significant difference in olfactory dysfunction was found. However, a subgroup of tremor-dominant PD with a family history of tremor had less olfactory dysfunction than those without a family history or those with standard PD. The authors proposed that patients with a family history of tremor may represent

a different disease process even though, apart from differences in olfaction, they are clinically similar to other patients with tremor-dominant parkinsonism [546].

In my study there was missing data on 20 subjects (43%). This is mainly because these subjects were unable to complete the test. In the majority of cases it was the more severely affected subjects who were unable to complete the test and this potential bias needs to be considered. However, the olfactory function of the subjects with fPD in this study cohort was reduced and is similar to that identified in a cohort with IPD, perhaps indicating similar disease mechanisms between the two groups. The individual subject UPSIT-40 scores will be useful to correlate with molecular genetic findings later in this study.

3.6.3 Summary of demographic and clinical characteristics of fPD cohort

Whilst taking into consideration that this was not a population-based study, and that a relatively small number of subjects were studied, several important conclusions can be drawn from this part of the study. First I successfully recruited a large cohort of fPD patients with AD inheritance, along with appropriate demographic and clinical data. Second the demographics and clinical characteristics of my cohort appear very similar to those of the fPD cohorts which have been previously described in the literature, and indeed to those of the much larger cohorts of PD patients recruited as part of population based studies. The data also suggests that the typical phenotype of patients within my cohort is more similar to the sPD phenotype rather than the typical 'slowly progressive' fPD phenotype which has been reported with fPD associated with certain genetic variations, such as *Parkin*, *PINK1* and *DJ-1* [234], [235], [300], [236], [227], [320], [321], [342], [344], [345]. Therefore this cohort of subjects with fPD can be used for molecular genetic analyses and data will be generalisable.

3.7 Discussion of epidemiological survey of fPD

I will now consider data on epidemiological factors, which may be important for the aetiology of PD, which was collected as part of my study of fPD, these results can be found in tables 3.6 and 3.7 above. Before discussing the data it is important to point out that unlike the other case-control epidemiological studies discussed here and in the introduction, my study was designed as a clinical and molecular genetic study of fPD, and not as a population-based study of epidemiological factors in PD. The epidemiological data was collected primarily for future reference to any genetic variations which might be identified in this cohort, and therefore discussion of the data in this section can only be observational, looking for potential trends.

Aside from caffeine consumption, which is covered separately below, up to 15% of data for each of the categories is missing or not recorded, in the majority of instances this was because the subject was unable to give accurate answers.

3.7.1 Smoking and caffeine consumption

Smoking is one of the most studied risk factors for PD with an apparent reduced risk of PD amongst smokers [200], [201], [202]. However, 41% of the probands in my study were smokers or ex-smokers, with an average 16.9 pack years. Data from the 2003 Health Survey for England showed that 27% of men and 24% of women were current smokers [547]. Further, self-reported cigarette smoking figures may underestimate true smoking prevalence by approximately 2.8% [548]. Therefore the percentage of smokers or ex-smokers amongst patients with fPD in my study is perhaps higher than would be expected.

Whilst less well studied than smoking, there is some data suggesting an inverse relationship between caffeine consumption and risk of developing PD [201], [526], [527], [528], [529],

[530]. One might therefore expect to find a low proportion of tea or coffee drinkers in my cohort of fPD patients. Of the available data, virtually all the probands were tea or coffee drinkers. This is the opposite of the trend that might be expected from previous studies, but given that 63% of the data on caffeine consumption is missing it is impossible to draw any firm conclusions. The data is missing as enquiry into caffeine consumption was not added to the study protocol until after a number of the subjects had already been recruited.

3.7.2 Occupation

Occupation can give important information as to which potentially hazardous environmental factors a subject may exposed. For example farming may be surrogate marker for pesticide exposure and welding may be a surrogate marker for heavy metal exposure. My data shows that 59% of subjects had non-manual occupations (e.g. clerical work, teacher). There were only a few subjects who were farmers, or worked on farms. However, there is likely to be a bias in this cohort with regard to occupation. This is the result of the bias of recruitment from the West Midlands, and also the methods of recruitment, such as via the internet, which might significantly influence the socio-economic composition of the group. Some of the individual exposures are dealt with separately below, but these data appear to suggest that in my cohort of fPD subjects occupational-related environmental exposures could be important aetiological factors in about one third of subjects. This may be important when considering the results of genetic analyses of these subjects, but it is not really possible to generalise these findings even to other similar cohorts of AD fPD subjects.

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3.7.3 Rural living, farming, well water consumption and toxin exposure

As outlined in the introduction, there is overlap with these three risk factors, which makes individual analysis difficult. There is some evidence in support of an increased risk of PD in subjects exposed to pesticides [155], [158]. The evidence in support of an increased risk of PD from other environmental toxins is less clear [173], [174].

In my fPD cohort, only 22% reported living in the country for most of their lives and 11% reported to have lived in both town and country equally. These findings tally with the finding that relatively few probands were farmers. Furthermore, only 17% of probands reported drinking well water for more than 6 months and only 24% of probands reported toxin exposure. Patients were questioned about any exposure that they might regard as potentially toxic, but in most cases it was pesticide exposure that was reported. Thus within this cohort there was a minority of patients who through rural living, well water consumption, and occupation, may have been at exposed to putative toxic compounds.

Recall bias may be important, particularly with regard to well water consumption and toxin exposure. Indeed, the geographical bias towards the West Midlands is also a potentially crucial factor in explaining these findings. Once again, however, this data may prove important later when putting the findings of genetic analyses into the context of the whole disease process.

3.7.4 Head injury and infections

In my study only 3 (6%) probands reported a head injury that had knocked them unconscious. Furthermore there was not a single subject who reported a history of cerebral infection. Thus, whilst interpreting the data with caution, head injury and infection do not appear to be

important environmental risk factors in the development of PD in this cohort of subjects with fPD.

3.7.5 Summary of the epidemiological survey of fPD

Whilst taking into consideration that this was not a population-based study, the over-representation of subjects from the West Midlands, and the recall bias which is inherent in this type of retrospective study, some conclusions can be drawn from this epidemiological survey. First, that head injury and cerebral infection do not appear to be significant risk factors for PD in this cohort. Second, similar proportions of subjects reported a history of toxin exposure, rural living and well water consumption, ranging from 17-24%. At least in the context of this fPD cohort, if these risk factors are important in the aetiology of PD they would only appear to be so in up to a quarter of cases.

3.8 Discussion of sPD study

I will now consider the demographics of four cohorts of patients recruited from around the UK with PD, unselected for family history of the condition, and also the clinical characteristics of one of these cohorts. The majority of these patients had apparently sPD.

3.8.1 Demographics

There have been a number of studies of PD in which the basic demographics of large cohorts of patients have been discussed. The cohorts of patients discussed in this section are from four different studies and the results have been listed separately. As such the data will be

considered and put into the context of the literature as four separate collections, but discussed together below.

3.8.1.1 Age at evaluation and symptom onset

The average age at evaluation is 62.6 years for the PD GEN study, 62.7 years for the Birmingham PD study, 67.5 years for the NDD study and 73.1 years for the PINE study. The average age at symptom onset is 67.2 years for the PD GEN study, 57.2 years for the Birmingham PD study, 55.6 years for the NDD study and 71.9 years for the PINE study. There were statistically significant differences between the four cohorts for both age at evaluation and age of disease onset (see tables 3.9 and 3.10). Whilst the age at symptom onset is comparable to those reported in the literature (see table 3.11) for both the Birmingham PD and NDD studies, it is 6.2 years older than the next highest figure for the PD GEN collection and 10.9 years older than the next highest figure for the PINE study [17]. Indeed comparison of the age at symptom onset between the PD GEN cohort and that reported in the largest population based study (61.0) revealed a statistically significant difference ($P<0.0001$).

The subjects in all four studies were not recruited on the basis of age or family history. The observed differences in age at symptom onset, and age at evaluation, of these cohorts is likely to be due to the nature of the clinics from which the patients are recruited. The subjects in the PD GEN study were recruited from general neurology clinics, movement disorder clinics and the clinics of elderly care physicians from throughout the UK. So this cohort of subjects should be most representative of a community-based sample of PD patients.

The subjects in the Birmingham PD and NDD studies were for the most part been recruited from specialist movement disorder clinics at two major neuroscience centres in the West

Midlands and are less representative of a community based sample. It is expected that at least some of these subjects may have younger onset of disease and perhaps even atypical, or difficult to diagnose disease.

The PINE cohort was recruited from a single movement disorder clinic at a major neuroscience centre in Aberdeen. The relatively late age of symptom onset may be explained by the small numbers of patients in this cohort or may be a peculiarity of the demographic composition of that single clinic.

3.8.1.2 Gender

The percentage of male subjects was 66% in the PD GEN study, 59% in the Birmingham PD study, 60% in the NDD study and 52% in the PINE study. No statistical difference was detected between the four cohorts. These figures are all comparable to those from the literature (see table 3.11). Indeed the difference in proportion of males between the PD GEN cohort and the largest population based cohort (68%) was not statistically significant (p=0.46).

The reasons for the apparent predominance of males in these studies are unclear, it may be that male gender itself is a risk factor or it may be a surrogate for another risk factor to which men might have a greater exposure. Another explanation comes from the *in vitro* evidence that oestrogen may be neuroprotective [508]. Furthermore, the isolation of a PD susceptibility gene on the X chromosome could potentially explain the higher risk of PD in men [364]. Finally mitochondrial dysfunction has been reported in PD [549], [550], [551], [323] and as the sex ratio is similar to that in Leber's hereditary optic neuropathy, which results from disruption of the mitochondrial electron transport chain, mitochondrial dysfunction might be a possible explanation.

3.8.1.3 Ethnicity

Virtually all the subjects were Caucasian, ranging from 95% in the NDD study to 100% in the PINE study. No statistical difference was detected between the four cohorts. There is evidence that ethnic variations in incidence of PD may exist, with prevalence rates lower in Asians than whites (non-hispanic or hispanic) and lower rates in blacks than those in non-hispanic whites [509], [510], [511], [512], [513], [514], [515]. Most recently Van Den Eeden et al. found that age and gender-adjusted incidence rates were highest amongst Hispanics, followed by non-Hispanic whites, Asians and then blacks [515]. Given that at the 2001 census 7.9% of the UK population, and 11.2% of the West Midlands population, was non-white [543] there may still be an underrepresentation of non-Caucasians in these cohorts, which could represent a bias in subject recruitment due to language difficulties.

3.8.1.4 Family history of PD

In the PD GEN study 15% of subjects report a family history of PD, of which 98% have one, and 2% have two other affected family members. Of these other affected family members, 60% are first degree relatives and 40% are other more distant family members. In the PINE study 24% of subjects report a family history of PD, of which 57% have one, and 43% have two other affected family members. Unfortunately there is some missing data for the Birmingham PD and NDD studies. However, 31% of subjects in the Birmingham PD study and 23% of subjects in the NDD study report a family history of PD. Indeed there was a statistically significant difference detected between the PD GEN and Birmingham PD cohorts (p<0.0001). The figures on reported family history of PD in the literature range from 16-19% [17], [41]. The higher figures reported particularly in the Birmingham PD study, may reflect in

part the recruitment from specialist movement disorder clinics at major neuroscience centres, where a higher proportion of patients might be expected to have a positive family history of PD.

3.8.1.5 'Community-based' sample?

When studying sPD one of the major aims is to ensure that the study cohort is as representative of a 'community-based' IPD cohort as possible. The major reason for this is to try to ensure that when genetic analyses are performed any variations which may be identified are as relevant as possible to the 'typical' IPD seen by general practitioners, physicians and neurologists rather than the more 'atypical' cases that might be seen in specialist movement disorder clinics.

To this end the PD GEN collection, recruited from many non-specialist clinics throughout the UK, may be most appropriate for this type of study. It is interesting that the diagnosis re-evaluation rate, to date, for subjects in the PD MED study is 2.2% (35 out of 1592 cases) [552]. This is much lower than the expected, and widely quoted, 8-10% rate [553], [554], suggesting a high diagnostic accuracy rate.

The demographics of the PD GEN cohort are in general terms similar to those previously reported in the literature. Indeed the mean age at evaluation in a recent community-based study of PD in London was also 72 years, with a mean age of disease onset of 69.2 years [76]. Statistical analysis revealed no significant difference between the ages at evaluation (p=0.5488), although I was unable to perform statistical analysis of age at disease onset between the PD GEN cohort and this publsihed data. Thus generalisable data can be generated from this study of sPD.

3.8.2 Clinical characteristics

Clinical data is available for the PINE study, with initial motor symptoms listed in table 3.8. The most common symptom was reported as tremor (59%) followed by other (17%). From the literature, tremor has also been reported as the most common initial motor symptom in two previous studies (49% and 59% respectively) followed by bradykinesia (21% and 16% respectively) [17], [41]. Therefore whilst only relatively few patients are being considered, the most common initial motor symptom tallies with that reported in the literature.

3.8.3 Summary for the sPD study

Taking into consideration the relatively small numbers of subjects in the NDD and PINE cohorts, several conclusions can be drawn from this part of the study. First the demographics of all four cohorts appear similar to those which have been previously described in the literature. Second the 357 subjects from the PD GEN study and are most representative of a 'community-based' sample of IPD with appropriate demographic data available. This cohort can be used for molecular genetic analyses of sporadic PD and the data will be generalisable.

3.9 Comparison of PD GEN and fPD cohorts

A brief comparison of the demographics of the sPD and fPD cohorts to be used in the genetic analyses in future chapters may be informative, as presented in table 3.13.

	PDGEN study	**fPD study**	**P value**
	Mean (SD)	*Mean (SD)*	
Age at evaluation	72.6 (9.1)	68.0 (11.3)	=0.006
Age at symptom onset	67.2 (9.2)	57.9 (12.6)	<0.0001
	Number (%)	*Number (%)*	*P value*
Gender Male	236 (66)	27 (59)	=0.3276
Female	121 (34)	19 (41)	
Ethicity Caucasian	346 (97)	45 (98)	=1.000
Other	11 (3)	1 (2)	

Table 3.13 Comparison of the demographics of the PD GEN and the fPD cohorts.

On comparing the data between the PD GEN and fPD cohorts, there is a statistically significant difference in both age at evaluation and symptom onset between the two groups, with both being younger in the fPD group. The small observed differences in gender and ethnicity between the two cohorts are not ststistically significant.

A useful comparison can be made with data from three studies which compared cohorts of patients with fPD and sPD, although in the first two of these studies below the two cohorts were age matched (also gender matched in the Papapetropoulos et al. study). Carr et al. (2003) found that 69% of their fPD patients were male and the mean age of disease onset was 60.1, 67% of their sPD patients were male [233]. Papapetropoulos et al. (2007) found that 58% of their fPD patients were male and the mean age of symptom onset was 61.1 [520]. Baba et al.

(2006) found that 66% of their sPD patients were male, and 65% of their fPD patients (from two large fPD kindreds) were male, with a mean age of symptom onset of 56.8 years (range 25-73) in the first kindred and 52.9 years (range 31-71) in the second kindred [519]. Therefore there were also some observed differences between the sPD and fPD cohorts in these studies.

The compositions of the PD GEN and fPD cohorts, the potential biases involved and the methodological considerations, have previously been discussed separately. However it may be important to re-consider the differences in demographic characteristics between the two cohorts later when discussing data from genetic analyses.

3.10 Conclusions

The aims of this chapter were to obtain and discuss demographic, phenotypic and epidemiological data on a large cohort of subjects with fPD and to discuss demographic data on several cohorts of subjects with sPD. Overall the demographics and clinical characteristics of the fPD and sPD cohorts collected for this study appear similar to those previously described in the literature and thus the results are generalisable.

I have explained that the fPD cohort is self-referred rather than population based. Nevertheless, the cohort provides a valuable resource for phenotype-genotype correlations in fPD. The PD GEN cohort appears to be closely representative of a 'community-based' PD collection and the data presented here justifies the use of this cohort for phenotype-genotype correlations in sPD.

4 A Novel *GTP Cyclohydrolase 1* Mutation in a British Kindred with Variable Phenotypes Ranging from Parkinson's Disease to Dopa-Responsive Dystonia

4.1 Introduction

In chapter 3 data was presented and discussed on 46 families with 3 or more affected members, compatible with autosomal dominant (AD) inheritance, that were successfully recruited to this study. In this chapter a more detailed study of a single family is presented. In this family there is variety and overlap of clinical features, presentations and diagnoses. Affected subjects display phenotypes ranging from PD to slowly progressive parkinsonism to Dopa responsive dystonia (DRD). The different clinical phenotypes in this family may be due to variable expression of a novel missense mutation within the GTP cyclohydrolase 1 gene. The first section of the chapter provides some background information on the clinical and genetic features of DRD and the role of functional neuro-imaging. As pathological gastrointestinal tissue has been examined in two subjects, the pathological changes in the enteric nervous system which are associated with PD are also outlined.

4.1.1 Dopa responsive dystonia (DRD)

The conditions which can mimic IPD have already been outlined in the introduction. One of these is the movement disorder of Dopa-responsive dystonia (DRD). Dystonia is condition that causes sustained muscle contractions, repetitive twisting movements, and abnormal postures of the trunk, neck, face, or arms and legs [555]. Tremor can also be present. Dystonia

can be classified in several ways, including on the basis of the genetic factors involved. The loci DYT1 to DYT15 have been identified to date [556].

DRD is an inherited metabolic disorder, now classified as DYT5, which results in dopamine deficiency in the basal ganglia. It was first described by Segawa in 1976 as an AD dystonia [557] and is also sometimes referred to as hereditary progressive dystonia with marked diurnal fluctuation (HPD). The incidence has been estimated to be between 0.5 and 1 per million, although this may be an underestimate due to misdiagnosis [558], and it is reported as being two to four times more common in females. DRD usually presents in childhood, most commonly between the ages of 4-8, although it has also been reported to manifest as late as the sixth decade. The typical presentation of DRD is of a lower limb dystonia with diurnal variation, although the spectrum of symptoms can be broad, even occurring as a predominantly parkinsonian condition [559], [560], [558], [561], [562], [563], [564], [557]. Indeed the presentation tends to be less severe with a later age of onset [565]. Interestingly, marked intra-familial variability of the clinical manifestations of DRD have also been observed [566], [567].

Most subjects with DRD have an excellent and sustained response to low dose Levodopa (50-100mg), and this is an important clinical diagnostic test. In comparison to PD there is also a very low incidence of dyskinesias and the 'on-off phenomena' associated with long-term Levodopa therapy [16]. This suggests a functional rather than anatomical lesion [568] and indeed post-mortem data on subjects with DRD indicates that there is little morphological change in the substantia nigra and striatum [569], [570]. Positron emission tomography (PET) studies have also shown minimal abnormalities in pre- and postsynaptic functioning neurons [571], [572].

Heterozygous mutations in the *GTP cyclohydrolase I* gene *(GCH1)* are the most common cause of AD DRD [573], [574], [575] and are responsible for approximately 50% of cases. *GCH1* is located on chromosome 14 (14q22.1-q22.2) and encodes the 32-kDa (250 amino acids) guanosine 5'-triphosphate cyclohydrolase 1 (GTPCH1) protein (EC3.5.4.16). This is the rate-limiting enzyme in the synthesis of tetrahydrobiopterin (BH4) from GTP [576], see figure 4.1 below. BH4 is a co-factor for tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine. The resulting deficiency of the BH4 cofactor presumably results in decreased phenylalanine hydroxylase activity, although serum phenylalanine levels are not usually elevated in DRD [577]. In subjects with DRD an oral phenylalanine-loading study can be used as a clinical diagnostic test. It increases the requirement for phenylalanine-hydroxylase activity, thus unmasking a partial BH4 deficiency and leading to elevated serum phenylalanine levels [578], [579]. Homozygous mutations in *GCH1* have been recognised for some time [580], [581], [582] and cause a more severe phenotype of a progressive neurological condition resembling atypical phenylketonuria [565].

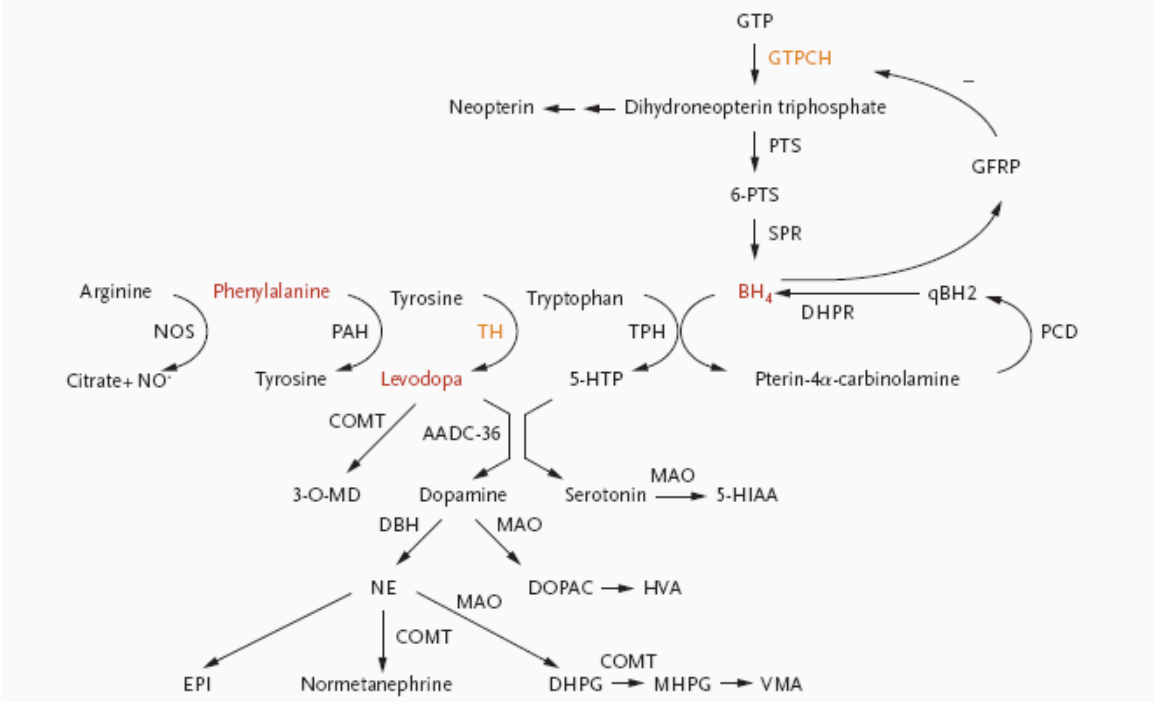

Figure 4.1 Synthesis and catabolism of the neurotransmitters catecholamine and indolamine. GTPCH1 is the rate-limiting enzyme in the synthesis of tetrahydrobiopterin (BH4). BH4 has a central role as cofactor in metabolic pathways, including the synthesis of dopamine, in the hepatic metabolism of phenylalanine to tyrosine, and in the production of the serotonin precursor 5-hydroxytryptophan (5-HTP). Therefore, the downstream consequences of GTPCH1 deficiency include alterations in a number of pathways that are important in biological and neurological homeostasis and function. A deficiency of dopamine underlies the movement disorder seen in dopa-responsive dystonia and can explain the remarkable clinical response to dopamine replacement therapy. The multiple metabolic consequences, secondary to GTPCH1 deficiency, may explain the more complex phenotype often seen in dopa-responsive dystonia and beyond that explained by simple dopamine deficiency. Abbreviations: PTS denotes pyruvoyl-tetrahydropterin synthase, GFRP-GTPCH feedback regulatory protein, SPR sepiapterine reductase, DHPR dihydropteridine reductase, NOS nitric oxide synthase, qBH2 quinoid dihydropterin, NO nitric oxide, 5-HTP 5-hydroxytryptophan, PCD pterin, COMT catechol-*o*-methyltransferase, AADC aromatic L-amino acid decarboxylase, B6 pyridoxine, 3-O MD 3-O-methyldopa, 5-HIAA 5-hydroxy-3-indole acetic acid, NE norepinephrine, DOPAC dihydroxyphenylacetic acid, HVA homovanillic acid, EPI epinephrine, DHPG dihydroxyphenyl glycol, MHPG 3-methoxy,4-hydroxyphenolglycol, and VMA vanilmandelic acid. Taken from Venna et al. 2006 [16].

There are reports of the nuclear localisation of GCH1 [583], as well as the involvement of GCH1 and BH4 in cellular differentiation [584], apoptosis [585] and mRNA stability [586]. BH4 also appears to increase the release of dopamine into neuronal synapses at high

concentrations [587]. Therefore it is likely that there are still proteins and cellular processes to be discovered that depend upon GCH1 and BH4 for activity or that control the sub cellular localisation of GCH1 or BH4, or both.

GCH1 contains six exons, and about 100 different mutations (missense, nonsense and frameshift) in the coding region or exon-intron junctions have been identified to date from different populations [588], [16], [589], [590]. Some of these mutations are shown below in figure 4.2. The overall reported frequency of point mutations varies from 50-60% of cases of clinically definite DRD [591], [562], [592], [593], [594]. Of these, approximately half were missense mutations, up to 17% were nonsense mutations, and up to 15% were frameshift mutations and [594], [595].

Many subjects with *GCH1* mutations do not have a family history of DRD and there appears to be a high new mutation rate [596]. Incomplete penetrance of *GCH1* mutations has also been identified, with estimates varying from 15% to 38% in men and 45% to 87% in women [597], [563], [596].

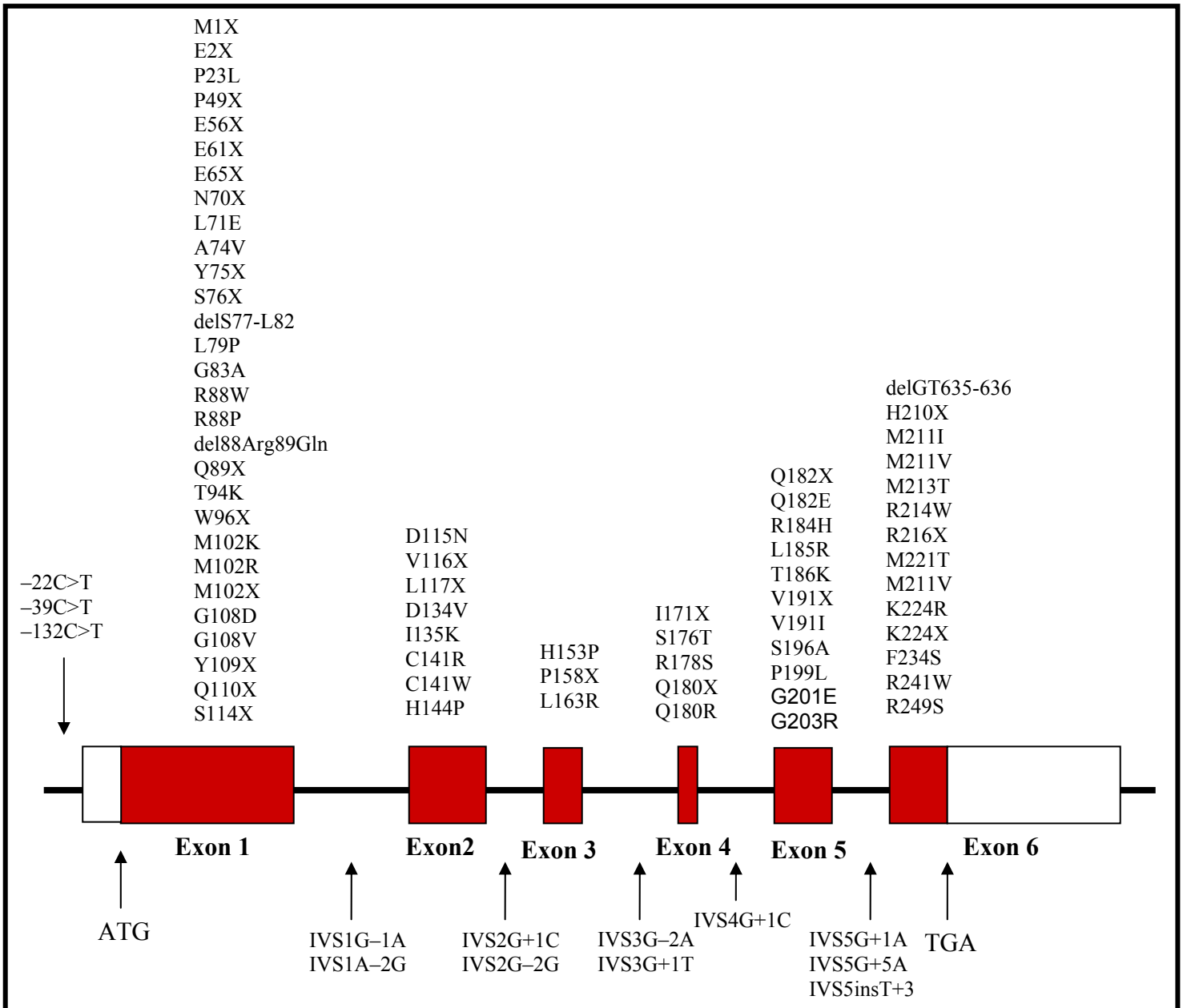

Figure 4.2 Mutations in the *GCH1* gene associated with DRD. The genomic organisation and location of mutations in the human *GCH1* gene on chromosome 14q22.1-q22.2. Red indicates exons. Adapted from Venna et al. 2006 [16].

AD GCH1 deficiency has been modeled in the GTP cyclohydrolase deficient *hph*-1 mouse, which shows many similar biochemical features as in human DRD [598], although the mice do not demonstrate an overt movement disorder.

There is an AR form of DRD with several missense mutations described in the gene encoding tyrosine hydroxylase (*TH*), in European subjects. The associated phenotype is generally more severe than the AD form, with an earlier age of onset [599]. Interestingly, the phenotype associated with the homozygous Leu205Pro mutation in *TH* was early onset parkinsonism [600]. There is also a single report of AD DRD caused by a mutation in the sepiapterine reductase gene (*SPR*) [601].

In summary, the nigrostriatal DA neurons appear susceptible to decreases in GCH1 activity, BH4 level and TH activity. DRD can therefore be considered as the phenotype resulting from dopamine deficiency without neuronal death, in contrast to PD which occurs with neuronal cell death [600].

4.1.2 Dopamine Transporter (DAT) Imaging

DAT imaging is a demonstration of *in vivo* striatal dopamine activity and yields results which are in keeping with other observations of the dopamine system such as autopsy studies [123], [124], [125]. DAT is a sodium chloride-dependent protein on the pre-synaptic dopaminergic nerve terminal which controls dopamine levels by active re-uptake of dopamine after its interaction with the postsynaptic receptor [127]. The DAT ligands for SPECT, including [^{123}I]FP-CIT (DaTSCAN) [128] have all shown significantly reduced striatal uptake in PD [129], even in early cases [130]. The abnormal uptake progresses from the putamen to caudate, and matches contralaterally to the side of the body more affected clinically. DAT

imaging is reproducible with test/retest variability in healthy controls and subjects with PD of 7% for DaTSCAN [131].

In some large studies of PD 11-15% of subjects have been found to have normal nigrostriatal uptake of presynaptic ligands [132], [133], [134], [135], [136]. In the ELL-DOPA study, 21 (14.7%) of patients scanned with β-CIT SPECT had normal initial scans. Follow-up scans remained normal in patients rescanned after 4 years. [602], [132]. The diagnosis in these patients remained unclear, and they have since been referred to as SWEDDs (Scans Without Evidence of Dopaminergic Deficit) [137].

These observations are important because they stimulated a debate as to whether it is possible to have PD with a normal presynaptic PET or SPECT scan, and are also of relevance to my data which is discussed later in this chapter. It has been proposed that at least some patients with SWEDDs have been misdiagnosed with PD, and may actually have an alternative diagnosis, such as essential tremor [137]. It has also been proposed that at least a proportion of these patients may have dystonic tremor or tremor associated with dystonia [136]. Alternatively these patients may indeed have a form of parkinsonism without true akinesia and a normal DaTSCAN.

As would be expected from its known aetiopathogenesis, studies of DAT imaging in DRD have consistently shown normal dopamine transporter density, whilst activity was abnormal in PD [603], [604], [605], [606]. Thus it has been proposed that where there is diagnostic difficulty, such as a negative family history or atypical presentation of DRD, DAT imaging can be a useful diagnostic test.

4.1.3 Dopaminergic neuronal loss in the enteric nervous system

PD has traditionally been regarded as a disease of the dopaminergic neurons in the substantia nigra. Gastrointestinal dysfunction, ranging from abnormal salivation to constipation, is a prominent non-motor feature of PD. Dysfunctional motility is the likely pathophysiological mechanism which underlies at least some of the gastrointestinal symptoms. Control of gastrointestinal motility arises from both the local intrinsic enteric nervous system (ENS) and central locations. The ENS consists of a deep myenteric plexus (Auerbach's plexus) and a more superficial submucosal plexus (Meissner's plexus), as depicted in figure 4.3. Central control arises from the autonomic inputs into the ENS. Parasympathetic innervation originates in the dorsal motor nucleus of the vagus (DMV) in the medulla and generally promotes increased motility, sympathetic input originates in paravertebral and sacral ganglia, and generally serves to inhibit gastrointestinal motility [607].

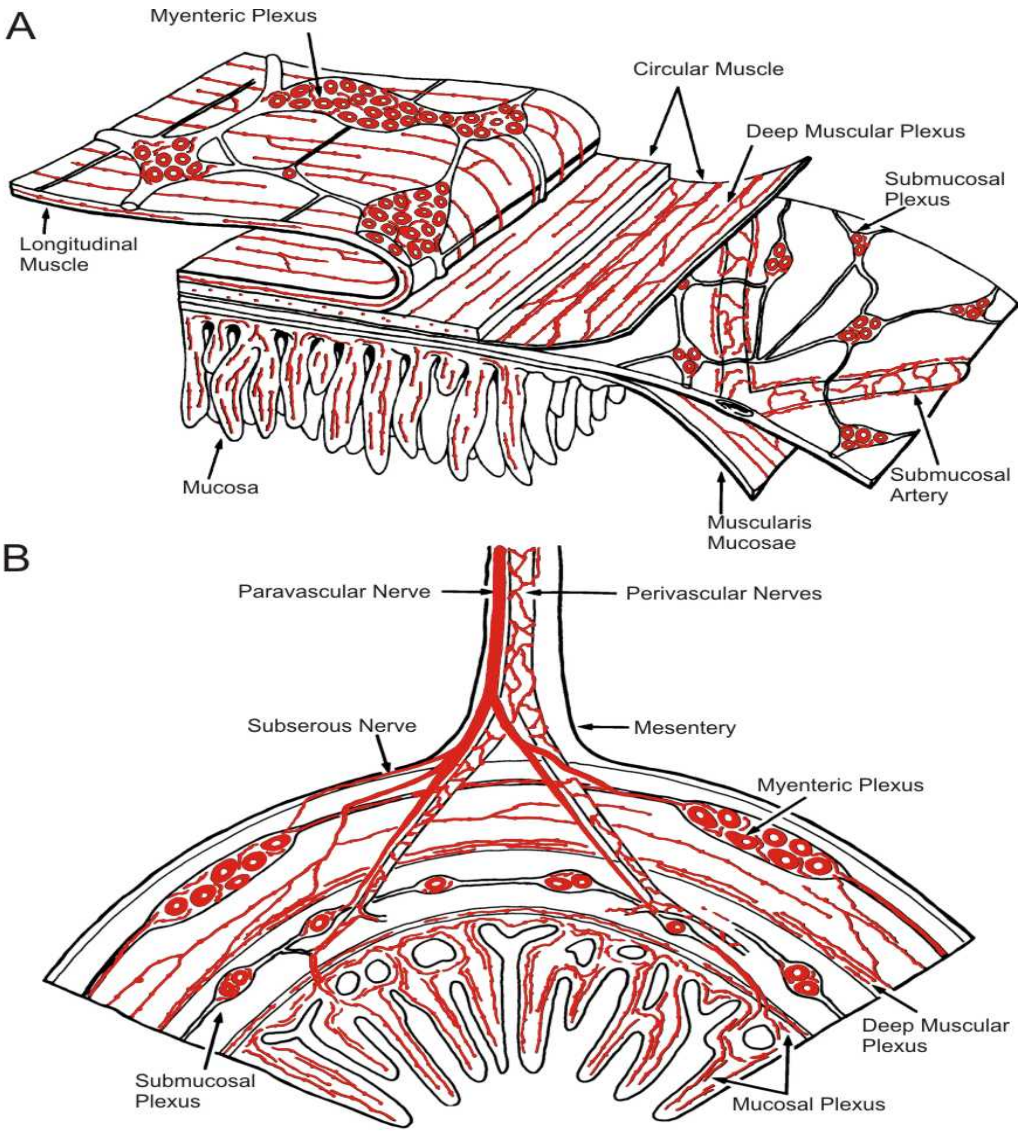

Figure 4.3 Arrangement of the enteric nervous system, depicted here for the small intestine. Panel A: appearance in separated layers. The deep myenteric plexus (Auerbach's plexus), which consists of numerous ganglia and connecting nerve fibre bundles, lies between the longitudinal and circular muscle layers. A second ganglionated plexus is in the submucosa (Meissner's plexus). These plexuses provide nerve fibre plexuses in the muscle, in the mucosa and around arterioles. Panel B: The enteric plexuses shown in a cross section of the intestine. Taken from Furness 2007 [8].

Pathological analyses of gastrointestinal samples in subjects with PD have revealed neuronal loss. Lewy bodies (LBs) and Lewy neurites (LNs) have also been found in both the myenteric and submucosal plexuses of the ENS in various portions of the gastrointestinal tract including the colon [607], [608], [609], [610], [611], [612], [613], [614], [615]. The majority of inclusions were found in cellular processes and cell bodies of vasoactive intestinal polypeptide (VIPergic) neurons [610], [616]. Anderson et al. have recently shown that there is also loss of enteric dopaminergic neurons, with associated changes in colon motility, in an MPTP mouse model of PD [617].

The central areas associated with gastrointestinal motility (DMV) have also been implicated [607]. One study suggests that the DMV is the earliest central site affected in PD, as α-synuclein pathology in the DMV was found in human brains without damage to the substantia nigra [107]. Braak even raised the intriguing question as to whether an environmental agent that induces aggregations could move by retrograde transport from the ENS to the CNS [607].

4.2 Aims

The aims of this detailed study of one family were firstly to describe the variety and overlap of clinical features, presentations and diagnoses within this family and to demonstrate the difficulties that can occur in coming to an accurate diagnosis. Secondly to present and discuss the results of imaging, molecular genetic and pathological studies that have aided elucidation of an accurate diagnosis in the various affected members of this family and to describe how this information may further understanding of the conditions involved.

4.3 Subjects and methods

4.3.1 Subjects

I identified family 6 for further specific study from a total of 46 families containing three or more members affected by PD, where the inheritance in the family appeared to be AD, which had been recruited to this study as described in section 2.1.1.

Nine members of this family were studied. All subjects underwent a clinical assessment, in their 'on state' if on anti-PD medication, by me to confirm the clinical features. For six subjects I assessed olfactory function by use of the University of Pennsylvania Smell Identification Test (UPSIT-40) (Sensonics, Haddon Heights, NJ) and olfaction was categorised as normal or pathologic according to normative data in relation to gender and age [482], [483]. Clinical assessment was performed prior to functional neuro-imaging, molecular genetic and pathological studies.

4.3.2 Methods

4.3.2.1 Functional neuro-imaging

To evaluate basal ganglia function six subjects from family 6 (III:1-III:5 and IV:1) underwent dopamine transporter SPECT scanning using [^{123}I]-FP-CIT SPECT (DaTSCAN, Amersham Health) at Sandwell and West Birmingham Hospital NHS Trust. In two of the subjects a second DaTSCAN was performed at a period of 18 months after the first scan. These images were reviewed by a consultant radiologist, Dr Edward Rolfe, Department of neuro-radiology, University Hospital Birmingham, experienced in the interpretation of these images, who was blinded to the demographic and clinical data on these subjects.

4.3.2.2 Pathological studies

In two subjects (III:1 and III:4) healthy bowel tissue was available from previous hemi-colectomies, performed for large bowel malignancies. This enabled examination of neural tissue from the mesenteric plexus. The tissue was studied by an experienced neuro-pathologist, Dr Martyn Carey, Department of Pathology, University of Birmingham, for the presence of neuronal loss and evidence of Lewy bodies. Tissues were prepared using standard protocols, and immunohistochemical staining was performed using a monoclonal antibody to alpha-synuclein (Novocastra) to detect the presence of Lewy bodies.

4.3.2.3 DNA extraction and molecular genetic studies

I extracted DNA from venous blood samples taken from family members as described in section 2.3. The DNA samples were then sent to the laboratory of Dr Vincenzo Bonifati, Erasmus MC Rotterdam, The Netherlands, where molecular genetic screening in this family was performed by Erik Simonds.

In six members of the family (III:1-III:5 and IV:1) screening for known and novel mutations was performed by PCR and sequencing of the entire coding sequence, and intron-exon boundaries, of the genes *Alpha-synuclein*, *Parkin*, *DJ-1*, *PINK1, LRRK2* and *GCHI*. Multiple ligation-dependent probe amplification (MLPA) was used to detect the presence or absence of copy number variation in these genes, using MLPA kits P051 and P052 (MRC Holland). MLPA is a well validated and high resolution method to detect copy number changes of up to 45 loci in one relatively simple, semi-quantitative PCR-based assay [618], [619], [620]. Subsequently, three unaffected family members (IV:2-IV:5) and 159 UK control samples from

the PD GEN study were screened by Erik Simonds for the novel *GCH1* mutation identified in subjects III:1, III:4, III:5 and IV:1.

4.4 Results

4.4.1 Clinical studies

Of the nine members of this family who have been studied, two subjects had slowly progressive PD, one subject had benign parkinsonism and two subjects had DRD. The four remaining members were unaffected. The median age of disease onset was 50 with median disease duration of 20 years. All available clinical details on affected and unaffected members of these families are given in detail in this section. All the studied members of this family are Caucasian.

The pedigree of this family is shown in figure 4.4 and a summary of the clinical features of the nine studied members of this family can be found in table 4.1.

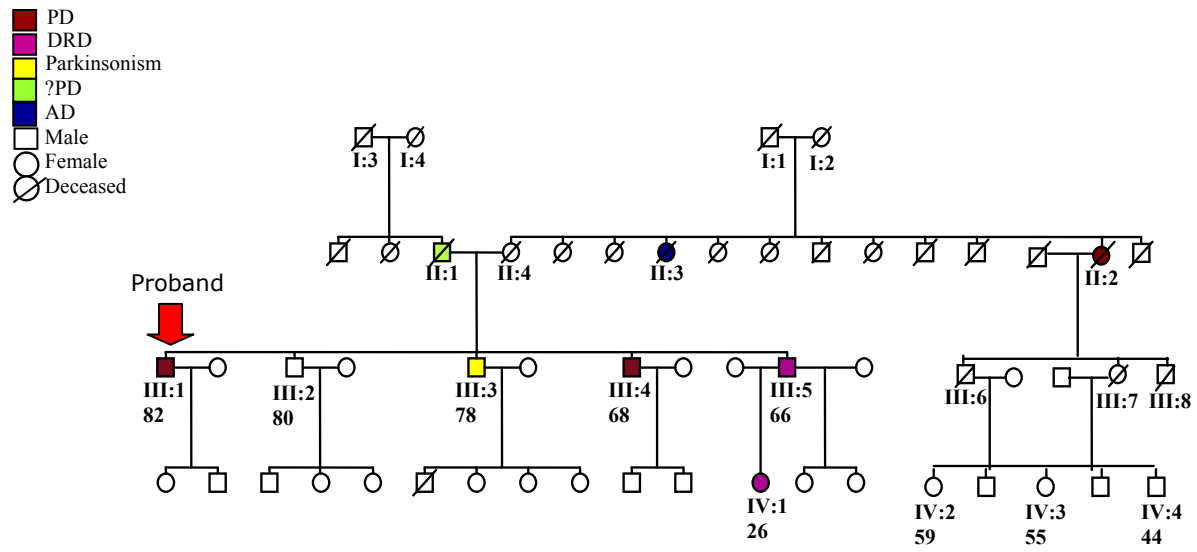

Figure 4.4 Pedigree of family 6. The ages at evaluation of the nine family members studied in detail are given.

4.4.1.1 Subject III:1

The proband case III:1 is a right-handed male, he was aged 82 at the time of evaluation and had onset of symptoms aged 58. His initial symptom was a resting tremor of the right hand. A diagnosis of PD was made and he was started on Levodopa (Sinemet) with significant treatment benefit. His symptoms have progressed slowly since diagnosis and his anti-parkinsonian medication has been increased.

At the time of evaluation he had had intermittent 'off-period' dystonia of the right leg for about 5 years and had also had treatment-induced dyskinesias of the right arm, as well as of the head and neck, for approximately 10 years. He did not have any significant problem with 'off-time' or fluctuations in his motor symptoms. His past medical history included hypertension as well as colon and skin cancer. He was retired and had worked as a chef for 26 years. He had never smoked and had lived most of his life in the town.

Neurological examination, on treatment, revealed features typical of PD with mask-like facies, asymmetrical rest tremor, bradykinesia and cog wheeling rigidity, all worse on the right side, as well as postural instability with retropulsion on the pull test and dyskinesias. His gait was slow with reduced arm-swing on the right side. His Hoehn and Yahr stage was 3, with a UPDRS III score of 31. The remainder of his neurological examination was normal. There was no evidence of significant cognitive deficit with a MMSE score of 27. His anti-parkinsonian medication at time of evaluation consisted of Levodopa (Sinemet) at a total daily dose of 650mg.

CT scanning of his brain, aged 84, showed cerebral atrophy, commensurate with his age.

The clinical diagnosis in this subject was a slowly progressive form of PD.

4.4.1.2 Subject III:2

He is a left-handed male, was aged 80 at the time of evaluation and was asymptomatic. His past medical history included hypertension and osteoarthritis. He was retired and had been a railway worker for 50 years. He was an ex-smoker with a 5 year pack history and had lived most of his life in the town. Neurological examination revealed no features of PD. There was no evidence of significant cognitive deficit with a MMSE score of 27.

4.4.1.3 Subject III:3

He is a right-handed male, was aged 78 at the time of evaluation and had onset of symptoms aged 75. His initial symptoms were a resting tremor of the right hand and slow gait. A diagnosis of parkinsonism was made, although his symptoms had not worsened since their onset and at the time of evaluation he had not received any anti-parkinsonian therapy. He was

reported to have had two cerebrovascular accidents (CVAs); the first was at the age of 65 when he had transient balance problems and the second episode was at the age of 71, when he had developed left arm weakness and dysarthria. His past medical history included angina and atrial fibrillation. He was retired and had worked as a hairdresser for 50 years. He was an ex-smoker with a 20 year pack history and had lived most of his life in the town.

Neurological examination revealed some features of parkinsonism, with an asymmetrical rest tremor, bradykinesia and cog wheeling rigidity, all worse on the right side. His gait was slow but was within normal limits. His Hoehn and Yahr stage was 1, with a UPDRS III score of 9. The remainder of his neurological examination was normal. There was no evidence of cognitive deficit with a MMSE score of 30.

Neither a CT scan of his brain, aged 73, nor an MRI scan of his brain, aged 78, had revealed any evidence of a CVA.

The clinical diagnosis in this subject was parkinsonism as he did not meet the UKPDS brain bank diagnostic criteria (see appendix A) for definite PD.

4.4.1.4 Subject III:4

He is a right-handed male, was aged 68 at the time of evaluation and had onset of symptoms aged 50. His initial symptoms were a resting tremor of the left hand and slow gait. A diagnosis of PD was made and he was initially commenced on Levodopa (Sinemet) 150mg daily, with significant treatment benefit. His symptoms had progressed slowly since diagnosis and were well controlled on a small dose of anti-parkinsonian medication. His past medical history included angina, hypertension and erectile dysfunction. He was retired and had worked as an

exhibition designer for over 30 years. He had never smoked and had lived most of his life in the town.

Neurological examination, on treatment, revealed features suggestive of PD, namely an impassive face, asymmetrical rest tremor, bradykinesia and cog wheeling rigidity, all worse on the left side. His gait was slow and he had poor arm swing, again worse on the left side. He also had evidence of postural instability with retropulsion on the pull test. His Hoehn and Yahr stage was 2, with a UPDRS III score of 19. The remainder of neurological examination was normal. There was no evidence of significant cognitive deficit with a MMSE score of 28. His anti-parkinsonian medication at time of evaluation consisted of Levodopa (Sinemet) at a total daily dose of 150mg.

He underwent an MRI scan of his brain, aged 69, which revealed some areas of non-specific increased signal in the right lentiform nucleus and around the posterior horns of both lateral ventricles, consistent with long-standing hypertension.

The clinical diagnosis in this subject was of a slowly progressive form of PD.

4.4.1.5 Subject III:5

He is a right-handed male, was aged 66 at the time of evaluation and had onset of symptoms aged 44. His initial symptoms were a shuffling gait, difficulty with gait initiation and tremor of both legs. He was initially diagnosed with PD and commenced on a dopamine agonist (Bromocriptine) with significant treatment benefit. His symptoms progressed slowly, although Levodopa was added to his therapy six years after diagnosis, at the age of 50, to give a total daily levodopa equivalent dose of 250mg.

At the time of initial evaluation, his symptoms were well controlled on treatment. His past medical history included erectile dysfunction but was otherwise unremarkable. He was retired and had worked as retail store manger for 30 years. He had never smoked and had lived most of his life in the town. His neurological examination at this time, on treatment, appeared to be normal with no evidence of the features of PD. There was no evidence of significant cognitive deficit with a MMSE score of 28. His anti-parkinsonian medication at time of evaluation consisted of dopamine agonist (Bromocriptine) and Levodopa (Sinemet) to give a total daily levodopa equivalent dose of 250mg.

This subject was subsequently re-evaluated by Dr David Nicholl, and given a trial period of one week off anti-parkinsonian therapy. During this period his symptoms worsened, he developed a feeling of akathisia and a resting tremor of both feet. He also developed some dystonic posturing of both feet, worse on the right, which tended to be worse at the end of the day. Neurological examination at this time revealed dystonic posturing of the right foot and resting tremor of both feet. On restarting anti-parkinsonian medication the symptoms improved.

The clinical diagnosis in this subject was DRD.

4.4.1.6 Subject IV:1

She is a right-handed female, was aged 26 at the time of evaluation and had onset of symptoms aged 6. Her initial symptoms were a clawing of the toes and in-turning of the ankle of the right foot. Her symptoms progressed slowly but she subsequently developed similar symptoms of the left foot. No specific neurological diagnosis was made. At the age of 24 she developed dystonic posturing of the right hand, especially when typing or writing. All of these

symptoms were worse in the evening or when stressed. Her past medical history was unremarkable. She was a secretary, had never smoked and had lived most of her life in the town.

Neurological examination at the time of evaluation, off treatment, revealed the presence of a dystonic tremor of the right hand with a tendency to claw and she developed dystonic writer's cramp whilst writing. Both feet were dystonic and were inverted. There were no other abnormal neurological features in the upper or lower limbs and allowing for the dystonic posturing of her feet her gait appeared within normal limits. There was no evidence of significant cognitive deficit with a MMSE score of 29.

She was also assessed by Dr David Nicholl and given a working diagnosis of DRD. MRI scanning of her brain revealed an apparent persistence of non-myelinated white fibres around the trigones, occipital and frontal horns. She was commenced on Levodopa (Sinemet), with significant treatment benefit, and the dose was titrated up to a total daily dose of 300mg.

The clinical diagnosis in this subject was DRD.

4.4.1.7 Subjects IV:2, IV:3 and IV:4

All three subjects were asymptomatic and neurological examination revealed no features of PD. Their ages at evaluation are listed in figure 4.4. Subject IV:3 has a history of hypothyroidism.

4.4.1.8 Olfactory testing

I performed olfactory testing on the five affected subjects and four unaffected subjects studied. The results were compared to the normative values and percentiles for age and gender

provided in 'The Smell Identification Test Administration Manual' to determine the olfactory diagnosis [482]. The UPSIT score was 21 for subject III:1 (Severe Microsmia, 40th percentile), 31 for subject III:2 (Mild Microsmia, 71st percentile), 21 for subject III:3 (Severe Microsmia, 23rd percentile), 28 for subject III:4 (Moderate Microsmia, 31st percentile), 26 for subject III:5 (Moderate Microsmia, 24th percentile) and 37 for subject IV:1 (Normosmia, 40th percentile).

4.4.1.9 Other subjects

According to family reports other known affected members of the family were: (i) subject II:1. He had been observed by the family to have had a shuffling gait and upper limb tremor, which started in his 50's, and was wheelchair bound for the last 6-7 years of his life. A formal diagnosis of PD was not made, and although he was treated with levodopa it is unclear whether he responded to this therapy. There was no reported significant cognitive deficit. His occupation was as a railway worker. He died at the age of 82. (ii) Subject II:2. She had developed tremor of both arms in her 70's and was diagnosed with PD. She had received treatment with levodopa to which she responded. Her condition was slowly progressive, but she remained independently mobile on treatment. There was no reported significant cognitive deficit. She died at the age of 91. She had three children, subjects III:6- III:8, all of whom were asymptomatic. (iii) Subject II:3. She was reported to have had Alzheimer's disease.

Other family members who were known to be asymptomatic include: (i) the mother of the proband, subject II:4, who died at the age of 94. (ii) The maternal grandfather of the proband, subject I:1, who died in his 70's. (iii) The maternal grandmother of the proband, subject I:2, who died in her 70's and was reported to have suffered from epilepsy. (iv) The paternal

grandfather of the proband, subject I:3, who died in his early 80's. (v) The paternal grandmother of the proband, subject I:4, who died in her early 50's.

4.4.2 Functional neuro-imaging

The DaTSCAN images on five affected subjects (III:1, III:3-III:5 and IV:1) and one unaffected subject (III:2) are shown below in figures 4.5-4.12. Subjects III:3 and III:4 had a second DaTSCAN performed approximately 18 months after the first scan due to the inconclusive result of the first scan and atypical disease phenotype. When detected, reduced uptake was regarded as suggestive of reduced basal ganglia function.

Subject III:1 had an abnormal scan with bilateral reduced uptake, worse on the left side. Subject III:2 had a normal scan. Subject III:3 had two scans, the first was found to be abnormal with reduced uptake on the right side, whilst the follow-up scan 18 months later was found to be normal. Subject III:4 had two scans, both scans were abnormal with bilateral reduced uptake, worse on the right side. Subject III:5 and IV:1 had normal scans. These findings are summarised in table 4.1.

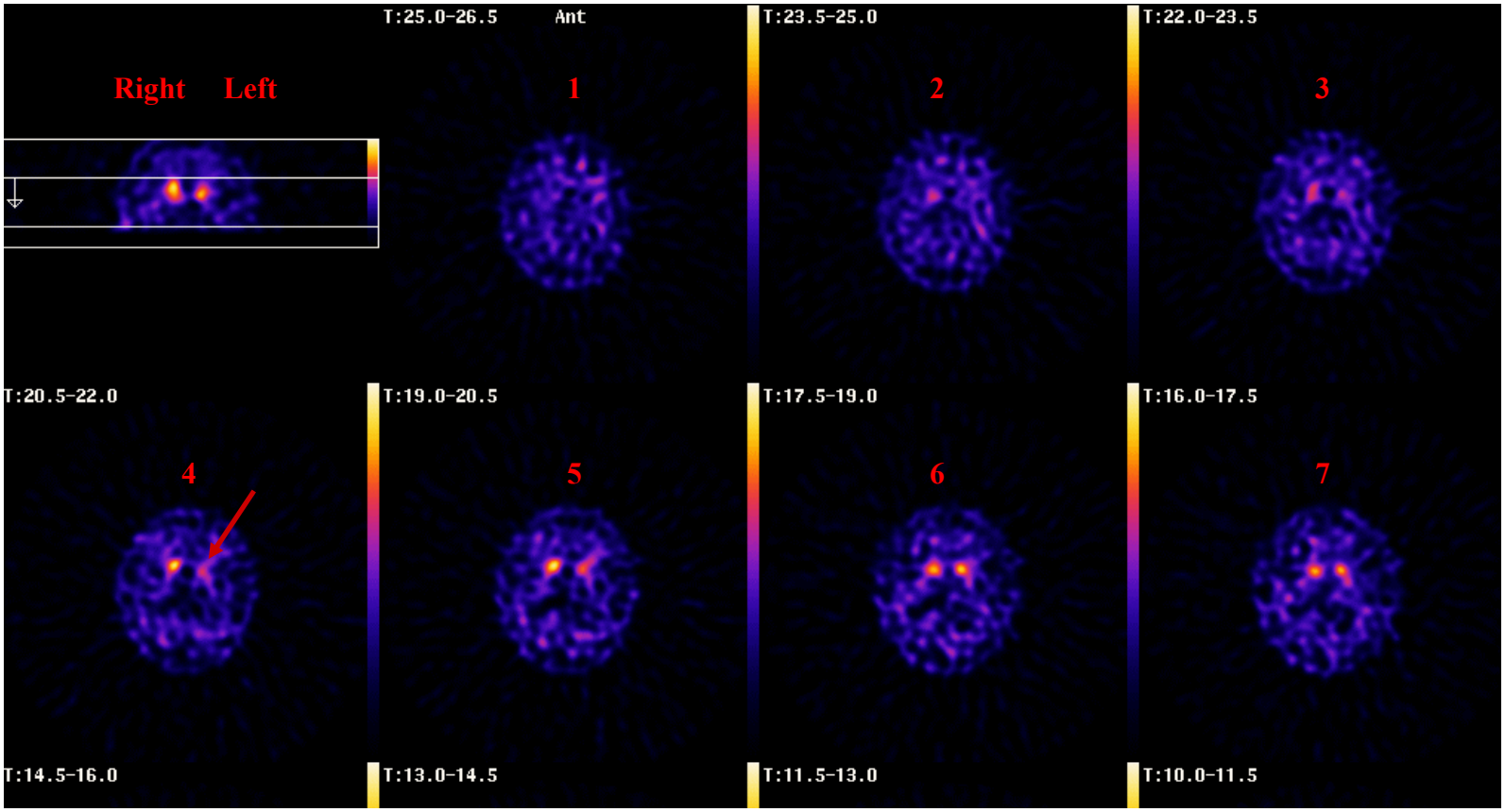

Figure 4.5 DaTSCAN of subject III:1. An abnormal scan, which shows bilateral reduced striatal uptake of [^{123}I]FP-CIT, worse on the left side, as demonstrated by the arrow. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

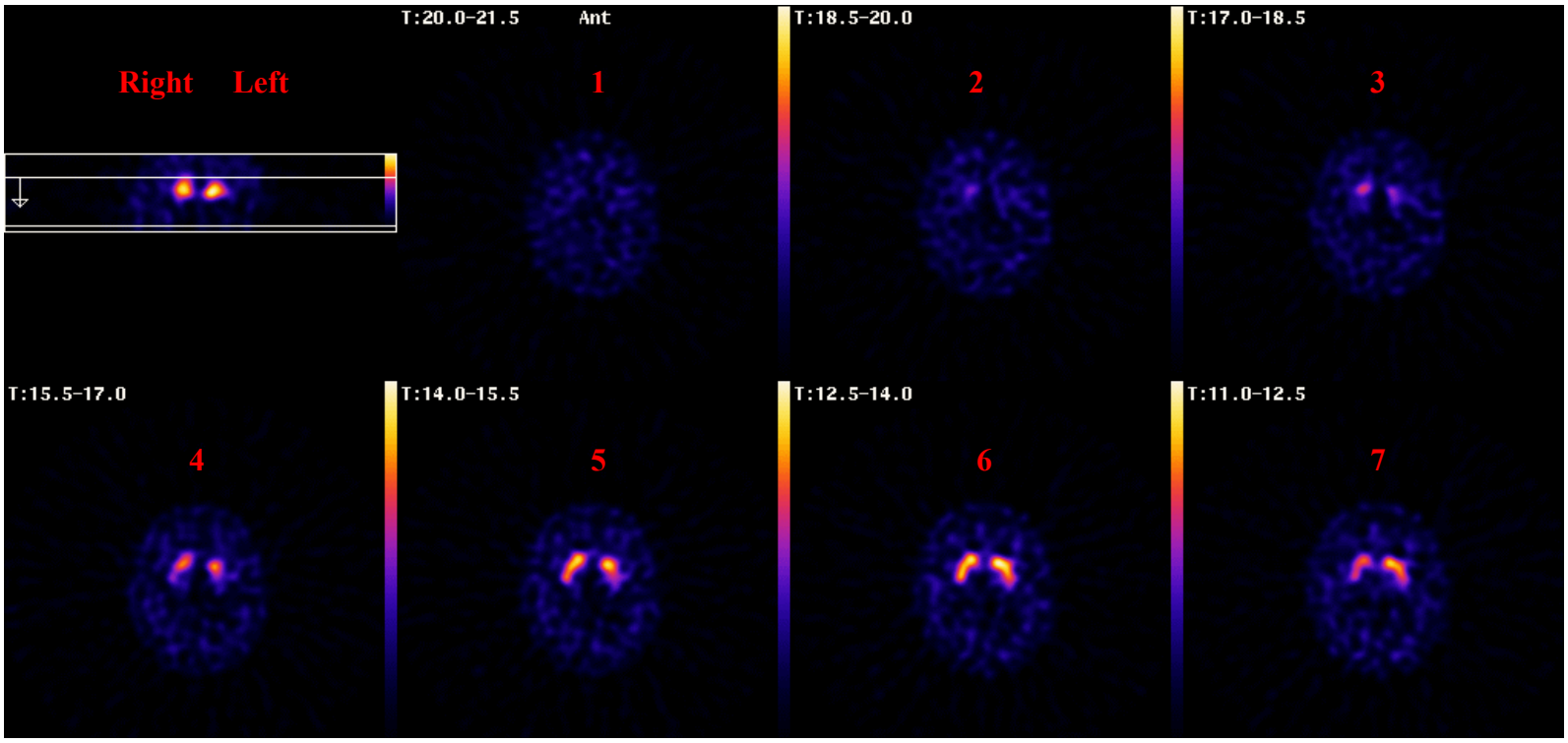

Figure 4.6 DaTSCAN of subject III:2. A normal scan, showing normal striatal uptake of [^{123}I]FP-CIT bilaterally. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

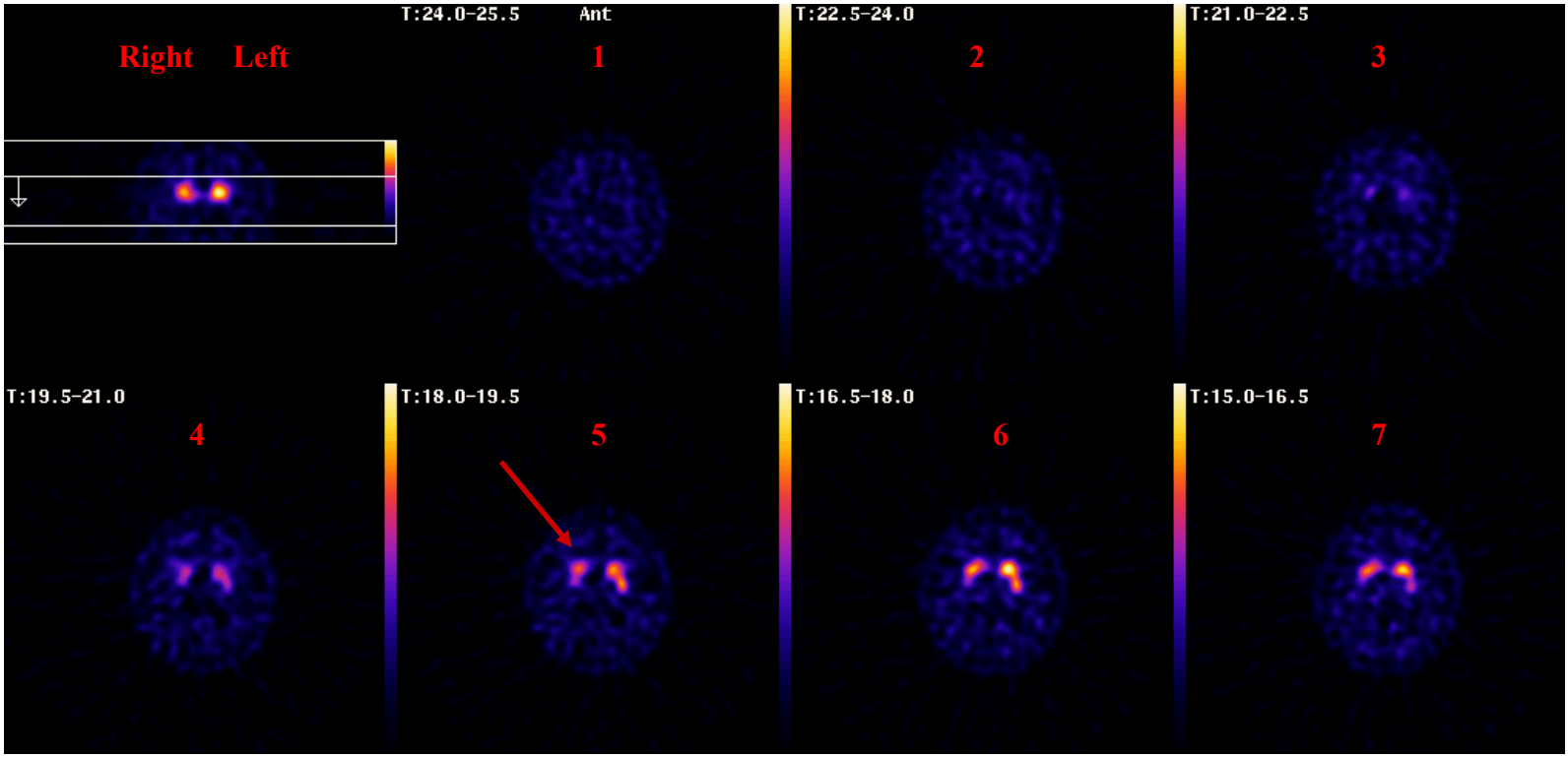

Figure 4.7 DaTSCAN 1 of subject III:3. An abnormal scan, showing reduced striatal uptake of [^{123}I]FP-CIT on the right side, as demonstrated by the arrow. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

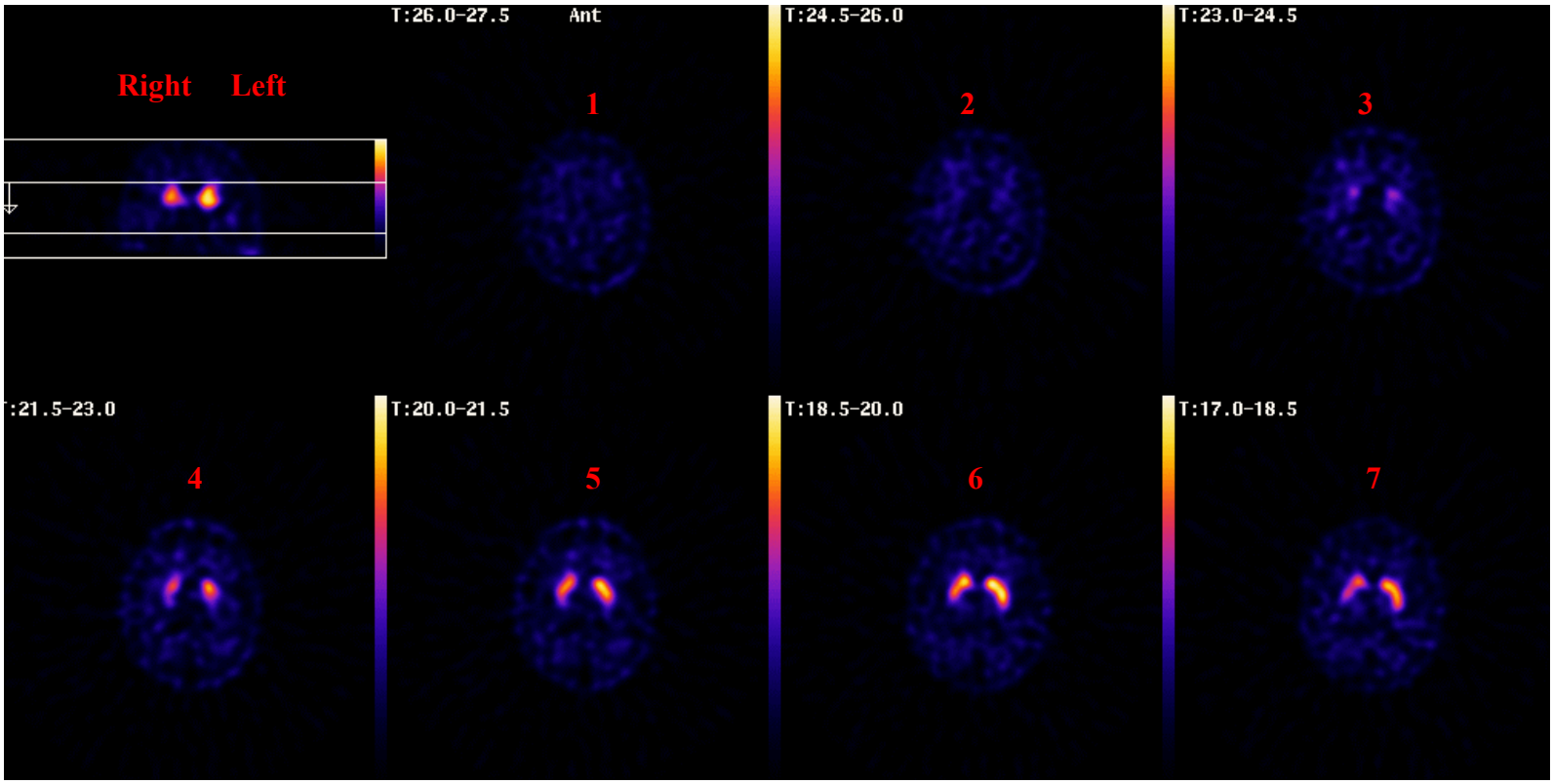

Figure 4.8 DaTSCAN 2 of subject III:3. Follow-up scan 18 months after first scan, a normal scan showing normal striatal uptake of [^{123}I]FP-CIT bilaterally. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

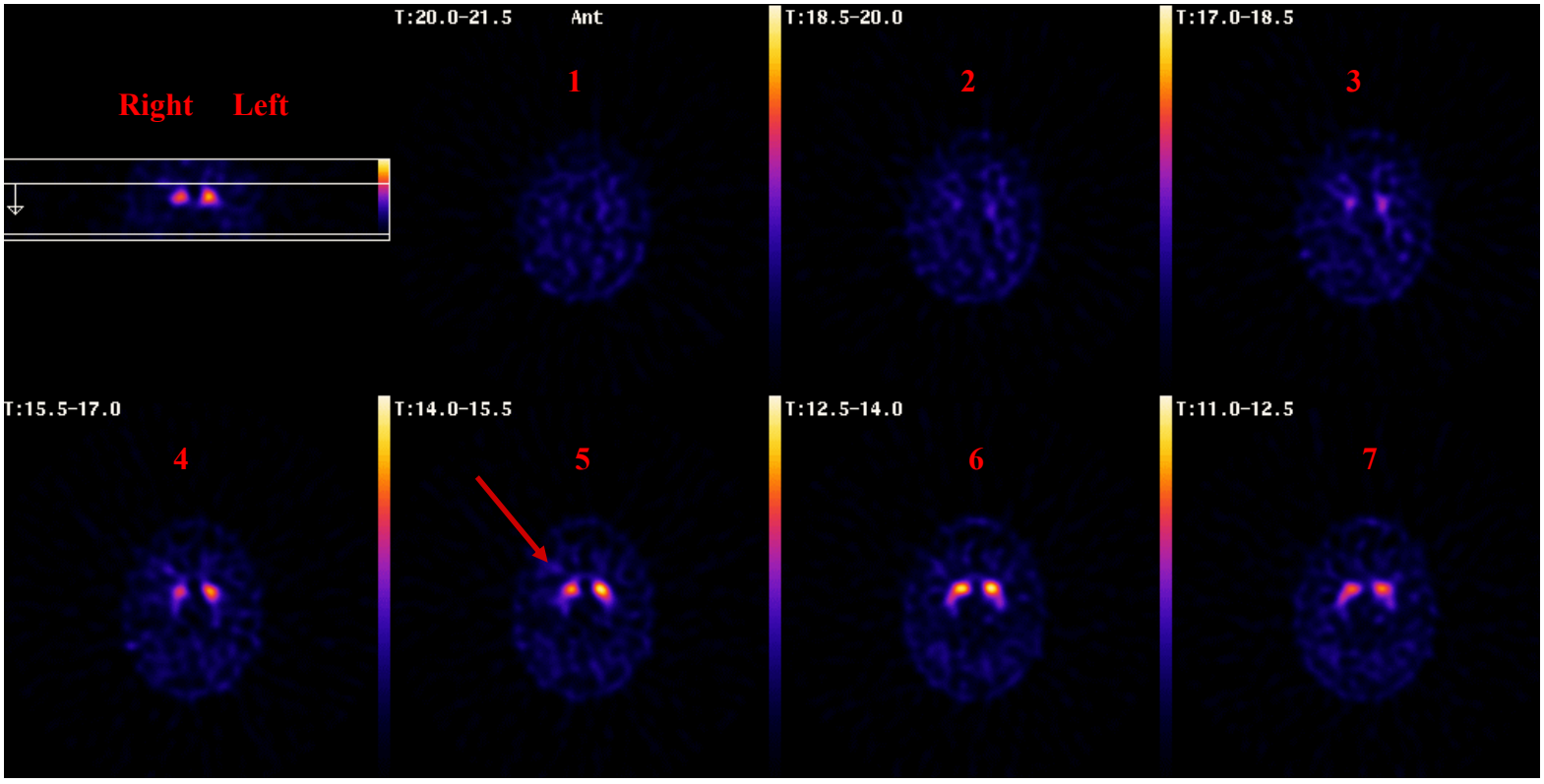

Figure 4.9 DaTSCAN 1 for subject III:4. An abnormal scan, showing bilateral reduced striatal uptake of [^{123}I]FP-CIT, worse on the right side, as demonstrated by the arrow. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

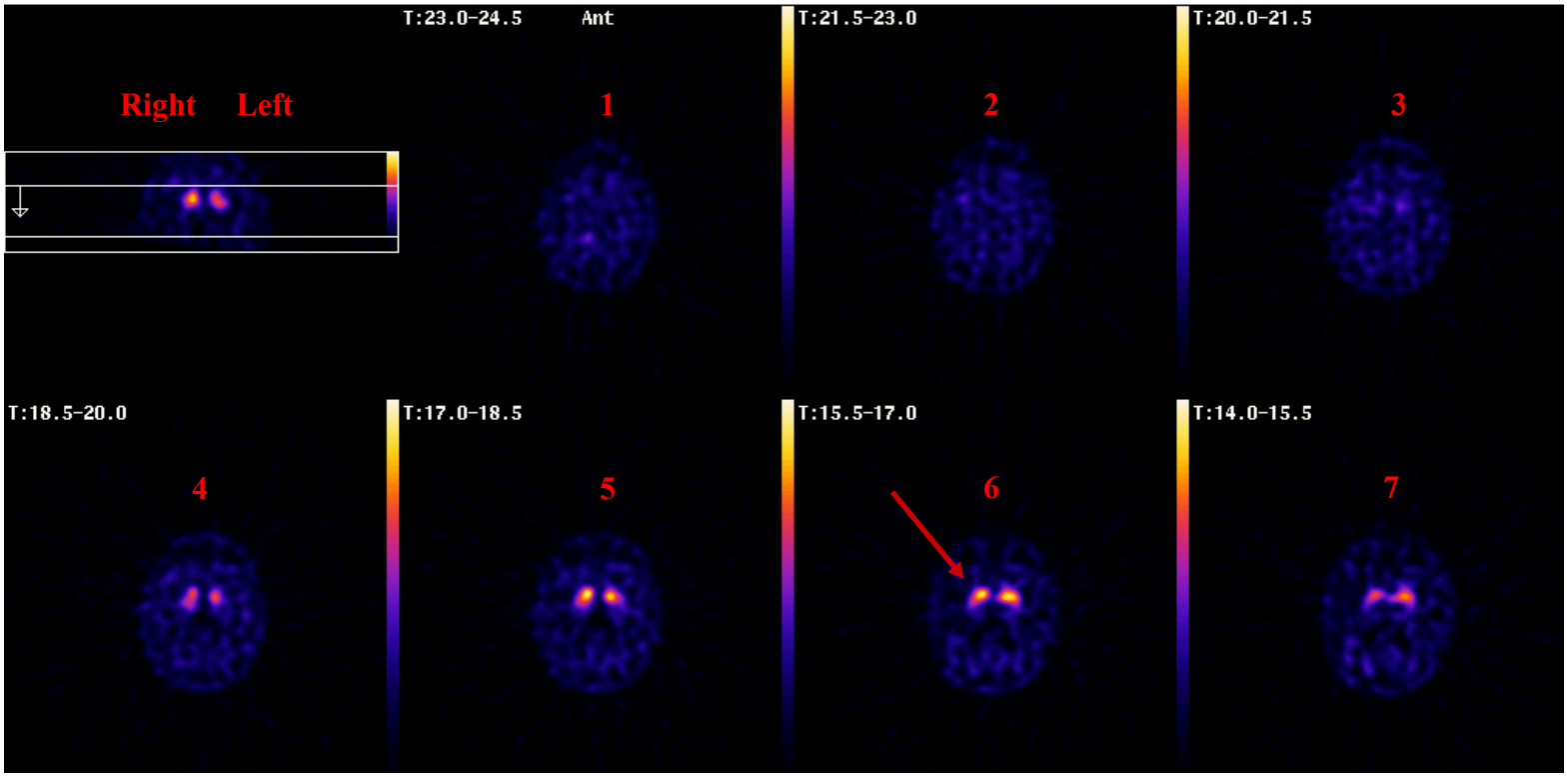

Figure 4.10 DaTSCAN 2 for subject III:4. Follow-up scan 18 months after first scan, an abnormal scan showing bilateral reduced striatal uptake of [^{123}I]FP-CIT, worse on the right side, as demonstrated by the arrow. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

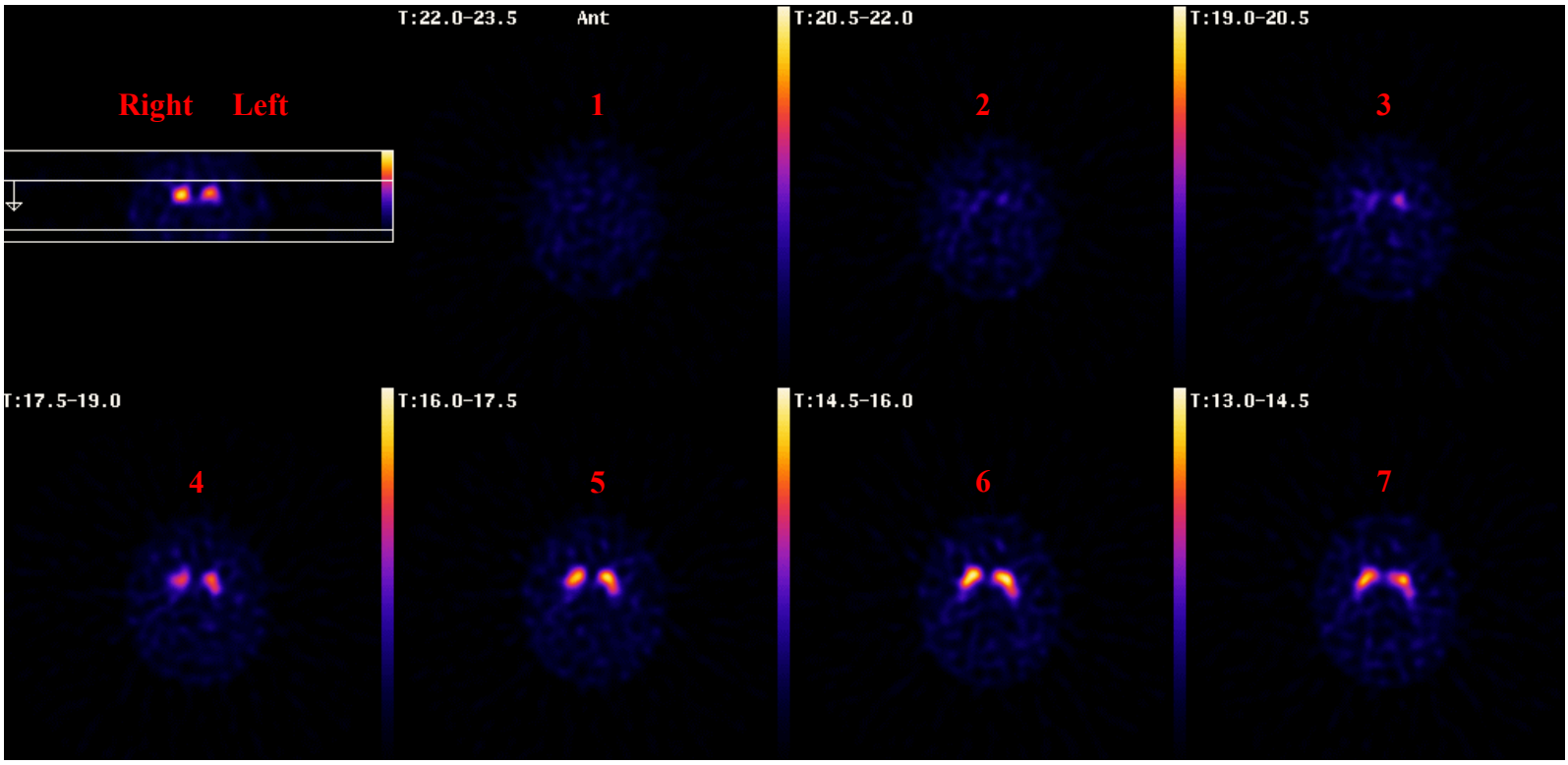

Figure 4.11 DaTSCAN of subject III:5. A normal scan, showing normal striatal uptake of [^{123}I]FP-CIT bilaterally. Images 1-7 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section.

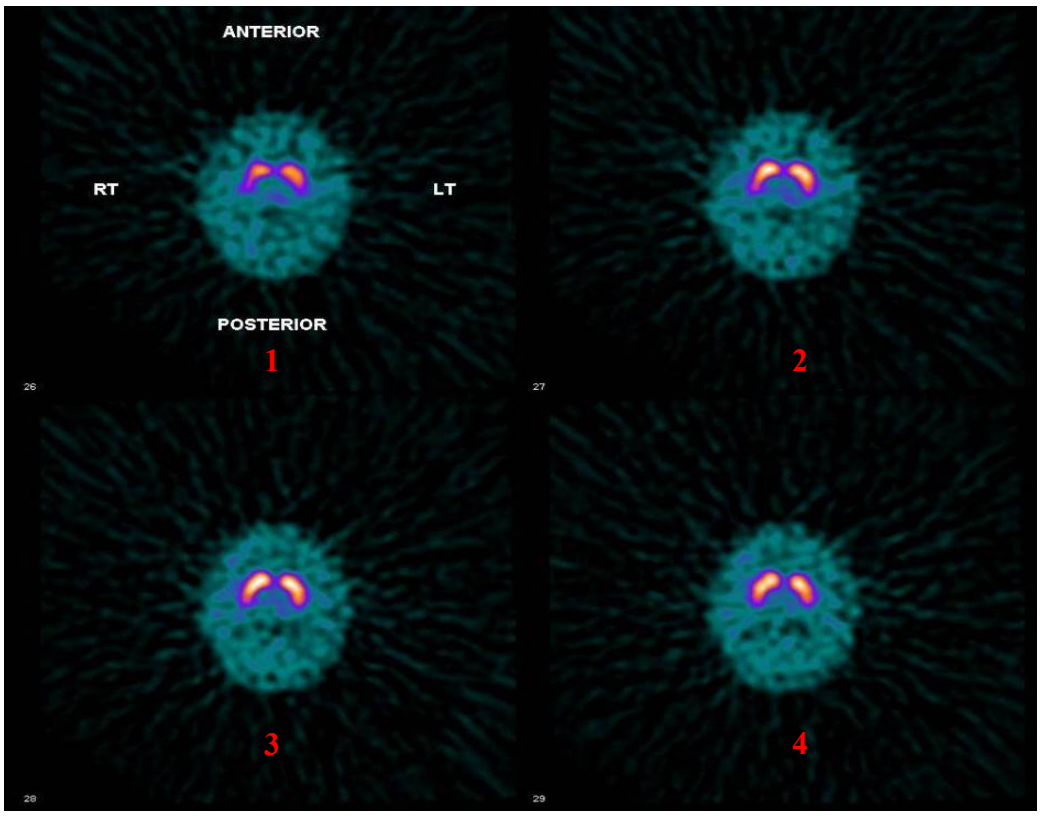

Figure 4.12 DaTSCAN of subject IV:1. A normal scan, showing normal striatal uptake of [^{123}I]FP-CIT bilaterally. . Images 1-4 represent transverse sections through the brain, where 1 is the most superior and 4 the most inferior section. RT= right, LT= left.

4.4.3 Molecular genetic studies

The entire known coding sequences and intron-exon boundaries of *Alpha-synuclein*, *Parkin*, *PINK1*, *DJ-1* and *LRRK2* were sequenced in subjects (III:1-III:5 and IV:1). No novel or known mutations were identified in the coding sequences of these genes.

A novel heterozygous missense mutation was identified in the first exon of *GCH1*, see figure 4.13, affecting the fifth nucleotide of the open reading frame (c.A5G). This mutation is predicted to replace glutamic acid with glycine (p.E2G). As given in table 4.1, the novel *GCH1* mutation was identified in four of the affected members of the family, but not in subject III:3 or the other unaffected subjects III:2 and IV:2-IV:5. This novel *GCHI* mutation was also not identified in 300 UK control chromosomes, from the PD GEN control cohort (see appendix B).

MLPA did not reveal any copy number variation in the PD genes *Alpha-synuclein*, *Parkin*, *PINK1*, *DJ-1* and *LRRK2* or *GCH1*

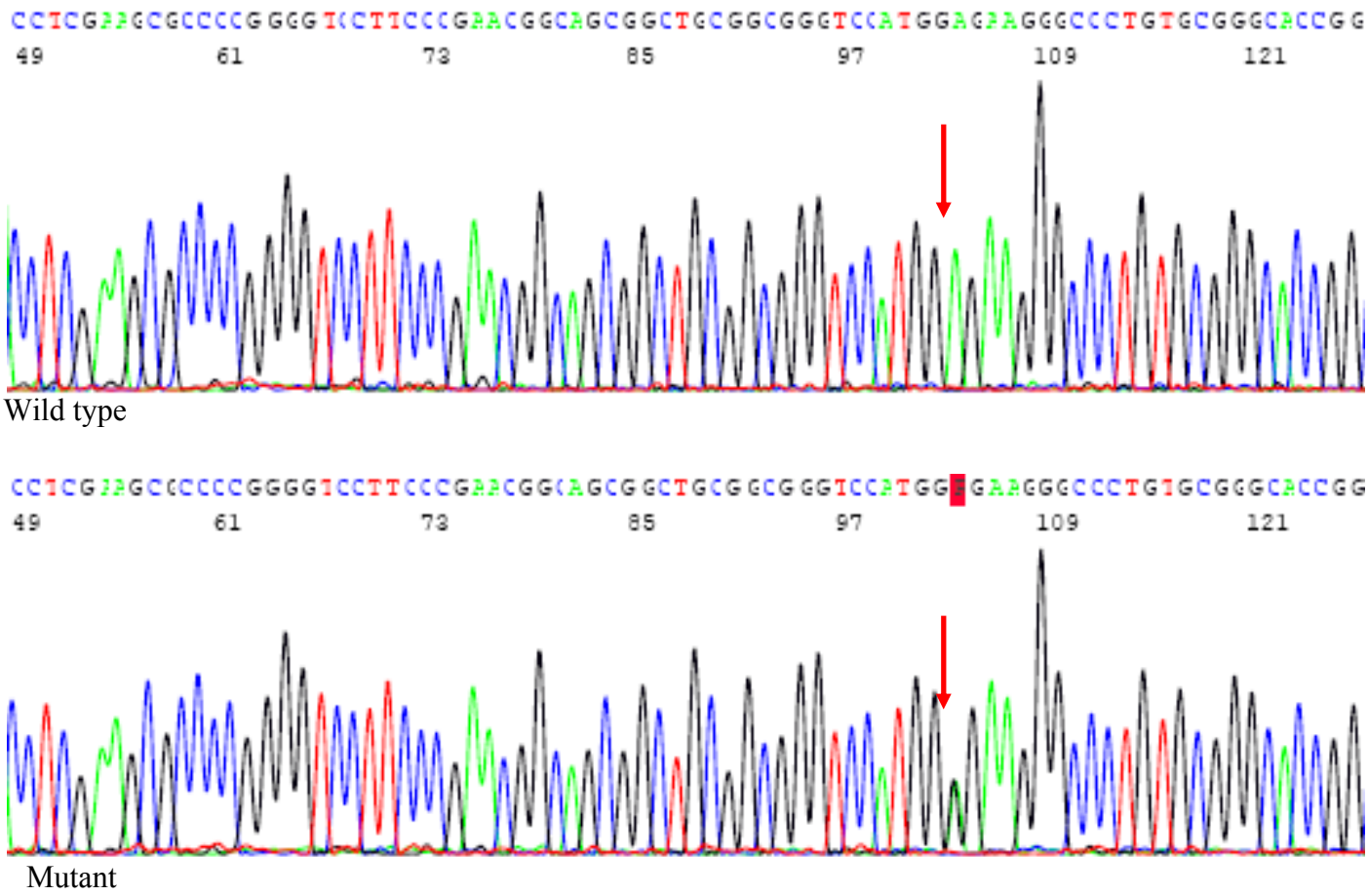

Figure 4.13 Electropherograms from subjects who are wild–type and heterozygote for the novel *GCH1* mutation c.A5G. Arrow indicates position of nucleotide variation.

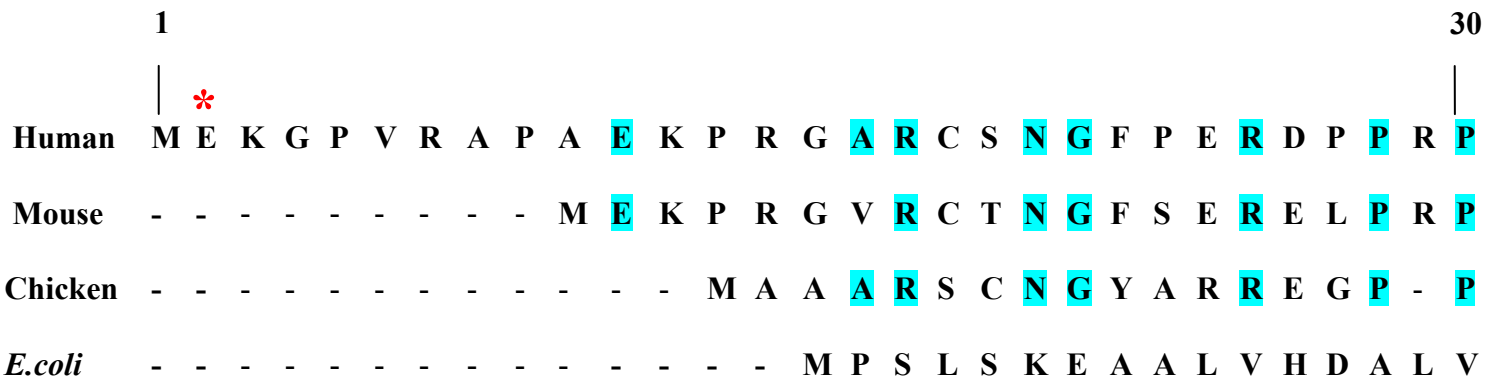

Figure 4.14 Human GTPCH1 protein aligned with other orthologs from mouse, chicken, zebrafish and *E.coli*. Amino acid number is based on the human protein. GenBank accession number human P30793, mouse Q05915, chicken P50141 and *E. coli* P27511. * Indicates position of amino acid substitution E2G. Adapted from Pelletier et al. 2001 [21].

4.4.4 Pathological studies

Histopathological study of the ENS from healthy bowel tissue, performed by Dr Martyn Carey, in subjects III:1 and III:4 did not reveal the presence of any neuronal loss, Lewy bodies or any other pathology suggestive of PD.

Subject & gender	Age at eval	Age at sympt. onset	Clinical Diagnosis	L-Dopa Response	H&Y Stage	MMSE	UPSIT score (percentile)	DaT SCAN	*GCH1* mut. p.E2G	Path.
III:1 M	82	58	PD	Y	3	27	21 (40th)	A	Y	Norm
III:2 M	80	N/A	Unaffected	N/A	N/A	27	31 (71st)	N	N	N/A
III:3 M	78	75	Parkinsonism	No Rx	1	30	21 (23rd)	1 A 2 N	N	N/A
III:4 M	68	50	PD	Y-low dose	2	29	28 (31st)	1 A 2 A	Y	Norm
III:5 M	66	44	DRD	Y-low dose	N/A	28	26 (24th)	N	Y	N/A
IV:1 F	26	6	DRD	Y-low dose	N/A	29	37 (40th)	N	Y	N/A
IV:2 F	59	N/A	Unaffected	N/A	N/A	N/A	N/A	N/A	N	N/A
IV:3 F	55	N/A	Unaffected	N/A	N/A	N/A	N/A	N/A	N	N/A
IV:4 M	44	N/A	Unaffected	N/A	N/A	N/A	N/A	N/A	N	N/A

Table 4.1 Summary of the clinical, functional neuro-imaging, molecular genetic and pathological data on nine subjects from family 6. PD denotes Parkinson's disease; DRD dopa responsive dystonia; N/A not done or not appropriate; MMSE mini mental state examination score; UPSIT University of Pennsylvania Smell Identification Test; DaTSCAN dopamine transporter imaging. Age and sex corrected percentile values for UPSIT scores are presented in brackets.

4.5 Discussion

The results section of this chapter described the clinical characteristics of family 6 as well as functional neuro-imaging, molecular genetic and pathological data on affected and unaffected family members. A novel missense mutation within the GTP cyclohydrolase 1 gene coding sequence was identified in affected subjects only.

4.5.1 Clinical studies

There was a wide variation of phenotypes within this family, ranging from PD to DRD. Given the atypical nature of some of these phenotypes, it is not surprising that accurate diagnosis was challenging in several members of the family. I determined the clinical phenotypes before the results of functional neuro-imaging, molecular genetic and pathological studies were available. The clinical symptoms and signs observed in the proband subject III:1, and subject III:4, excluding the positive family history, fitted the UKPDS brain bank diagnostic criteria for definite PD (see appendix A). For both subjects their symptoms responded well to Levodopa therapy, and subject III:1 also had levodopa-induced dyskinesias. However in both cases their conditions were much more slowly progressive than would normally be expected in IPD. The reported rates of progression of PD vary greatly even amongst cohorts, but both pre-levodopa era studies, and more recent work, have shown similar results. The pre levodopa results showed that the proportion of subjects severely disabled (Hoehn and Yahr stage 4 or 5) or dead at 5 years was 28%, with this figure rising to 61% at 5-9 years follow up and 83% at 10-15 years [13]. More recently the proportion of subjects severely disabled (Hoehn and Yahr stage 4 or 5) or dead at 4 years was 22%, with this figure rising to 71% at 10 years follow up. Approximately 10% of subjects followed a more benign course [621].

Subject III:3 had very mild features of parkinsonism which were not disabling and did not require treatment, his clinical signs did not reach the UKPDS brain bank diagnostic criteria for definite PD. Subjects III:5 and IV:1 exhibited a much different phenotype to the other affected family members, with predominantly lower limb symptoms and signs of dystonia, with diurnal fluctuation, which responded well to Levodopa therapy and were slowly progressive. The clinical diagnosis in both of these subjects was DRD with no parkinsonian features evident. The major difference between the two was that the symptoms in subject IV:1 started at a much younger age, and were more severe. This could suggest genetic anticipation of an inherited mutation, as previously suggested [622]. However, there are a number of reports of a more severe phenotype when symptoms begin at an earlier age [623], [574], [565]. Whilst the onset of DRD in subject III:5 is much later than the typical childhood onset there are reports of subjects presenting as late as the sixth decade, with less severe symptoms [565].

There are multiple affected members of this family who exhibit various features consistent with a movement disorder. Although there is too little clinical information available on affected subjects from previous generations, i.e. subjects II:1 and II:2, to make definitive conclusions, the reported phenotype of subject II:1, father of the proband, does appear to be similar to that of the proband and subject III:4.

Whilst it was possible that two separate conditions may be inherited within this family, the working hypothesis was of a single AD inherited condition that provides the unifying diagnosis to explain all the observed phenotypes i.e. a single genetic variation with variable expression. Given the pattern of symptoms and signs, and negative clinical investigations such as cerebral MRI scans, there were only two realistic differential diagnoses to be considered. The first was fPD and the second was DRD. This is discussed further below in section 4.5.3.

There was also another potentially important pattern noted in the phenotypic presentation in this family, which is that the subjects with an older age of onset (III:1, III:3, III:4 and II:1) present with predominantly parkinsonian features, whilst the subjects with a younger age of onset (III:5 and IV:1) present with features typical for DRD. Assuming that the affected subjects in this family all have the same genetic variation, this suggests that either gene-environment interactions are affecting the presenting phenotype at different ages or that the effect of the genetic variation causing the condition is mitigated by a second genetic variation, thus affecting the phenotype at different ages.

4.5.1.1 Olfactory testing

Olfactory dysfunction is found in 70-100% of PD patients [108], [109], [110] and is associated with neuronal loss and Lewy body deposition in the olfactory pathway [112]. Furthermore, olfactory dysfunction is markedly reduced in PD from the early stages (UPSIT mean scores of 20-21) [113], [108], [114], [115]. Given that DRD is a result of dopamine deficiency without neuronal cell death [600], the UPSIT-40 data would be expected to be normal in patients with this condition.

In my study, the mean UPSIT-40 score for the affected subjects was 26.6 (SD 6.6). In a recent study of olfaction in PD, the mean score in 18 British subjects with IPD was 17.1 and in 27 age-matched controls it was 27.6 [118]. The individual subject scores were compared to the normative values and percentiles for age and gender [482]. The individual UPSIT score from this study were 21 for subject III:1 (Severe Microsmia, 40th percentile), 31 for subject III:2 (Mild Microsmia, 71st percentile), 21 for subject III:3 (Severe Microsmia, 23rd percentile), 28 for subject III:4 (Moderate Microsmia, 31st percentile), 26 for subject III:5 (Moderate

Microsmia, 24th percentile) and 37 for subject IV:1 (Normosmia, 40th percentile). Therefore, apart from the youngest subject IV:1 all the affected subjects had at least moderately reduced olfactory function, but there was no clear correlation of loss of smell to a particular phenotype within the family. Indeed, even the score for subject III:1, who was most severely affected by parkinsonian symptoms and had a symptom duration of 24 years, was not as low as that of the average score of 17.1 from the previous study of IPD [118]. This data indicates that the olfactory pathway is only moderately affected by the underlying condition in this family, which is perhaps not surprising given that the symptoms in the affected members of this family have been so slowly progressive. However it does suggest that there may be some associated PD-type CNS pathology.

4.5.2 Functional neuro-imaging

DaTSCANs were performed in this study to provide further supportive evidence for the clinical diagnoses, particularly given the atypical phenotypic features in this family. Indeed the data from these scans supported the clinical diagnoses in most cases.

The scans for subjects III:1 and III:3 are abnormal as would be expected for subjects with PD or parkinsonism [128], [129], [130]. The scans for subjects III:2, III:5 and IV:1 are normal, as would be expected for an unaffected subject and for subjects with DRD, in which dopa uptake, dopa decarboxylation and dopamine storage in the nigrostriatal dopaminergic terminals should be normal [603], [604], [605], [606].

The DaTSCAN data for subject III:3, who had a three year history of slowly progressive parkinsonism, is more difficult to explain. His first scan was abnormal, as would be expected

for a subject with parkinsonian. However, the second scan performed 18 months later was normal.

As outlined in the introduction, there is a category of subjects with parkinsonism who have normal DaTSCANs, so called SWEDDs [137]. 11-15% of subjects with PD have been found to have normal nigrostriatal uptake of presynaptic ligands [132], [133], [134], [135], [136]. It has been proposed that some of these patients with SWEDDs may have been misdiagnosed with PD, and may actually have an alternative diagnosis, such as essential tremor [137] or dystonic tremor [136].

However, this would not explain why for subject III:3 his DaTSCAN had changed from being abnormal to normal. There are several possible explanations for this, first that there was a technical problem. However, this seems unlikely as both scans were performed using the same technique on the same scanner. Second, a change in medication or use of CNS stimulants can affect the scan. However there was no change in the subject's medication and no reported use of medications such as CNS stimulants (for example amphetamines or sympathomimetics). Perhaps the most likely explanation may be test/rest variability and indeed DaTSCAN test/retest variability in healthy controls and PD has been reported as 7% [131]. The use of another form of functional neuro-imaging, which has traditionally been regarded as the gold standard for assessing the function of the dopaminergic nigrostriatal pathway, 18F-fluorodopa PET [624], may be able to clarify whether there is indeed any abnormality within the dopaminergic neurons in the basal ganglia of this subject.

In summary, whilst the DaTSCANs in this family do in general correlate with clinical phenotype, what is really intriguing is that both abnormal and normal scans have been found in subjects carrying the same *GCH1* mutation, as is discussed below.

4.5.3 Molecular genetic studies

4.5.3.1 PD genes

The coding sequence of the five genes for Mendelian PD for which there is strongest evidence: *alpha-synuclein*, *Parkin*, *PINK1*, *DJ-1*, and *LRRK2* were screened for mutations. There were no novel or known mutations identified in the coding sequence of *alpha-synuclein*, *Parkin*, *PINK1*, *DJ-1* or *LRRK2*.

Given the phenotypes observed in this family the negative screening is perhaps not surprising, as apart from *Parkin* they differ markedly from the phenotypes associated with mutations in the other PD genes. The phenotypes associated with the AD genes *alpha-synuclein* and *LRRK2* are very similar to that of IPD, although there is a relative paucity of tremor, younger onset, and longer disease course, reported with *alpha-synuclein* [242], [239], [288], [283], [289], [290], [285], [625].

Of the recessive genes, *Parkin*-related PD is the most common, with homozygous mutations responsible for approximately half of patients with autosomal recessive fPD with disease onset before the age of 45 years [304]. The typical associated phenotype shows early onset of parkinsonism with slow disease progression, good levodopa response, and levodopa-induced dyskinesias. Dystonia at onset is also seen more frequently than in IPD and indeed the phenotypical spectrum overlaps with classical PD for late-onset cases, and with DRD [234], [235], [300], [236], [626]. Interestingly, pseudodominant inheritance has also been reported with *Parkin*-related PD [301], [627], [628], [629]. This is a description of an inheritance pattern where a parent and child are affected by a phenotype that is normally inherited in a recessive pattern. This inheritance pattern may occur when a phenotype, which is normally inherited in a recessive pattern, does not reduce reproductive fitness and the carrier rate is high [630], [631]. PD associated with *PINK1* and *DJ-1* mutations is much rarer than *Parkin* related

PD but with a similar phenotype to that of typical *Parkin* PD [227], [320], [321], [342], [344], [345].

Therefore of all the PD genes, a mutation within *Parkin* was most likely to explain the wide range of phenotypes seen within this family. Although no mutations were identified in the coding sequences, it was important to test gene dosage especially for the *Parkin* gene. This data is discussed below in section 4.5.3.2

4.5.3.2 GCH1

The novel heterozygous missense mutation in the first exon of *GCH1* (c.A5G) identified in four affected family members is not listed as a known mutation (www.biopku.org/dbsearches/BIOMDB_Results.asp), or a polymorphism (www.ensembl.org/Homo_sapiens/geneseqview?db=core;gene=ENSG00000131979). It was also not detected in 300 UK control chromosomes. This novel *GCH1* mutation is predicted to replace glutamic acid with glycine (p.E2G) in GTPCH1, a change predicted to alter function in the protein as one of the larger hydrophilic amino acids is replaced by a small hydrophobic one.

The first exon of *GCH1* is the area of the gene that mutations are most commonly found in association with DRD (see figure 4.2). The M1X and E2X mutations are of particular interest as they occur so close to the site of our novel mutation. The M1X mutation was identified in a 36 year old female, and her asymptomatic mother, who had symptoms of gait difficulty and limb dystonia since the age of 8. At the age of 20 she began to complain of a postural tremor of her hands, her symptoms showed diurnal fluctuation and responded to Levodopa therapy. This subject was heterozygous for a base substitution (c.G3C) in the initiation codon, which

results in a frameshift and a small 46 amino acid peptide translation product [632]. The E2X mutation was identified in two individuals from a family (mother and daughter). They were heterozygous for a two base insertion (c.3insGG) which results in a frameshift and production of a truncated protein [574].

Our novel mutation does not cause a frameshift, but knowledge of the structure and functional domains of GTPCH1 may provide clues as to how the mutation could be pathogenic for DRD. GTPCH1 is a homodecamer assembled from five dimmers into a ring-like structure that has the appearance of two pentamers aligned face to face [633]. Only the GTPCH1 decamer has enzyme activity [634]. The C-terminal portion of GTPCH1, which is directly involved in catalysis, has evolved conservatively with 55% of the terminal 100 amino acids being identical in human and bacterial proteins [635], [636]. However, the N-terminal region of GTPCH1 shows a high degree of species diversity, with primates having a nine amino acid N-terminal extension (see figure 4.14). This suggests that that the N-terminus may be involved in functions unrelated to catalysis, such as the binding of accessory proteins [637]. This region may also be involved in the interaction with the GTPCH1 feedback regulatory protein (GFRP), by stabilizing the protein-protein complex [637]. It is interesting to note the large number of apparently pathogenic mutations identified in this region, indicting that any change in the amino acid composition of the N-terminal region could significantly effect the normal functioning of the protein.

It has been proposed that most GCH1 mutations exert a dominant negative effect, with the mutant polypeptide interacting with the wild-type, to form dysfunctional GTPCH1 heterodecamers. The activity of GTPCH1 has been shown to be reduced to less than 20% that of normal controls in phytohemagglutinin-stimulated lymphocytes [574] and biopterin

concentrations in the putamen of two autopsied subjects with DRD were also found to be reduced to 16% of that of age-matched control subjects [570]. More recent co-transfection experiments with wild-type and mutated GCH1 cDNA also supported this theory [638]. However, formation of heterodecamers was not demonstrated for two other DRD missense mutations where aberrant subunits were not able to interact with the wild-type subunits [639]. Whilst these mutations could also exert a dominant-negative effect at the transcriptional level, the recent findings of deletions of *GCH1* suggests that at least in these cases haploinsufficiency of GTPCH1 may be the molecular cause of signs and symptoms of DRD [640].

Further evidence in support of a pathogenic role of the novel *GCH1* mutation identified in the current study is provided by the results of the MLPA screening which did not reveal any copy number variation in *GCH,* or in any of the PD genes *alpha-synuclein*, *Parkin*, *PINK1*, *DJ-1* and *LRRK2.* MLPA is a recently developed technique which has been shown to be as reliable and more time- and cost-efficient in detecting copy number variation as quantitative real-time PCR [640], [641].

4.5.4 DRD or PD or overlap?

The identification of a novel missense mutation in *GCH1* in four affected subjects suggests that the AD inherited movement disorder within this family is indeed a variant of DRD. Subjects III:5 and IV:1 displayed a typical DRD phenotype, although an age of onset of 44 for subject III:5 was substantially later than the usual childhood or adolescent presentation. However, symptoms in subjects III:1 and III:3 started later and they displayed a predominantly parkinsonian variant of DRD.

Whilst DRD typically manifests as lower limb dystonia with diurnal variation the spectrum of symptoms can be broad, even occurring as a predominantly parkinsonian condition [559], [560], [623], [558], [561], [642], [562], [563], [564], [557], [643], [16]. Indeed, with a later age of onset subjects appear more likely to present with parkinsonian features rather than dystonia and diurnal fluctuation [626], [644], [575], [645]. Furthermore, marked intra-familial variability of the clinical manifestations between affected members with the same mutation has been reported previously [566], [567].

There are several previous reports in which affected subjects within the same family have typical and atypical presentations of DRD, although in most instances molecular genetic analyses were not performed. Nygaard et al. described a family in which the proband had typical DRD whilst three members over the age of 50 developed a parkinsonian syndrome without dystonia. 18F-fluorodopa PET scans performed on the proband and two of the subjects with parkinsonian presentations were normal [646]. The investigators proposed that the parkinsonism was an expression of the disease and that their findings did not support consideration of the DRD gene as a risk factor for PD [646]. Harwood et al. reported a family in which some members presented in childhood with a typical dystonia whilst others presented as adults with a parkinsonian syndrome [647]. Most recently Grimes et al. reported that a female member of large DRD family, with a heterozygous 18 base pair deletion in exon 1 of *GCH1,* presented with parkinsonism in the absence of dystonia at the age of 56 [644].

Interestingly, the proband case (III:1) in our study displayed treatment-induced dyskinesias, but a low incidence of dyskinesias and the ‘on-off phenomena’ associated with long-term Levodopa therapy in PD, have previously been reported in DRD [16]. Indeed this difference has been considered to distinguish DRD from fPD, particularly associated with *Parkin*

mutations. The reason for this difference is unknown, but may be due to differences in nerve terminal integrity and/or postsynaptic receptor changes. However, there are now several reports in which subjects with *GCH1* mutations have been shown to display levodopa-induced dyskinesias [626], [644], [648], [649], [650].

This raises the question as to whether *GCH1* mutations may in fact be the cause of some cases of fPD and to my knowledge there is only one study in which subjects with PD were screened for *GCH1* mutations. This study was of 87 Danish subjects with early onset PD, including both familial and sporadic disease, no *GCH1* mutations were identified [651].

4.5.4.1 Phenocopy or more than one disease?

The novel *GCH1* mutation is not carried by subject III:3 and would not therefore explain his symptoms. The simplest and perhaps most likely explanation is that he is a phenocopy and has mild form of sporadic PD. Phenocopies in *GCH1* mutation positive families have previously been reported [644], [652]. Furthermore, this subject had the oldest age of onset of symptoms (75) and from previous studies of large families it has been suggested that the later the disease onset the less likely that the individual is truly affected by DRD [644].

The other more complex explanation is that there is in fact more than one inherited movement disorder within family 6. The father of the proband (II:1) was reported to have displayed symptoms of a condition which started in his 50's and appeared to be very similar to the condition displayed by subjects III:1 and III:4. Therefore one could hypothesize that he may have carried the novel *GCH1* mutation which he passed on to his offspring. A maternal aunt of the proband (II:2) was reported to have had PD which was diagnosed in her 70's and indeed her symptoms appeared more typical of IPD. Her grandchildren, subjects IV:2-IV:4 who are

unaffected, do not carry the *GCH1* mutation. Therefore one could speculate that subject II:2 carried a separate mutation for late onset PD which has as yet not been identified from the molecular genetic screening performed on family 6. If this mutation shows incomplete penetrance, as has been reported in other forms of AD PD [283], [287], [281], [279], [248], [653], it may also have been carried by subject II:4 who passed it onto her offspring. Therefore subject III:3 may carry this unknown mutation which has resulted in him developing late onset PD. Furthermore, whilst the predominantly parkinsonian variant of DRD displayed by subjects III:1 and III:4 could be caused by the novel *GCH1* mutation alone, it is possible that they also carry this unknown mutation and it is the combination of the two mutations which has resulted in their atypical phenotypes. How this interaction would occur to alter the clinical expression of the DRD from a dystonia to parkinsonism is unknown. Following this theory through, subjects III:5 and IV:1, who displayed the more typical DRD phenotype, may have inherited the novel *GCH1* mutation alone. The only way to prove this theory would be to identify another AD inherited gene mutation within this family.

4.5.4.2 Functional neuro-imaging in DRD

The theory of two separate AD inherited movement disorders within this family is based on a number of assumptions, most notably reduced penetrance of an unknown mutation. The abnormal functional neuro-imaging data obtained on subjects III:1 and III:4 might provide support for the coexistence of DRD and PD within these two individuals. Indeed, this has been investigated in previous studies. Hjermind et al. presented a case report concerning a 38 year old subject with a combination of dystonic and parkinsonian signs which began at the age of 28. He was found to have a point mutation in exon 5 of *GCH1*, an abnormal DAT scan and

treatment induced dyskinesias. Although the subject did not carry a *Parkin* mutation, the authors concluded that he may either have a variant of DRD or alternatively DRD and early onset PD [650]. Postuma et al. reported the case of a subject with onset of DRD aged 15 years, who presented with a prominent isolated leg tremor and carried a point mutation in exon 5 of *GCH1*. Although her 18F-fluorodopa PET scan showed that uptake on both sides was within normal limits, there was some asymmetry noted. However, she was also found to have polymorphisms of the *Parkin* gene in a compound heterozygous state. The authors speculated that either of the *Parkin* polymorphisms could change its *in vivo* activity and alter the clinical expression of her DRD [654].

Kikuchi et al. reported a 54 year old subject with adult onset DRD and parkinsonism (aged 39) who carried a point mutation in exon 5 of *GCH1*, with no mutations detected in the coding region of *Parkin*. Motor fluctuations were noted several years after starting treatment. He was also found to have an abnormal 18F-fluorodopa PET scan [648]. Furthermore, Turjanski et al. reported abnormal 18F-fluorodopa PET scans on six subjects with adult-onset dystonia-parkinsonism, which appeared similar to those of 12 age matched subjects with PD [571], although these subjects were not analysed genetically.

Therefore whilst functional neuro-imaging in DRD has previously been reported to be normal [603], [604], [605], [606], the data from the last two studies suggests that the DaTSCAN abnormalities observed in subjects III:1 and III:4 with atypical DRD could be due to DRD alone.

4.5.4.3 Variable expression of GCH1

If the phenotypes in this family are indeed due to the novel *GCH1* mutations alone a mechanism to explain the variable expression of this mutation is required, particularly the association of parkinsonian features with a later age of onset. One proposed mechanism is that age-dependent differences in the function of the basal ganglia-thalamocortical motor loop may influence the development of parkinsonian features in adult-onset DRD [655]. Another possible explanation is that chronically decreased GCH-I activity may result in impaired synaptic function in the nigrostriatal pathways [570], [569], [563]. It has also been proposed that dopamine transporters are decreased in the putamen, possibly reflecting a regression of nerve terminals derived from nigral dopaminergic neurons [570], [569].

4.5.5 Pathological studies

Study of the ENS in subjects III:1 and III:4 did not reveal the presence of any pathology suggestive of PD. Neuronal loss (with LBs and LNs) has previously been identified in the ENS in subjects with PD, although no lesions were found to occur in the ENS in the absence of immunoreactive inclusions in the lower brainstem [607], [608], [609], [610], [611], [612], [613], [614], [615].

The gastrointestinal specimens investigated in this study were from affected subjects III:1 and III:4 who both exhibited the PD phenotype. These findings do not support the hypothesis that the movement disorder exhibited by these two subjects is associated with a typical PD-type pathology. Therefore given that both of these subjects carry a *GCH1* mutation this would appear to suggest that their phenotype is caused by a parkinsonian-type variation of DRD.

There are a number of possible factors which must be considered which discussing this data. Firstly, there was only limited tissue available for study from one section of the gastrointestinal tract and had more tissue been available underlying PD-type pathology may have been identified. Furthermore, due to sectioning of the tissue PD-type pathology in parts of the specimen which were not examined may have been missed. Indeed, in previous studies the majority of pathological changes have been observed in the cellular processes and cell bodies of a specific neuronal type, the VIPergic neuron [610], [616]. Secondly, fPD can be associated with various types of pathology. Indeed in *LRRK2*-associated PD pathology ranging from neurofibrillary tangles to pure nigral degeneration without LBs has been reported [27], [271], [279], [656], [284], [657], [658], [659], [625]. Therefore, a pathological process specific to the basal ganglia, for example without the presence of LBs, might not be evident on examination of the ENS only. Finally, because there are relatively few reports on the pathological findings in the ENS in subjects with PD, it is not known how common even typical PD-type pathology is in the ENS. Hence, the chance of identifying the presence of PD-type pathology in an individual subject, or even two subjects, may in fact be very low.

Therefore, despite study of the gastrointestinal tissue on two affected members of this family it is not possible to make definitive conclusions as to the underlying pathological process in this family, and to be certain of this post mortem brain tissue is required.

4.5.6 Future work

There are clinical, functional neuro-imaging and molecular genetic studies that can be done to further our knowledge on both this unique family and the implications of this novel *GCH1* mutation for our understanding of DRD.

The first is to assess the proband subject (III:1) off his anti-parkinsonian therapy to further clarify the phenotypes associated with the novel *GCH1* mutation. If other family members become affected, assessment of their phenotype would also help with this clarification. The second is to screen other unaffected family members for the *GCH1* mutation to allow an estimate of the penetrance of this mutation. This information will be crucial for future genetic counselling of family members. The third is to clarify the apparently contradictory functional neuro-imaging results for subject III:3. As discussed in section 4.5.2, this could be done with the use of a 18F-fluorodopa PET scan. The fourth is to screen for the novel *GCH1* mutation in the UK fPD cohort which I have recruited (see chapter 3). Given the phenotype of late onset PD in this family, it is important to determine if the novel *GCH1* mutation may also be important in fPD. The fifth is to determine whether the functional effects of this novel mutation are similar to those previously reported in DRD. Bonafe et al. have shown that in cytokine-stimulated skin fibroblasts intracellular neopterin and biopterin concentrations were low and GTPCH activity was reduced. They concluded that this technique was helpful in diagnosing subjects with DRD [660]. In collaboration with Professor Malcolm Taylor, in the Institute for Cancer Studies, University of Birmingham, I have generated fibroblast cultures for subjects III:1, III:2, III:4 and III:5, which will allow us to repeat this work in these four family members with varying phenotypes. The sixth is to screen for any novel gene mutations associated with PD, which may be identified in the future by other investigators, in the five affected members of the family. This would allow further clarification as to whether the wide range of phenotypes could be due to the subjects carrying mutations in more than one gene associated with parkinsonism.

4.6 Conclusions

The aims of this chapter outlined in section 4.2 have largely been met. The clinical diagnosis in affected subjects from this unique family has proven challenging for over 20 years because of the atypical nature of the symptoms and signs. However, the identification of a novel heterozygote *GCH1* missense mutation, in affected subjects, has at last provided an explanation for the diverse clinical presentations observed in this family, and will allow more accurate genetic counseling.

The association of this *GCH1* mutation with such a wide range of phenotypes also furthers our understanding of the way late onset DRD can present. Furthermore, the association of this novel *GCH1* mutation with a late onset parkinsonian phenotype and abnormal functional neuro-imaging, suggests a potential role for the *GCH1* gene in PD, which requires further investigation.

5 Comprehensive Molecular Genetic Analysis of the *LRRK2* Gene in a UK Familial Parkinson's Disease Cohort

5.1 Introduction

In chapter 3 demographic and clinical data was presented and discussed on 46 UK families in which there were three or more members affected by PD, where segregation in each family was consistent with an autosomal dominant (AD) pattern. In this part of the study I screened the proband case from each of these families for known and novel coding sequence variations in all 51 exons of *LRRK2*.

The clinical phenotype associated with *LRRK2* mutations in subjects with both familial and sporadic PD, in several different populations, closely resembles that of idiopathic PD (IPD) [288], [283], [289], [290], [285], [625]. Indeed *LRRK2* has emerged as potentially the most relevant of the PD genes identified to date for our understanding of IPD.

5.1.1 LRRK2 Background

As has been outlined in the introduction, *LRRK2* is a large gene, located on chromosome 12 (12p11.2-q13.1) which contains 51 exons and encodes a protein of 2527 amino acids (286kDa) [25], [27]. Sequence analysis indicates that the LRRK2 protein contains several discrete domains (see figure 5.1). LRRK2 is predominantly a cytoplasmic protein and is associated with cellular membrane structures such as mitochondria, Golgi apparatus, endoplasmic reticulum and cytoskeleton [429], [428], [661], [656], [662], [312]. At the protein level, LRRK2 expression has been shown in brain and peripheral organs [656], [663].

The presence of the protein-protein interaction domains (ankyrin, LRR and WD40) suggests that LRRK2, in addition to protein kinase and GTPase activities, may also serve as a scaffold to a multiprotein complex. The physiological function of LRRK2 is not yet established. However, there is evidence from *in vitro* as well as *in vivo* over-expression and mutagenesis experiments to suggest that the protein is likely to regulate neurite maintenance and neuronal survival. MacLeod et al. used a rodent model in which they generated a mouse/human chimera. Over-expression of PD-associated mutant *LRRK2* alleles (G2019S and I2020T) in the kinase domain led to reduced neurite complexity, the formation of tau-positive inclusions, lysosomal abnormalities and apoptotic cell death [664].

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5.1.2 Molecular genetic studies of LRRK2-related PD

The past three years has seen an explosion in the number of studies performed on *LRRK2* on a number of different populations of PD patients throughout the world. Five missense mutations, which are believed to be definitely pathogenic, have been identified: R1441C, R1441G, Y1669C, G2019S and I2020T [18], [278]. However, a number of other variations have been identified, the significance of which are still being evaluated. Overall, *LRRK2* mutations appear to account for approximately 10% of familial PD (fPD) cases with AD inheritance [279], [280], [665], [26], [293], [666].

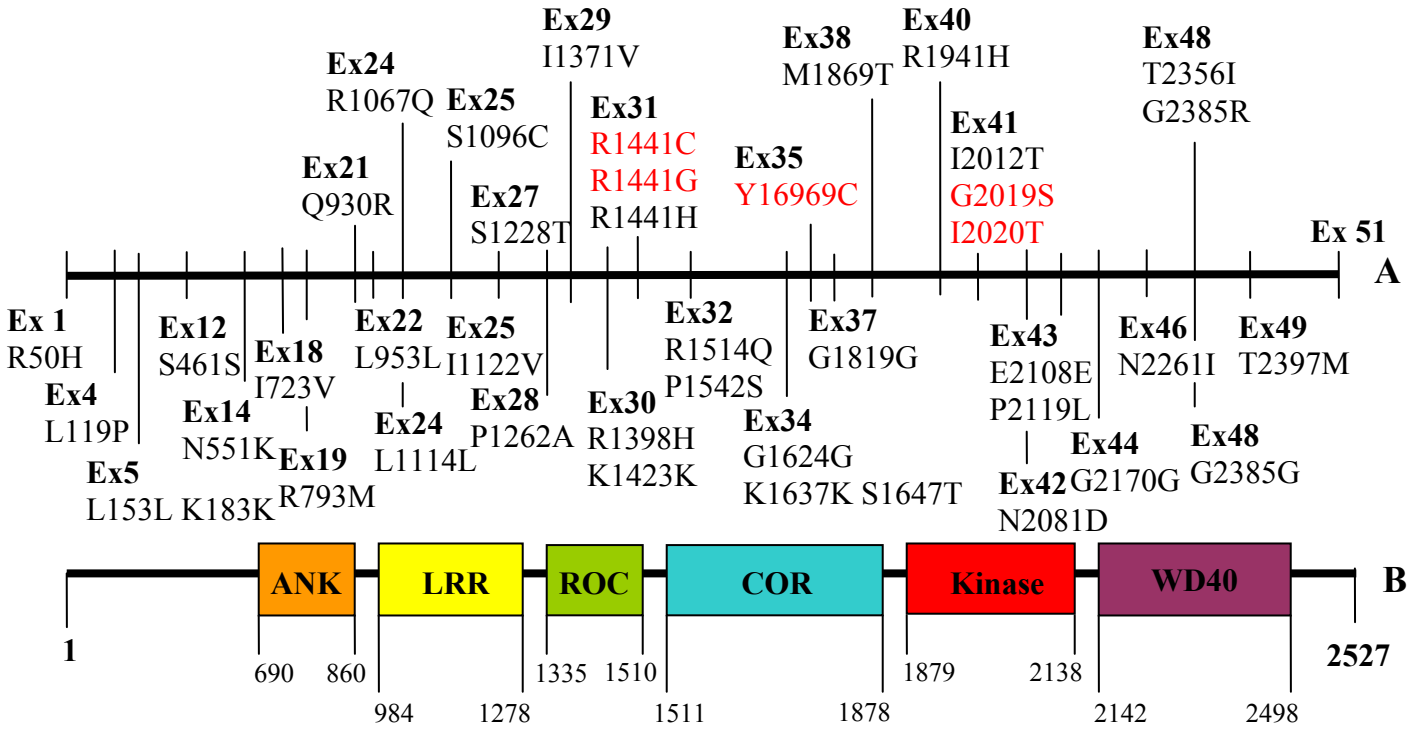


Figure 5.1 Schematic representation of LRRK2 protein domain structure and *LRRK2* coding sequence variants identified to date. Panel A= Schematic representation of *LRRK2* exons 1-51 with amino acid variants described to date. Putatively pathogenic amino acid substitutions are shown above, with those segregating with disease in red. Variants believed to be polymorphisms are shown below. Corresponding exon numbers are shown in black. Panel B= schematic representation of LRRK2 protein structure from residue 1-2527, with estimated domain boundaries indicated by residue numbers beneath. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Adapted from Mata et al. 2006 and Nichols et al. 2007 [18], [26].

One of these *LRRK2* mutations, G2019S (c.6055G>A) in exon 41, appears to be particularly important as it alone accounts for up 5% to 6% of familial and 1% to 2% of sporadic PD (sPD) cases [282], [283], [284], [285], [281], [282], [667]. Furthermore, this mutation is found in sPD at even higher frequencies in certain populations, including Askenazi Jewish (18%) [668]

and North African Arab subjects (41%) [287]. The discovery of the G2019S mutation in apparently sPD raises the possibility of a link between fPD and sPD.

It had been proposed that subjects of European origin, Ashkenazi Jews and North African Arabs carrying the G2019S mutation all inherited the mutation from a single common Middle Eastern founder [669], [670], [281], [671], [672]. However Zabetian et al. observed two distinct haplotypes; haplotype 1 was present in families of Ashkenazi Jewish and European ancestry, whereas haplotype 2 occurred in European American families. They estimated that the families with haplotype 1 shared a common ancestor 2,250 years ago, whereas those with haplotype 2 appeared to share a more recent founder [673]. Two Japanese patients heterozygous for the G2019S mutation shared a haplotype distinct from that observed in Europeans, thus suggesting that G2019S originated from separate founders in Europe and Japan [674], [673].

5.1.3 Clinical studies LRRK2-related PD

The clinical phenotype of most *LRRK2* mutation positive PD patients appears indistinguishable from that of typical IPD, including a good response to Levodopa [27], [288], [283], [289], [290], [285], [625]. A recent large study reported that the age of onset of *LRRK2*-related PD is approximately 60 years [675], although a variable age at onset for subjects carrying the G2019S mutation, ranging from 28 to 86 years, has been reported [287], [282], [676], [677], [678], [290], [679], [279], [292], [286], [284], [281], [680], [293], [675]. It is interesting that there does not appear to be a gene dose effect associated with the G2019S mutation. This has been demonstrated by the finding that subjects with homozygous G2019S mutations have an age of onset (30-82 years) almost identical to those of heterozygous

G2019S mutation carriers [669], [681]. Furthermore, Ishihara et al. described the phenotypes of 26 PD subjects with homozygous G2019S mutations, from an international consortium of studies. Their phenotypes were indistinguishable from subjects with heterozygous G2019S mutations [681].

As has been outlined in the introduction, functional imaging with SPECT ligands for the dopamine transporter ([^{123}I]FP-CIT, [^{123}I]β-CIT, [^{123}I]IPT, and [^{99m}Tc]Trodat) provides a marker for presymptomatic neuronal degeneration, and striatal uptake correlates with disease severity, in particular bradykinesia and rigidity [126]. There have been some functional imaging studies on subjects with *LRRK2* mutations, mostly in subjects with fPD, but also some in sporadic disease. In general the pattern of nigrostriatal dopaminergic dysfunction seen in heterozygous carriers of *LRRK2* mutations was similar to that observed in IPD [682], [683], [279], [684], [290], [685], [686]. We await functional imaging studies on subjects who are homozygous carriers of *LRRK2* mutations.

5.1.4 Comprehensive screening for LRRK2 mutations

Despite the intense research into *LRRK2*-PD over the last 3 years there are relatively few comprehensive screens of the whole coding region of the gene. This is understandable because of the large size of the gene, and the complexity and cost involved of screening all 51 exons. Therefore most studies have focused on testing for mutations within the known 'hot-spot' exons, such as exon 41, or even simply looking for known pathogenic mutations. Therefore the frequency of *LRRK2* mutations may have been underestimated and other potentially important variations missed.

Including one of the original studies in which *LRRK2* was identified as the PARK8 gene, to date there are only twelve studies that have performed a comprehensive analysis of the whole coding region of the *LRRK2* gene in large cohorts of subjects with PD [27], [292], [279], [293], [294], [280], [26], [665], [687], [666], [688], [689]. These studies had cohort sizes ranging from 23 to 272 subjects. In the most part these subjects had been recruited from tertiary referral centres. In another study analysis was limited to 29 exons [690]. In four other studies in which a comprehensive analysis of the coding region of the *LRRK2* gene was carried out, this was only performed on small numbers of families, ranging from 1 to 15 [283], [290], [281], [659].

The overall findings from these studies were that pathogenic *LRRK2* mutations were detected in 5-12% of subjects where segregation of PD in their families was consistent with an AD pattern, the associated disease phenotypes appeared similar to IPD, and the mutations with unequivocal disease segregation were clustered in the C-terminal half of the protein [27], [292], [279], [293], [280], [26], [665], [687], [666], [688].

5.1.5 Functional effects of the pathogenic LRRK2 mutations

There has now been some work performed on the five mutations which are believed to be pathogenic on the basis of co-segregation data: R1441C, R1441G, Y1669C, G2019S and I2020T [18], [278]. [278]. *In vitro* expression of several mutant LRRK2 proteins (R1441C, Y1669C, G2019S and I2020T) has shown to cause toxicity in cultured cells [661], [457], [691], [664], [312]. Biochemical studies on cultured cells transfected to express LRRK2 have demonstrated a modest (0.5- to 3-fold) but consistent increase in kinase activity for the G2019S mutation [429], [661], [691], [692]. The effect of the other *LRRK2* mutations on

kinase activity is more controversial. The functional effects of *LRRK2* mutations identified in this thesis are covered in more detail in chapter 7.

5.2 Aims of the chapter

The first aim of the work in this chapter was to screen all 51 exons of *LRRK2* for known and novel mutations in proband subjects from 46 UK families with three or more affected members, where segregation of PD in the family was consistent with an AD pattern.

The second aim of this chapter was to screen for any novel potentially pathogenic variations detected in the coding sequence of *LRRK2* in other affected and unaffected members of the relevant proband's family, and within cohorts of subjects with sPD and controls. This was to determine whether any novel variations segregate with the disease and to assess their possible role in sPD.

5.3 Subjects and methods

5.3.1 Subjects

The first part of this chapter involved the study of subjects with familial PD (fPD) from around the UK. I recruited a total of 46 proband cases from families where segregation of PD was consistent with an autosomal dominant pattern, with three or more PD cases in at least two consecutive generations, between April 2005 and March 2008, as covered in section 2.1.1. In addition, from these 46 families there were seven other members affected by PD and 16 unaffected family members who I also recruited to the study.

I assessed olfactory function in all subjects by use of the University of Pennsylvania Smell Identification Test (UPSIT-40) (Sensonics, Haddon Heights, NJ). Olfaction was categorised as normal or pathologic according to normative data in relation to gender and age [482], [483].

The second part of this chapter involved the screening of other cohorts of familial and sporadic PD for novel *LRRK2* variations I had detected. The following cohorts of PD patients and controls were used: (i) PD GEN cohort of PD patients (see section 3.5); (ii) NDD cohort of PD patients (see section 3.5); (iii) PINE cohort of PD patients (see section 3.5); (iv) Queen Square PD patient and control collection (see appendix B) (iv) PD GEN control cohort (see appendix B); (v) PINE cohort of controls (see appendix B); (vi) NDD cohort of controls (see appendix B); (vii) Birmingham MND control collection (see appendix B).

5.3.2 Methods

I screened all 51 exons of *LRRK2* by PCR and sequencing for known and novel mutations in the 46 proband subjects. In addition, where pathogenic mutations or novel coding sequence variations were identified in proband subjects, and other affected or unaffected family members had been recruited to the study, I also screened for these specific variations by PCR and sequencing of the relevant exon. Subsequently, I designed specific assays to screen for novel *LRRK2* coding sequence variations detected in this study in cohorts of patients with sporadic and familial PD, and controls.

5.3.2.1 DNA extraction

I extracted DNA from venous blood as described in section 2.3. DNA was also extracted from flash frozen brain tissue by Cathy Woodward, according to standard protocols, in the laboratory of a collaborator Professor Nicholas Wood, Institute of Neurology, London.

5.3.2.2 PCR and sequencing

I studied the whole coding sequence and exon-intron boundaries of the *LRRK2* gene by PCR using primers and PCR temperatures as described in previous studies [283], [280]. Primers and reaction conditions are detailed in appendix C, each PCR was optimized as described in section 2.4.

In addition, I designed specific primers for screening for novel *LRRK2* variations detected within exons 42 and 49 by using the Primer3 program (http://fokker.wimit.edu/primer3/input.htm). Primers selected and reaction conditions are detailed in appendix C. Each PCR was optimized as described in section 2.4.

I performed all sequencing using the dideoxy DNA sequencing method as described in section 2.7. The consequences of mutations on the amino acid sequence were predicted according to *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511). The effects on protein structure and function were predicted using the Protein Sequence Analysis Launcher (ProSAL)- (http://xray.bmc.uu.se/subnet/prosal.html), the Oriel Gene Mining Tools- (http://bioinfo.itb.cnr.it/oriel/spilce-view.html) and the Exonic splicing enhancers finder (ESEfinder)- (http://rulai.cshl.edu/tools/ESE2).

5.3.2.3 Restriction fragment length polymorphism (RFLP) analysis

I used RFLP, as described in sections 2.6, to screen for the G2019S mutation within exon 41 of *LRRK2,* in the Birmingham PD study and PINE study patient and control collections. The PCR primers selected and reaction conditions can be found in appendix C, the PCR was optimised as described in section 2.4.

5.3.2.4 Agarose gel electrophoresis

I used gel electrophoresis with a 3% high resolution RESponse™ Research agarose (Geneflow) gel, as described in section 2.5.1, to screen for the novel *LRRK2* variation in exon 42, identified in this study, in the NDD cohort of PD patients and controls, the PINE cohort of PD patients and controls, the Queen Square PD patient and control collection and the Birmingham MND control collection. The PCR primers selected and reaction conditions are detailed in appendix C, the PCR was optimised as described in section 2.4.

5.3.2.5 Denaturing High Performance Liquid Chromatography (DHPLC)

I used DHPLC, as described in sections 2.9 and 6.3.2.3, to screen for *LRRK2* variations in six exons (31, 32, 38, 40, 41 and 42) in the PD GEN patient and control collections. This was performed using the Transgenomic WAVE system (Transgenomic Ltd).

5.3.2.6 PCR fragment analysis

I used PCR fragment analysis, as described in section 2.8, to screen for the novel *LRRK2* variation in exon 49, identified in this study, in the PD GEN cohort of PD patients and

controls, the NDD cohort of PD patients and controls and the PINE cohort of PD patients and controls.

5.3.2.7 Functional neuro-imaging

To evaluate basal ganglia function the proband subject from family 12 underwent a dopamine transporter SPECT scan using [^{123}I]-FP-CIT SPECT (DaTSCAN, Amersham Health) at Sandwell and West Birmingham Hospital NHS Trust. This scan was reviewed by a consultant radiologist, Dr Alp Notghi, Department of Radiology, Sandwell and West Birmingham Hospital NHS Trust, experienced in the interpretation of these images, who was blinded to the demographic and clinical data on this subject.

5.3.2.8 Statistical analysis

I performed statistical analysis using the SPSS for Windows release 15.0 (SPSS Inc, Chicago, Ill., USA). Observed genotypes were compared with the Fisher's exact test (two-tailed) and interpreted as significant if the distribution differed by $p<0.05$. However, as multiple hypothesis testing was performed on the subject and control data, the Bonferroni correction (or adjustment) for multiple comparisons was used, to safeguard against these multiple tests of statistical significance falsely giving the appearance of significance. The correction was calculated using the website: (http://www.quantitativeskills.com/sisa/calculations/bonfer.htm), giving an adjusted p value of <0.002. Power calculations were made using a program developed by Heather Cordell in Microsoft Excel [693], [694], and with the advice of a statistician, Dr Sayeed Haque, Department of Psychiatry, University of Birmingham.

Where control data on *LRRK2* variations was not available from my study, the recently published control data from a North American cohort of 275 unrelated neurologically normal individuals was used [666]. This is stated in the text and the demographics of this cohort can be found in appendix E.

5.4 Results

5.4.1 Comprehensive screening of LRRK2 in familial PD

5.4.1.1 Genetic studies of 46 proband cases

By using sequence analysis, I identified 61 *LRRK2* sequence variations in this study, 15 of which had not previously been reported. These 61 variations are all listed, with frequencies, in tables 5.1-5.4.

Of the *LRRK2* variations identified, 25 were in protein coding regions. Sixteen of these coding sequence variations are predicated to lead to non synonymous amino acid substitutions (see table 5.1). Three of these heterozygous variations which had not previously been described are: (i) c.4364delAT in exon 31 which results in a substitution of glycine for aspartic acid at position 1455 (p.D1455G); (ii) c.6187delCTCTA (p.L2063STOP) in exon 42; and (iii) c.7187insGT (p.T2397STOP) in exon 49. These three variations were identified in one proband case each, from families 48, 12 and 38 respectively. All three variations cause a shift in the reading frame and are predicted to lead to the introduction of a premature termination codon, with production of a truncated protein. The chromatograms for these changes are shown below in figure 5.2. Cross species alignment of D1455, L2063 and T2397 reveals that these regions are generally conserved (see figure 5.4).

Of the previously described *LRRK2* mutations for which there is data to suggest pathogenicity, including segregation with disease, three probands were found to be heterozygous carriers of the c.6055G>A (G2019S) mutation in exon 41 (families 10, 32 and 33). In addition, one proband was found to be a heterozygous carrier of the c.4321C>T (R1441C) mutation in exon 31 (family 20). The chromatograms for these changes are shown below in figure 5.3.

Of the previously reported putatively pathogenic *LRRK2* mutations, for which disease segregation data is not available, one proband was found to be a heterozygous carrier of the c.3683G>C (S1228T) mutation in exon 27 (family 1) and one proband was found to be a heterozygous carrier of the c.5606T>C (M1869T) mutation in exon 38 (family 3).

Nine of the coding sequence variations were synonymous, of which one heterozygous variation was novel: c.5412A>G (p.G1804G) in exon 37, listed below in table 5.2. This novel variation and was identified in one proband case only.

The remaining 36 *LRRK2* variations identified were non-coding (intronic) variations, of which 13 have not previously been described, these changes are listed below in tables 5.3 and 5.4.

There were 23 *LRRK2* variations detected with a minor allele frequency (MAF) of 3% or higher, for which control data was available. The genotype frequencies did not deviate from those predicted by Hardy-Weinberg equilibrium (HWE). The genotype and allelic frequency of these variations was tested against control data for evidence of association with disease. Control data was available from my study for the *LRRK2* variation c.4624C>T, one of the coding sequence variations which leads to a non synonymous amino acid substitution (control data contained in work outlined in chapter 6). The remaining control data was taken from the recently published study by Paisan-Ruiz et al. [666]. A similar genotype and allelic frequency was observed in patients and controls for all of these variations, and statistical analysis failed

to provide evidence of an association with disease for any of the variations (see tables E.2-E.4). However, this was not designed as an association study and the analysis must be interpreted with caution given the relatively small number of cases used, and the low minor allele frequency for the majority of variations investigated. For one of the more common variations, with a minor allele frequency of 0.4, this study had 73% power to detect a difference with an odds ratio of 1.8. For one of the rarer variations, with a minor allele frequency of 0.08, this study only had 29% power to detect a difference with an odds ratio of 1.8.

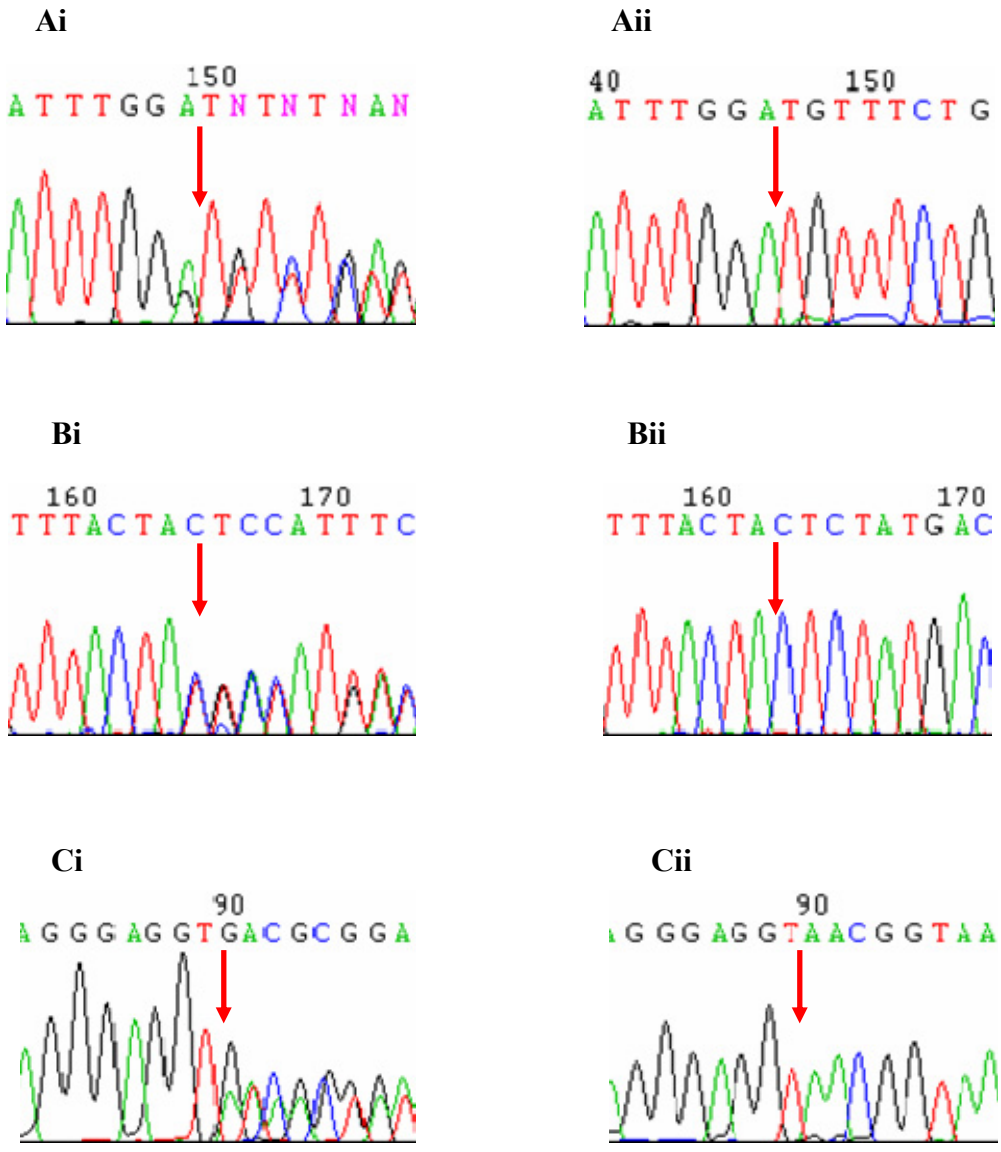


Figure 5.2 Mutant and wild-type chromatograms for three novel *LRRK2* variations identified in this study. Panels Ai and Aii- mutant and wild-type chromatograms for the c.4364delAT (D1355G) mutation in exon 31. Panels Bi and Bii- mutant and wild-type chromatograms for the c.6187delCTCTA (L2063STOP) mutation in exon 42. Panels Ci and Cii- mutant and wild-type chromatograms for the c.7187insGT (T2397STOP) mutation in exon 49. Arrows indicate position of nucleotide variations.

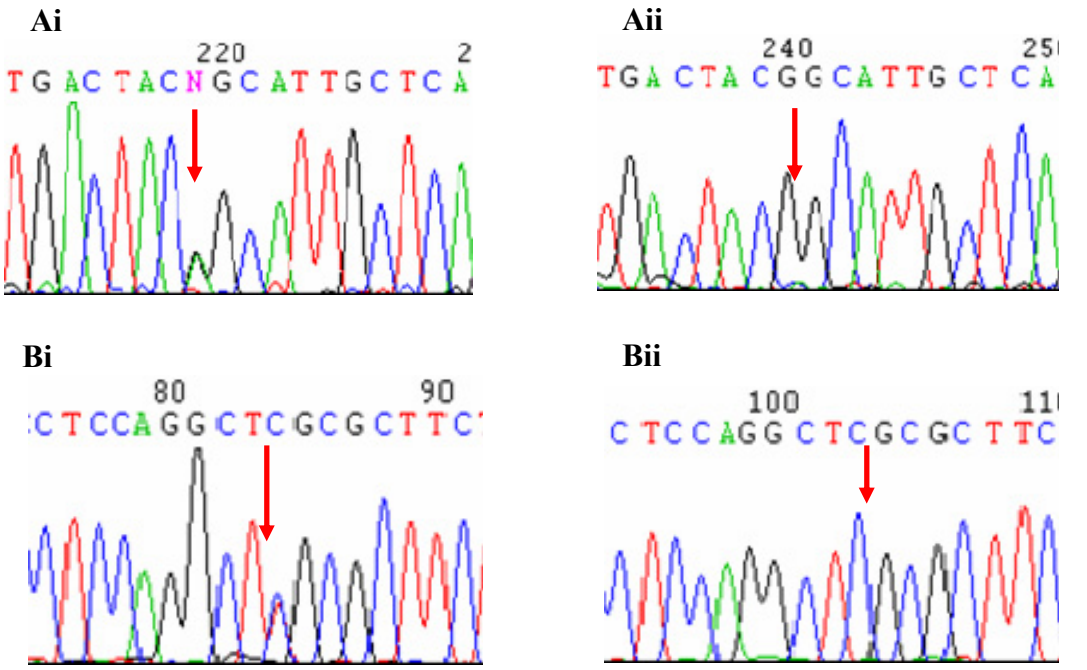


Figure 5.3 Mutant and wild-type chromatograms for pathogenic *LRRK2* mutations. Panels Ai and Aii- mutant and wild-type chromatograms for the c.6055G>A (G2019S) mutation in exon 41. Panels Bi and Bii- mutant and wild-type chromatograms for the c.4321C>T (R1441C) mutation in exon 31. Arrows indicate position of nucleotide variations.

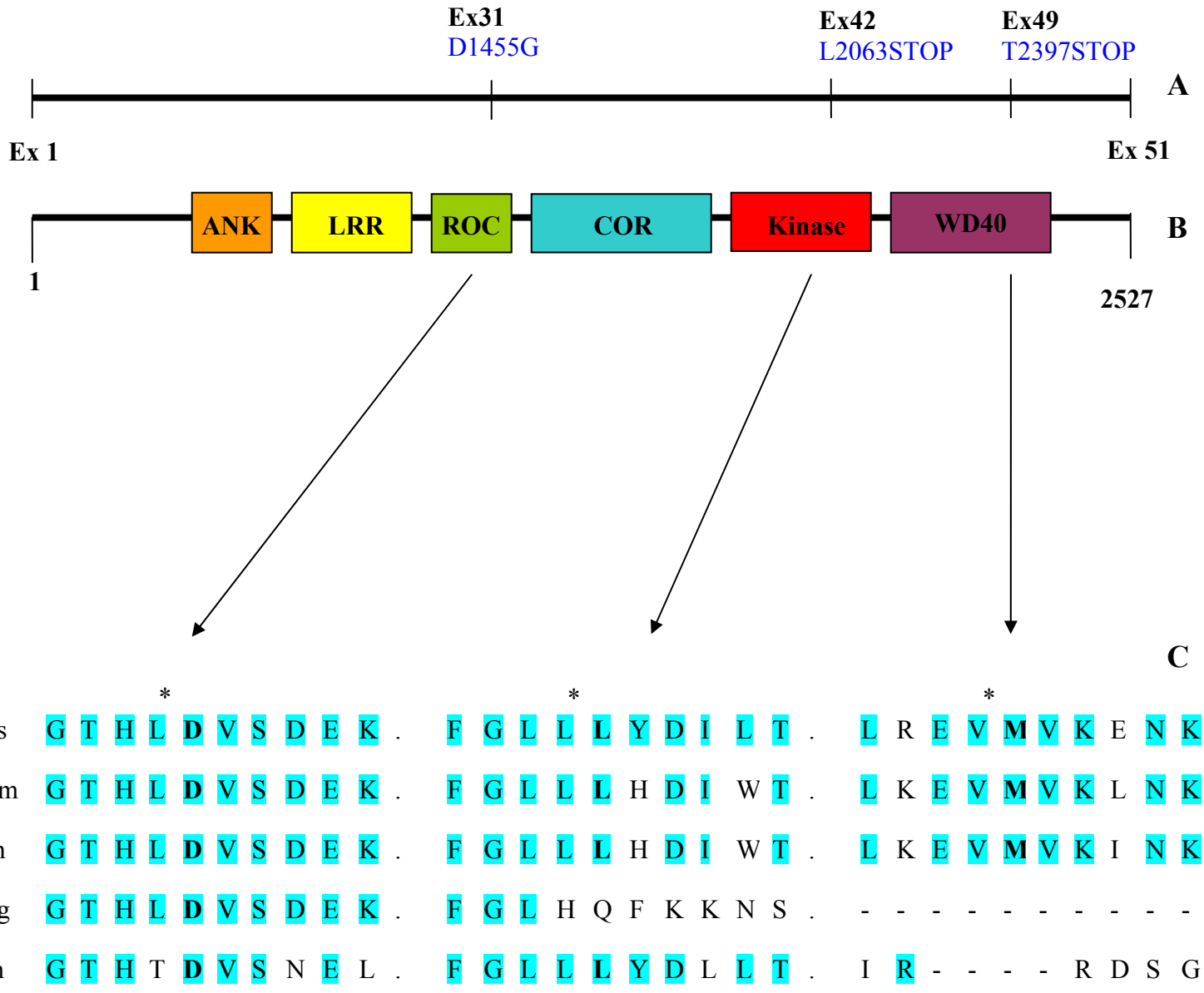


Figure 5.4 Schematic representation of LRRK2 protein domain structure, three novel *LRRK2* coding sequence variants identified in this study, and the cross species protein alignment of these variants. Panel A= Schematic representation of *LRRK2* exons 1-51 with three novel amino acid variants described in this study. Corresponding exon numbers are shown in black. Panel B= Schematic representation of LRRK2 protein structure from residue 1-2527. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Panel C= Cross species alignment of LRRK2. Human (*Homo sapiens, hs*) LRRK2 protein AAV63975.1 aligned with other orthologs from Mouse (*Mus musculus, mm*) NP_080006; Rat (*Rattus norvegicus, rn*) XP_235581; Chicken (*Gallus gallus, gg*) XP_425418.1; and Tetraodon (*Tetraodon nigrovirdis, tn*) CAG05593.1. * Represents amino acid of interest mutated in these three novel variants. X Represents conserved amino acid across species. Adapted from Zimprich et al. 2004, Mata et al. 2006 and Nichols et al. 2007 [27], [18], [26].

Nucleotide change	*LRRK2* exon	Genbank reference number	Predicted protein change	LRRK2 domain	Number of probands in which change identified
c.149G>A	1	rs2256408	p.R50H		46/46
c.356T>C	4	rs3399546	p.L119P		1/46
c.1653C>G	14	rs7308720	p.N551K		2/46
c.2167A>G	18	rs1087830	p.I723V	ANK	4/46
c.3683G>C	27	N/A	p.S1228T	LRR	1/46
c.4193G>A	30	rs7133914	p.R1398H	ROC	3/46
c.4321C>T	31	rs3393992	p.R1441C	ROC	1/46
c.4364delAT	**31**	**N/A**	**p.D1455G**	**ROC**	**1/46**
c.4624C>T	32	rs3395890	p.P1542S	COR	3/46
c.4541G>A	32	rs3550703	p.R1514Q	COR	1/46
c.4934T>A	34	rs1156414	p.S1647T	COR	29/46
c.5606T>C	38	rs3560279	p.M1869T	COR	1/46
c.6055G>A	41	rs3463758	p.G2019S	Kinase	3/46
c.6187delCTCTA	**42**	**N/A**	**p.L2063STOP**	**Kinase**	**1/46**
c.7187insGT	**49**	**N/A**	**p.T2397STOP**	**WD40**	**1/46**
c.7190C>T	49	rs3761863	p.T2397M	WD40	**22/46**

Table 5.1 Sixteen *LRRK2* coding sequence variations which lead to non synonymous amino acid substitutions identified in this study. Variants not previously described in the literature are shown in bold, pathogenic mutations are shown in red, other variants are known single nucleotide polymorphisms (SNPs).

Nucleotide change	*LRRK2* exon	Genbank reference number	Predicted protein change	LRR K2do main	Number of probands in which change identified
c.578C>T	5	rs10878245	p.L153L		25/46
c.2857T>C	22	rs7966550	p.L953L		10/46
c.4269G>A	30	rs11175964	p.K1423K	ROC	3/46
c.4872A>C	34	rs1427263	p.G1624G	COR	21/46
c.4911G>A	34	rs11176013	p.K1637K	COR	30/46
c.5412A>G	**37**	**N/A**	**p.G1804G**	**COR**	**1/44**
c.5457C>T	37	rs10878371	p.G1819G	COR	28/44
c.6324G>A	43	rs10878405	p.E2108E	Kinase	29/46
c.7155A>G	48	rs33962975	p.G2385G	WD40	13/46

Table 5.2 Nine synonymous LRRK2 coding sequence variations identified in this study. Novel variant shown in bold, other variants are known single nucleotide polymorphisms (SNPs).

Nucleotide change	*LRRK2* intron	Genbank reference number	Number of probands in which change identified
IVS5+33T>C	5	N/A	1/46
IVS16+39C>T	16	N/A	1/46
IVS17-114T>C	16	N/A	1/46
IVS19-9insT	18	N/A	2/46
IVS20-11insT	19	N/A	36/46
IVS20+21insA	20	N/A	2/46
IVS26-8delT	26	N/A	3/46
IVS30+28G>A	30	N/A	17/46
IVS34+67T>A	34	N/A	45/46
IVS36+59A>C	36	N/A	2/46
IVS36+68delA	36	N/A	44/46
IVS43+133C>A	43	N/A	1/46
IVS49+33T>G	49	N/A	1/46

Table 5.3 Thirteen non-coding (intronic) *LRRK2* variants identified in this study which have not previously been described in the literature.

Nucleotide change	*LRRK2* intron	Genbank reference number	Number of probands in which change identified
IVS2-56G>A	1	rs2723273	46/46
IVS3+45T>C	3	rs1352879	46/46
IVS4+38A>T	4	rs2131088	7/46
IVS5-44T>G	4	rs2723270	1/46
IVS6-125T>C	5	rs6581622	5/46
IVS8-160C>T	7	rs732374	33/46
IVS10-10C>A	9	rs7955902	34/46
IVS11+130G>A	11	rs7969677	11/46
IVS13+104G>A	13	rs28903073	1/46
IVS14+68G>C	14	rs10784462	33/46
IVS30-137C>T	29	rs11175963	1/46
IVS30-62A>T	29	rs7305344	40/46
IVS30+52insGT	30	rs10650388	35/46
IVS34-31T>C	33	rs1896252	36/46
IVS34+32A>G	34	rs11564205	16/46
IVS35-51A>T	34	rs10878368	38/46
IVS35+23T>A	35	rs70307276	38/46
IVS36+36T>C	36	rs7137665	22/46
IVS38+35G>A	38	rs17484342	2/46
IVS40+48C>T	40	rs2404834	9/46
IVS43+53G>A	43	rs11176143	11/46
IVS45+ 101G>A	45	rs890575	33/46
IVS47-9delT	46	rs11317573	39/46

Table 5.4 Twenty-three previously described non-coding (intronic) *LRRK2* single nucleotide polymorphisms (SNPs) identified in this study.

5.4.1.2 Screening for novel LRRK2 variations

Three novel non synonymous *LRRK2* exonic variations: c.4364delAT (D1455G), c.6187delCTCTA (p.L2063 STOP), and c.7187insTG (p.T2397STOP) were identified in the fPD cohort. I then screened for these variants in large cohorts of subjects with sporadic PD and controls, the demographics of these cohorts can be found in chapter 3 and appendix B. Screening was performed by agarose gel electrophoresis, denaturing high performance liquid chromatography (DHPLC), or PCR fragment analysis (examples of raw data from are shown below in figures 5.5-5.7).

The novel variations were not identified in any subjects from either the patient or control cohorts, the results are given below in table 5.5.

I also screened for the c.4364delAT (D1455G) variation in the affected brother of the proband case who carried the mutation (subject II:2, see section 5.4.2.1 and figure 5.8). He was found to be wild-type at this position.

***LRRK2* variants**	**Number of subjects carrying *LRRK2* variations (%) in each cohort tested**											
	PD GEN Pts	**NDD Pts**	**PINE Pts**	**B'HM PD Pts**	**QSPD Pts**	**Total Pts**	**PD GEN Cont**	**NDD Cont**	**PINE Cont**	**QSPD Cont**	**MND Cont**	**Total Cont**
L2063 STOP (Exon 42)	0/350 (0)	0/50 (0)	0/32 (0)	0/227 (0)	0/776 (0)	0/1435 (0)	0/221 (0)	0/47 (0)	0/53 (0)	0/31 (0)	0/136 (0)	0/488 (0)
T2397 STOP (Exon 49)	0/329 (0)	0/54 (0)	0/31 (0)	N/A	N/A	0/414 (0)	0/258 (0)	0/45 (0)	0/53 (0)	N/A	N/A	0/356 (0)
D1455G (Exon31)	0/349 (0)	0/55 (0)	0/32 (0)	N/A	N/A	0/436 (0)	0/143 (0)	0/46 (0)	0/53 (0)	N/A	N/A	0/242 (0)

Table 5.5 Summary of screening results for three novel non synonymous *LRRK2* exonic variations: D1455G, L2063STOP, and T2397STOP in cohorts of subjects with sporadic PD and controls. Screening was performed in a patient cohort from the Birmingham PD study, patient and control cohorts from the PD GEN study, NDD study, PINE study, and Queen Square (QSPD) DNA collection, and a control cohort from the Birmingham MND DNA collection. Abbreviations: Pts= patients; Cont= controls; B'HM= Birmingham.

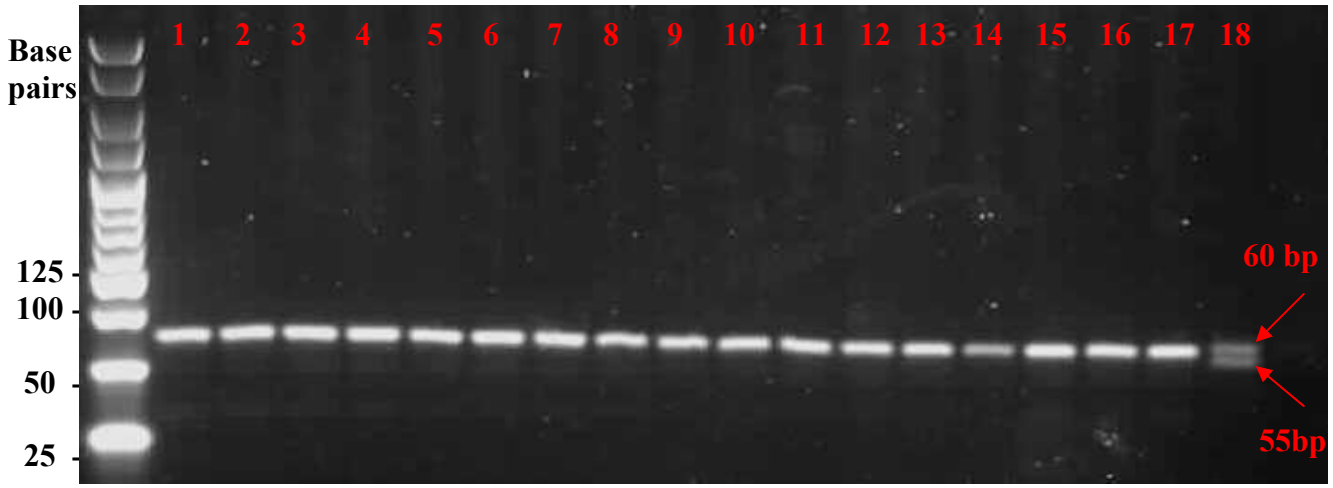


Figure 5.5 Screening for novel *LRRK2* variant L2063STOP by agarose gel electrophoresis of PCR products. PCR reaction was performed as described in section 5.3.2.4. The PCR products were then run on a 3% high resolution RESponse™ Research agarose (Geneflow) gel at 120V for 3 hours. The sizing ladder run on the gel was Hyperladder™ V (Bioline). The PCR product was 60 base pairs in length in wild-type and 55 base pairs if the variant was present, as indicated by arrows. Lanes 1-17 represent wild-type PCR products from wild-type subjects and lane 18 a PCR product from a subject heterozygous for the L2063STOP variant (proband subject II:1 from family 12). Abbreviations: bp= base pairs.

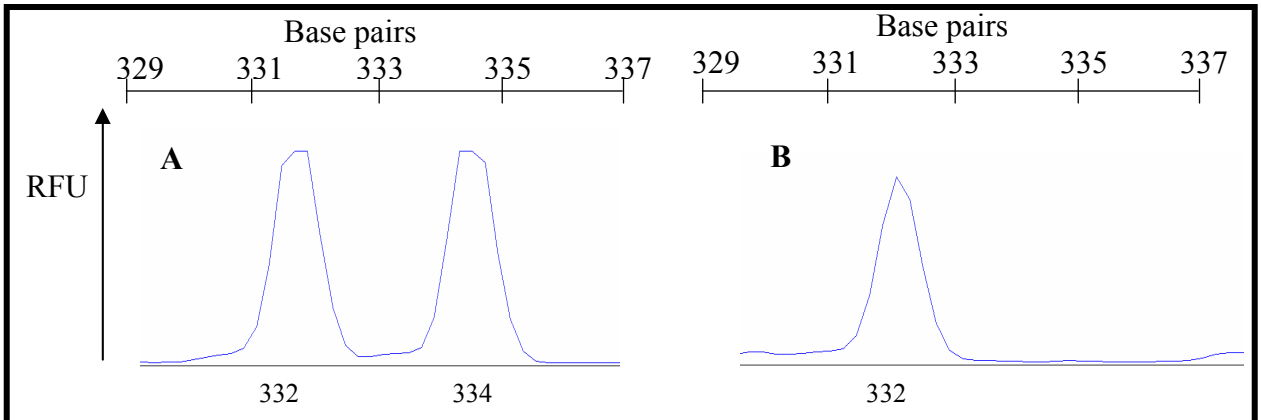


Figure 5.6 Screening for *LRRK2* variant c.7187insGT (T2397STOP) by fragment analysis of PCR products using Genescan®. Panels A and B show electrophoretograms of amplified fluorescent-labeled PCR products from DNA template for the c.7187insGT variation. Panel A represents DNA from subject carrying the variation and panel B represents DNA from a control subject. The wild-type PCR product is 332 base pairs and the mutant PCR product is 334 base pairs in size. Mutant PCR product is absent in the control subject. The y-axis represents the intensity of fluorescence, expressed as relative fluorescence units (RFU).

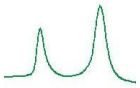
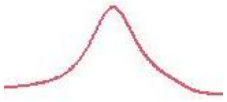
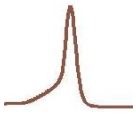
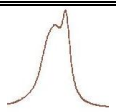
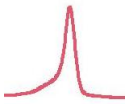
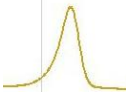
***LRRK2* variant and Exon**	**DHPLC column temperatures**	**DHPLC Chromatograms**	
		Wild Type	**Variant**
L2063STOP Exon 42	**54.7°C**		
	55.9°C		
	56.9°C		
	58.0°C		
D1455G Exon 31	**59.5°C**		
	60°C		
	61.5°C		

Figure 5.7 Screening for *LRRK2* variants identified in this study, L2063STOP and D1455G, by denaturing high performance liquid chromatography (DHPLC). PCR reactions were performed as described in section 6.3.2.4 and then DHPLC used to screen for variants. The figure demonstrates DHPLC chromatograms, at different column temperatures, for the two *LRRK2* variants from subjects who were known to be wild-type or heterozygous carriers (proband subjects II:1 from family 12 and II:1 from family 48) of the variants. The chromatograms clearly show one peak for the subjects who were wild-type, representing homoduplexes only, and two peaks for the subjects carrying the variants, representing a mixture of homoduplexes and heteroduplexes. This technique allows discrimination between wild-type and mutants for these variations, and is described in more detail in chapter 6.

5.4.2 Clinical studies

The clinical features and pedigrees of proband cases carrying the three novel exonic variations identified in this study: (i) c.4364delAT (D1455G) in exon 31, (ii) c.6187delCTCTA (p.L2063 STOP) in exon 42; and (iii) c.7187insGT (p.T2397STOP) in exon 49 are presented below. The clinical features and pedigrees of proband cases carrying the pathogenic (G2019S and R1441C) and the two putatively pathogenic mutations (S1228T and M1869T) are presented in appendix D. This data is summarised in table 5.6 below. All studied family members are Caucasian.

5.4.2.1 Family 48

One affected member of this family (II:1) was studied, although extensive clinical information is also available on his brother (II:2).

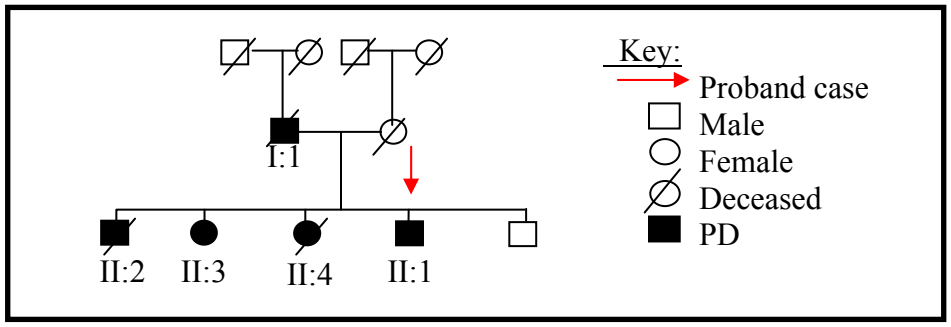


Figure 5.8 Pedigree for family 48

The proband case, II:1, carries the heterozygous D1455G mutation. He is right-handed, was aged 71 at the time of evaluation, and had onset of symptoms aged 60. His initial symptom was tremor of his left arm. His symptoms responded well to Levodopa therapy. He had

reported dystonia in the lower limbs for one year and on-state dyskinesias for two years before the date of evaluation. He had also reported increasingly unpredictable on/off phenomena, with on periods only lasting for approximately two hours. His anti-parkinsonian medication at time of evaluation consisted of Levodopa and Amantadine (levodopa equivalent dose of 450mg daily).

Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical tremor, rigidity and bradykinesia as well as postural instability. His Hoehn and Yahr stage was 4, with a UPDRS III score of 44. He did complain of some cognitive deficit, with a MMSE score of 20.

There were four other affected members of the family. The first is subject II:2, a brother of the proband. He had a definite diagnosis of PD in life and his brain was donated to Queen Square Brain Bank post mortem. He was right handed and had onset of symptoms aged 63. His initial symptom was clumsiness of the left hand. He had asymmetrical rigidity, bradykinesia and tremor. He responded well to Levodopa and developed on state dyskinesias three years after diagnosis. His final recorded anti-parkinsonian medication was Levodopa and dopamine-agonist (Pergolide) (levodopa equivalent dose of 700mg daily). He died aged 70. Pathological examination of his brain showed features typical of IPD- alpha-synuclein immunoreactive neurites and Lewy bodies (LBs) in the substantia nigra. LBs were either uniformly positive or showed a strongly positive halo with weaker or absent staining of the central core.

From family report the other affected family members were: (i) the father of the proband, subject I:1, he was diagnosed with ‘mild’ PD. (ii) Two sisters of the proband, subjects II:3 and II:4 had definite PD.

5.4.2.2 Family 12

One affected member of this family was studied (II:1).

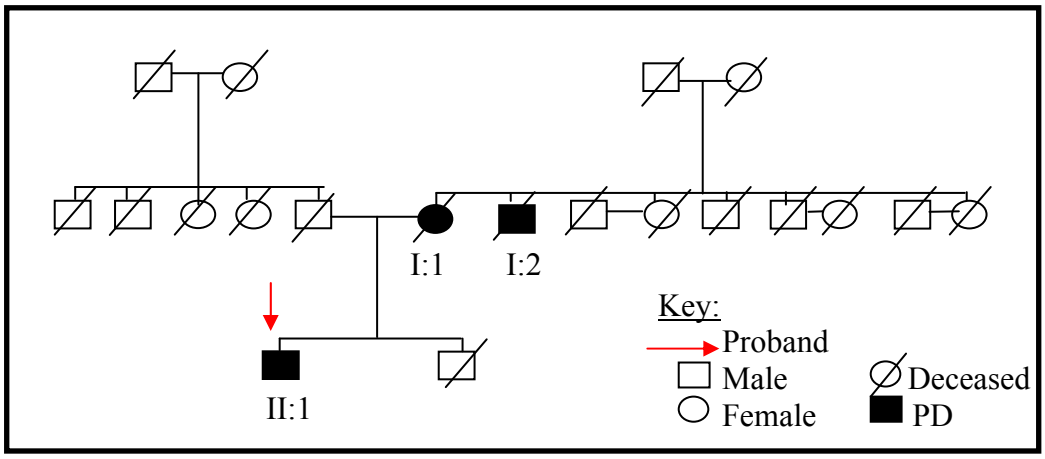


Figure 5.9 Pedigree for family 12

The proband case, II:1, carries the heterozygous L2063STOP mutation. He is right-handed, was aged 63 at the time of evaluation and had onset of symptoms aged 56. His initial symptom was tremor of his left arm. His symptoms responded well to dopamine agonist therapy (Pramipexole). His anti-parkinsonian medication at time of evaluation was a combination of Levodopa and a dopamine agonist (Pramipexole) (levodopa equivalent dose of 1400mg daily). Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical tremor and rigidity as well as postural instability. He was also mildly dyskinetic. His Hoehn and Yahr stage was 1, with a UPDRS III score of 11. He did not complain of any cognitive deficit and his MMSE score was 30.

From family report there were two other affected members of the family: (i) the mother of the proband, subject I:1. She was diagnosed with PD at the age of 75, responded well to Levodopa therapy and died at the age of 88. (ii) A maternal uncle, subject I:2. He was diagnosed with PD in his early 60's and died in his late 60's.

A DaTSCAN was performed on the proband, which revealed reduced uptake in the putamen bilaterally, consistent with parkinsonism (see figure 5.10).

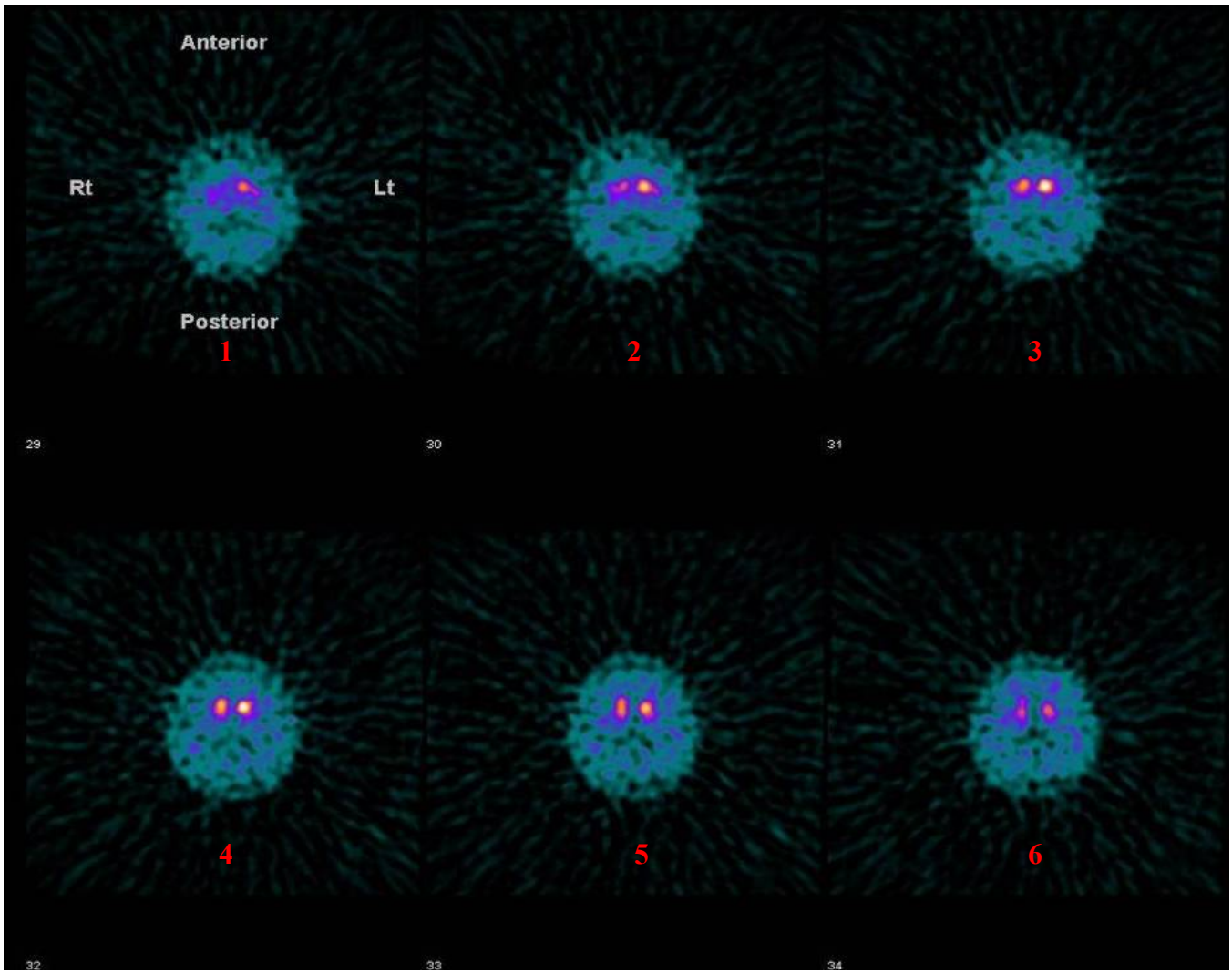


Figure 5.10 DaTSCAN for proband subject from family 12. An abnormal scan, showing bilateral reduced striatal uptake of [^{123}I]FP-CIT, as demonstrated by the arrows. Images 1-6 represent transverse sections through the brain, where 1 is the most superior and 7 the most inferior section. RT= right, LT= left.

Olfactory testing was performed on subject 12:1; the UPSIT score was 15.

5.4.2.3 Family 38

One affected member of this family was studied (III:1).

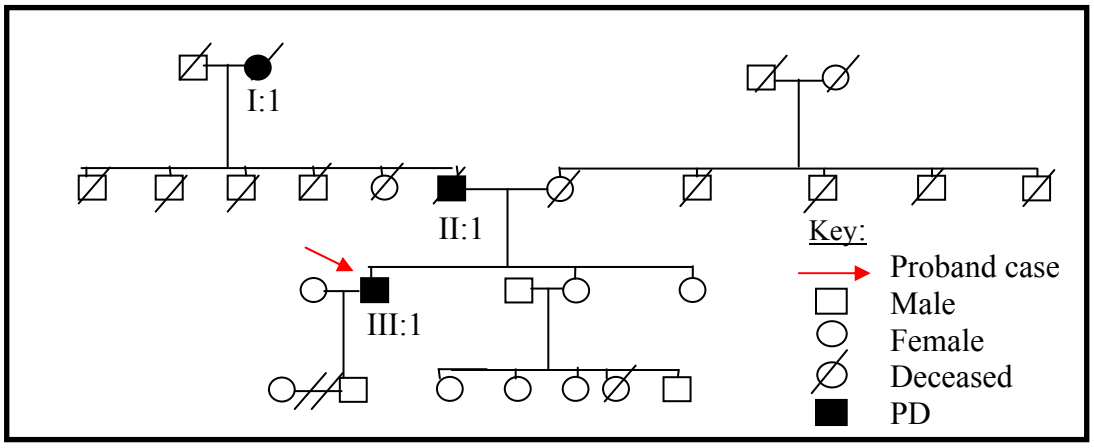


Figure 5.11 Pedigree for family 38

The proband case, III:1, carries the heterozygous T2397STOP mutation. He is left-handed, was aged 82 at the time of evaluation and had onset of symptoms aged 78. His initial symptom was tremor of his left arm. His symptoms responded well to Levodopa therapy. His anti-parkinsonian medication at time of evaluation consisted of Levodopa at a dose of 300mg daily.

Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical tremor, rigidity and bradykinesia as well as postural instability. His Hoehn and Yahr stage was 3, with a UPDRS III score of 26. Although he did not complain of any cognitive deficit, his MMSE score was 27.

According to family report there were two other affected members: (i) the father of the proband, subject II:1. He was diagnosed with PD at his late 70's. He had tremor, gait difficulty and some cognitive dysfunction, he died at the age of 86. (ii) The paternal grandmother of the proband, subject I:1. She developed a tremor and gait difficulty in her late 70's, although a

formal diagnosis of PD was not made. She died at the age of 80. Olfactory testing was performed on subject III:1; the UPSIT score was 12.

Family & Proband gender	Age at eval.	Age at onset	Initial symptoms	Hoehn & Yahr stage	UPDRS III score	L-Dopa response	L-dopa dose (mg/day)	*LRRK2* mutation (Het)
48 M	71	60	Unilateral arm tremor	4	44	Good	450	D1455G
12 M	63	56	Unilateral arm tremor	1	11	Good	1400	L2063STOP
38 M	83	78	Unilateral arm tremor	3	26	Good	300	T2397STOP
10 M	71	54	Unilateral arm tremor, bradykinesia	2	17	Good	550	G2019S
32 F	70	59	Unilateral bradykinesia, micrographia	2	29	Possible	Nil	G2019S
33 F	61	49	Unilateral rigidity	2	23	Good	800	G2019S
20 F	72	71	Gait difficulty	2	15	No treatment	Nil	R1441C
1 F	75	65	Neck rigidity, unilateral arm tremor	3	28	Good	525	S1228T
3 M	67	64	Unilateral arm tremor, bradykinesia, micrographia	1	12	No treatment	Nil	M1869T

Table 5.6 Summary of clinical data on proband cases carrying novel exonic *LRRK2* frameshift mutations identified in this study, as well as proband cases carrying previously described pathogenic and putatively pathogenic *LRRK2* mutations. Abbreviations: M= male; F= female; eval= evaluation; L-Dopa= levodopa; Het= heterozygous.

5.5 Discussion

In this chapter the coding sequence of *LRRK2* was screened for both known mutations and for variations which had previously not been described, in 46 proband cases from the UK fPD cohort described in chapter 3. Sixteen coding sequence variations in *LRRK2,* which lead to non synonymous amino acid substitutions, were identified. Of these three were novel heterozygous variations c.4364delAT (D1455G), c.6187delCTCTA (p.L2063STOP), and c.7187insGT (p.T2397STOP). These three novel variations were found in one proband case each and all result in a shift in the reading frame, with the introduction of a premature termination codon.

Furthermore, previously reported pathogenic *LRRK2* mutations, that have been shown to segregate with disease, were identified in four probands, three were heterozygous carriers of the G2019S mutation and one was a heterozygous carrier of the R1441C mutation. Taken together, the data suggests that previously reported pathogenic *LRRK2* mutations are associated with 8.7% of PD cases in this cohort. Of the previously reported putatively pathogenic *LRRK2* mutations, for which disease segregation data is not available, one proband was a heterozygous carrier of the S1228T mutation and one proband was a heterozygous carrier of the M1869T mutation. All of these *LRRK2* coding sequence variations are shown below in figure 5.12.

One novel synonymous coding sequence variation was identified, c.5412A>G (p.G1804G) in exon 37, in one proband case. Thirteen novel non-coding sequence (intronic) variations were also identified.

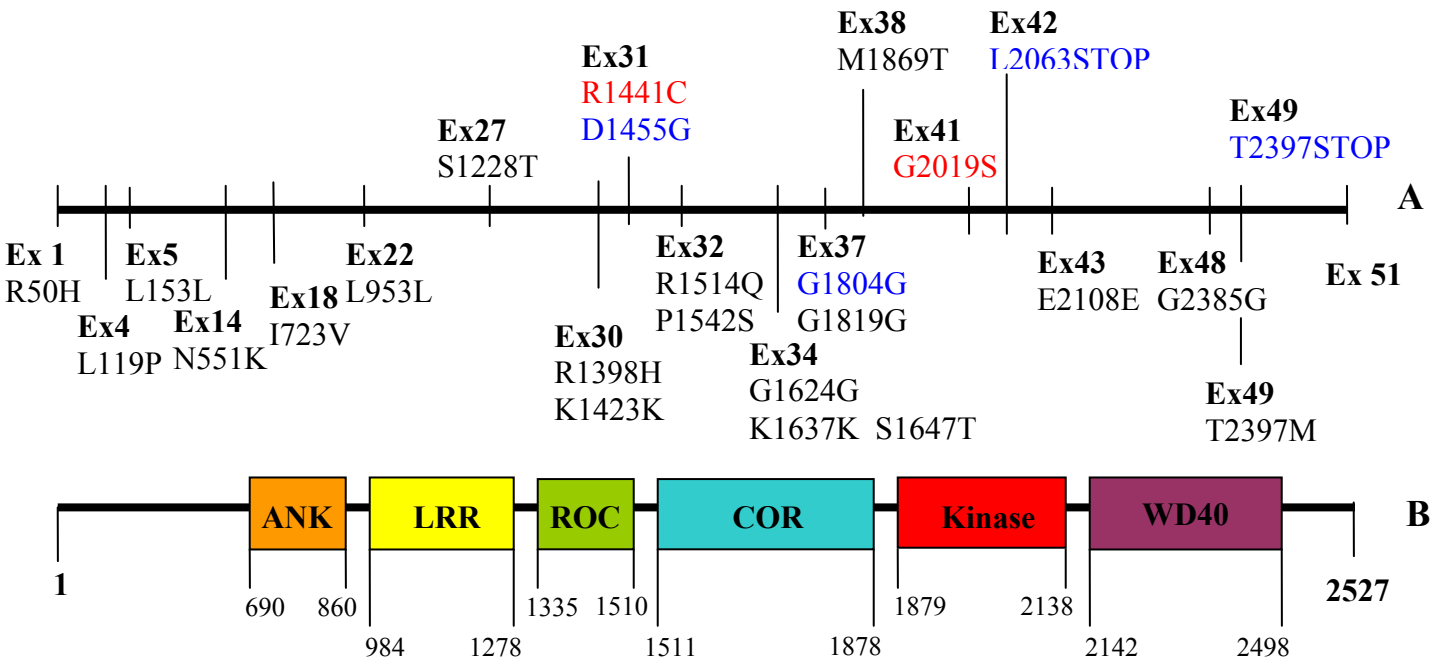


Figure 5.12 Schematic representation of LRRK2 protein domain structure and *LRRK2* coding sequence variants identified in this study. Panel A= Schematic representation of *LRRK2* exons 1-51 with amino acid variants described in this study. Putatively pathogenic amino acid substitutions are shown above, with those segregating with disease in red and the novel changes identified in this study in blue. Variants believed to be polymorphisms are shown below. Corresponding exon numbers are shown in black. Panel B= schematic representation of LRRK2 protein structure from residue 1-2527, with estimated domain boundaries indicated by residue numbers beneath. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Adapted from Mata et al. 2006 and Nichols et al. 2007 [18], [26].

The different categories of *LRRK2* mutations identified are discussed below individually in terms of phenotype-genotype correlation and importance for understanding of *LRRK2*-related PD. One factor which must be considered is that is possible that some of the proband cases do in fact carry *LRRK2* mutations in regions of the gene which were not analysed in my study,

such as the 5'or 3' untranslated or promoter regions. Furthermore, they may have a type of mutation that will not be detected by sequencing, such as copy number variation. This is considered again later.

In comparison to data generated from other comprehensive analyses of *LRRK2* from different populations around the world (Europe, Russia, Asia, North America and UK) the findings of this study appear quite similar. Pathogenic mutations have previously been identified in 5-12% of subjects from families with AD inheritance, mutations with unequivocal disease segregation have been noted to cluster in the C-terminal half of the protein and subjects carrying the mutations appeared to have typical late-onset PD [27], [292], [279], [293], [280], [26], [294], [665], [687], [689], [688], [666].

Three of these studies deserve further mention, the first because it was the largest cohort of subjects and controls. Paisan-Ruiz et al. screened 272 North American subjects with PD, 64 of whom had a positive family history of the condition, and 275 neurologically normal controls. In total the authors identified 90 variants (exonic and intronic) within *LRRK2* [666]. Four PD cases carried the G2019S mutation, and six novel *LRRK2* nonsynonymous disease specific variants (M712V, R1728L, R1728H, T2141M, R2143H, and L2466H) were also identified in six unrelated PD cases, and not found in 275 controls [666].

The study of Nichols et al. is noteworthy because of the unique approach taken to the selection of subjects. These authors screened one subject from each of 88 families who had the highest family-specific multipoint LOD score at the *LRRK2* locus from a cohort of 430 North American PD families without the G2019S mutation. They identified twelve coding sequence variants which lead to nonsynonymous amino acid substitutions, of which five had not previously been described (E10K, E334K, Q1111H, I1192V and L1795F) and seven

previously reported. Again the clinical features of subjects carrying the *LRRK2* mutations appeared typical for PD with an age at onset range of 57-75 [26].

The study by Khan et al. is important as it was the only other comprehensive screen of a large cohort of UK subjects with fPD. This group screened 117 subjects with PD (60 with a family history consistent with AD inheritance and 57 sibling-pairs). The G2019S mutation was identified in three subjects, with the R1941H and T2356I mutations each being identified in one subject. The clinical features were again those of typical IPD [279].

5.5.1 Novel Frameshift mutations

5.5.1.1 The D1455G mutation

The c.4364delAT (D1455G) mutation in exon 31 was identified in the proband case from family 48 and was absent in 484 UK Caucasian control chromosomes, supporting its case as a pathogenic change. I predict this heterozygous mutation to cause a shift in the reading frame, to result in a premature termination codon at position 1457, corresponding to a truncated protein of 1456 amino acids. Truncation of the protein at this position removes the kinase domain and would be likely to severely affect the function of the GTPase domain. The aspartic acid at position 1455 is highly conserved across species (figure 5.4) suggesting the importance of this region for proper function of LRRK2.

The phenotype of the proband appeared typical for IPD with onset of symptoms aged 60, good response to Levodopa therapy and treatment-induced dyskinesias (section 5.4.2.1 and table 5.6).

To ascertain segregation of this mutation in the family I was fortunate to be able to obtain DNA from this proband's deceased brother (individual II:2, figure 5.8) as described in

sections 5.3.2.1 and 5.4.2.1. However, on screening for the novel variation he was found to be wild type at this position. The phenotype of this subject appeared typical for IPD with good response to Levodopa, indeed very similar to that of the proband. Neuro-pathology was also typical for IPD. Although in the majority of the patients with *LRRK2* mutations Lewy body pathology typical of PD has been reported [27], [271], [279], [656], [284], [657], [658], [659], [625], this subject appears to be a phenocopy and his disease is likely to have been IPD. Phenocopies have been reported previously in *LRRK2*-related PD families [294], [290], [279], [282]. Another explanation for this finding could be a technical error, in that a sample mix up could have occurred. This could be investigated with DNA fingerprinting analysis to ascertain if they shared common parents.

Although the absence of the novel mutation in this subject does not appear to support a pathogenic role for the mutation, the predicted effect of the mutation is so dramatic that this should still be considered. Unfortunately other family members are unavailable for DNA studies, but functional evidence of the effect of the mutation would lend further support to this argument, as discussed in chapter 7.

5.5.1.2 The L2063STOP mutation

The c.6187delCTCTA (p.L2063STOP) mutation in exon 42 was identified in the proband case from family 12 and was absent in 976 UK Caucasian control chromosomes, suggesting that it is a pathogenic change. I predict this heterozygous mutation to cause a shift in the reading frame to result in a premature termination codon at position 2063, corresponding to a truncated protein of 2062 amino acids. This mutation is within the centre of the kinase domain

and again is predicted to severely affect protein function. The leucine at position 2063 is conserved across species, except chicken (see figure 5.4).

The phenotype of the proband appeared typical for IPD with onset of symptoms aged 56, good response to anti-parkinsonian therapy and treatment-induced dyskinesias (section 5.4.2.2 and table 5.6). Furthermore a DaTSCAN was performed which revealed reduced uptake in the putamen bilaterally, consistent with parkinsonism. There are previous reports of functional imaging studies on subjects with *LRRK2* mutations, mostly in subjects with fPD, but also some in sporadic disease. Isaias et al. used SPECT scanning and reported that in subjects with PD who carried the G2019S mutation, the pattern of reduced striatal uptake of [^{123}I]FP-CIT was similar to that observed in IPD [682]. Other brain imaging studies have involved the use of [^{18}F]fluorodopa positron emission tomography (PET) scans. The pattern of dopamine deficiency seen in PD subjects who carried *LRRK2* mutations appeared similar to that observed in IPD [683], [279], [684], [290], [685], [686]. Also of interest is that in some asymptomatic carriers of *LRRK2* mutations PET scans suggested early nerve terminal loss, whilst dopa uptake, decarboxylation and storage of dopamine were maintained and therefore prevented onset of symptoms [685], [686]. This may provide a unique opportunity to study the presymptomatic phase of PD.

5.5.1.3 The T2397STOP mutation

The c.7187insGT (p.T2397STOP) mutation in exon 49 was identified in the proband case from family 38 and was absent in 712 UK Caucasian control chromosomes, suggesting that it is a pathogenic change. I predict this heterozygous mutation to cause a shift in the reading frame to result in a premature termination codon at position 2397, corresponding to a

truncated protein of 2397 amino acids. The variation is within the WD40 domain. This domain can be involved in a variety of processes such as signal transduction, pre-mRNA processing and cytoskeleton assembly, but lacks enzymatic activity and regulatory function [277]. Therefore, by causing a truncation of the protein at this position this novel variation may alter the ability of LRRK2 to accurately coordinate protein-protein interactions, such as those that may exist between its substrates, or for LRRK2 dimerisation. A *LRRK2* variant (Gly2385Arg) in the WD40 coding domain has been observed in several Asian populations, and is significantly more common in patients than controls. The associated disease phenotype is indistinguishable from IPD and it has been proposed as a common risk allele for PD in those populations [293], [294], [279], [280], [695], [696], [697], [698], [699], [700], [701], [702]. Indeed *in vitro* studies have shown that both wild type and the Gly2385Arg variant LRRK2 protein localise to the cytoplasm and form toxic and aggregates and when put under oxidative stress, the Gly2385Arg variant was more toxic and associated with a higher rate of apoptosis [696].

Against a pathogenic role for the T2397STOP mutation is the finding that the methionine at position 2397 is only conserved across human, mouse and rat (figure 5.4). Furthermore, there is a common single nucleotide polymorphism (SNP) at c.7190T>C (39/45 fPD cases), carried in homozygous form by the proband from family 38, which results in the amino acid substitution p.T2397M. Thus the amino acid at position 2937 appears less critical for proper function of LRRK2.

The phenotype of the proband carrying the T2397STOP mutation appeared typical for IPD, with onset of symptoms later than the other two novel frameshift mutations, at 78 years, and he had good response to Levodopa therapy (section 5.4.2.3 and table 5.6).

5.5.1.4 Summary

This is the first time that frameshift mutations in the coding sequence of *LRRK2* have been described. The phenotype observed in all three novel variations described here appears to be typical for IPD, as has been observed in previous studies of *LRRK2* positive PD patients [27], [288], [283], [289], [290], [285], [625].

The are only three other reports of nonsense mutations. Recently a 4 base pair deletion in the splice donor site of exon 19 (IVS20+4delGTAA) was reported [665]. The affected subject had fPD (father and possibly mother also affected) and although his phenotype appeared quite typical for IPD, he did have onset of symptoms at 38 [665]. Two point mutations, the first one a known SNP, that are predicted to result in truncated proteins have also been described c.5175C>T (p.R1725STOP) in exon 36 and c.5620G>T (p.E1874STOP) in exon 38, but no clinical details are available on these subjects [293], [666].

The three novel mutations identified in this study appear to be rare familial mutations as they have not been found in 872, 1392 and 828 sPD chromosomes respectively. Indeed the p.L2063STOP mutation in exon 42 was not found in 160 chromosomes from the Queen Square Brain Bank fPD cohort. The pathogenicity of these variants is not yet certain, with other affected family members needed to confirm co-segregation with disease.

Most *LRRK2* mutations described to date are missense mutations and the working hypothesis of the mechanism of pathogenicity has been through a gain of function effect. The identification of these three novel frameshift mutations suggests that at least for these variations pathogenicity may be through a novel mechanism for *LRRK2*-related PD- loss of functional LRRK2 via haploinsufficiency, although a toxic gain of function from a mutant protein species also remains a possibility. These putative pathogenic mechanisms are discussed in more detail in chapter 7.

5.5.2 Previously described pathogenic mutations

In this study three probands were found to be heterozygous carriers of the G2109S mutation and one proband was found to be a carrier of the R1441C mutation. Therefore previously described likely pathogenic *LRRK2* mutations have been found in 8.7% of this cohort. These two mutations were not found in 180 and 242 age-matched UK controls respectively (table 6.3). The pathogenic roles of G2019S and R1441C have been established on the basis of absence in large number of control chromosomes, co-segregation with disease in several large pedigrees, cross species conservation of the relevant amino acids and predicted structural importance of the amino acids involved [27], [283], [290], [281], [289], [703], [292].

5.5.2.1 The G2019S mutation

The probands carrying the G2019S mutation from families 10 and 33 had typical clinical features of IPD and responded well to levodopa therapy. Indeed, the sister of the proband from family 10 (subject III:2) was also affected and carried the same mutation. She was aged 60 at disease onset and again had typical clinical features of IPD, although she was unable to tolerate anti-parkinsonian medication (see sections D.1 and D.3 and table 5.6). However, the proband carrying the G2019S mutation from family 32 had more atypical disease. Her symptoms began at the age of 59, however they were very slowly progressive and she had been unable to tolerate Levodopa. Twelve years after the onset of disease she remained mildly affected, without any treatment, and she had a Hoehn and Yahr stage of 2 (see section D.2 and table 5.6).

The mean age of onset of symptoms of the subjects carrying the G2019S mutation was 55.5 (SD 5.1), with a range of 49-60, and apart from the proband case in family 32 the associated

phenotype was typical for IPD. This is in general agreement with previously reported findings from studies in large cohorts of subjects with fPD [27], [292], [279], [293], [294], [280], [26], [665], [687], [666], [688], [689].

From the demographic, clinical and epidemiological data available on the subjects carrying the G2019S mutation, there is no clear explanation as to why the proband from family 32 appeared to have an atypical form of PD and subject III:2 from family 10 had an apparently poor response to Levodopa therapy. The previously reported clinical phenotype associated with the G2019S mutation has in most cases been very similar to IPD, although with a wide range of age at onset [282], [677], [704], [678], [705], [682], [706]. However, there are reports of the mutation being associated with slowed disease progression [282], [667] and recently a heterozygous carrier of the G2019S mutation was reported who had a more atypical phenotype. She had onset of symptoms aged 39, with hypokinesia only at the onset of disease, although she developed more typical features later [688].

Given that the pathology associated with G2019S mutations ranges from typical Lewy body pathology to neurofibrillary tangles through to nigral degeneration without Lewy bodies [656], [284], [657], [658], [707], [708] one might expect a wider range of associated phenotypes. Several studies have not identified G2019S mutations in other neurodegenerative conditions such as Alzheimer's disease, progressive supranuclear palsy (PSP), and multiple system atrophy (MSA) [709], [710], [711], [712], [713], [714], [657], [715]. However, there are two recent reports in which G2019S mutations have been associated with frontotemporal dementia and corticobasal degeneration [716], [717]. Therefore the finding of the G2019S mutation associated with a more atypical form of PD is interesting and warrants further clinical investigation as discussed below in section 5.6.

5.5.2.2 The R1441C mutation

The proband carrying the R1441C mutation had onset of disease aged 71, and had typical clinical features of PD. This has been reported previously, although a slower progression of the parkinsonism has also been noted [27], [703], [293], [280], [718], [666]. Recent studies have identified two major haplotypes for R1441C carriers, indicating a minimum of two independent founders [718], [280], [719].

The p.R1441 amino acid residue is the second most common location of pathogenic substitutions, after p.G2019S, indeed the p.R1441 residue appears to be prone to mutagenesis as the two pathogenic substitutions (p.R1441C; c.4321C>T and p.R1441G; c.4321C>G) and one putatively pathogenic substitution (p.R1441H, c.4322G>A) affect the same residue. This residue is highly conserved across species and even between the ancestral LRK1 within invertebrates and LRRK1 and LRRK2 in vertebrate radiations [18].

From the few reports of pathological findings in carriers of R1441C mutations, a broad spectrum ranging from Lewy body pathology to tau pathology was noted [27], [271]. Once again several studies have not identified R1441C mutations in other neurodegenerative conditions [711], [712], [713], [715], although there is a single PD patient with a R1441H mutation whose disease subsequently evolved into PSP [720].

5.5.2.3 Summary

In this study of UK fPD pathogenic *LRRK2* mutations were identified in 8.7% of probands. The mean age of onset of symptoms of the subjects carrying the pathogenic *LRRK2* mutations was 58.6 (SD 8.2), with a range of 49-71, and apart from the proband case in family 32, the associated phenotype was typical for IPD.

These findings raise the question as to whether screening for the pathogenic mutations (G2019S and R1441C) in fPD cases is something that should be considered in routine clinical practice. At the current time there is no therapy which prevents or slows neurodegeneration in the condition. Incomplete penetrance has also been reported in *LRRK2*-positive families [283], [287], [281], [279]. The relatively high frequency of the G2019S mutation has allowed some estimates of penetrance to be made, namely 15-17% at age 50, increasing to more than 50% by age 80, depending on cohort [281], [287], [721], [675]. The oldest clinically unaffected heterozygote reported is aged 89 years [722]. Furthermore, although the G2019S mutation shows clear familial segregation, rare phenocopies have been reported [282], [287], [290], [294]. Estimates of penetrance for the R1441C mutation suggest that less than 20% of carriers show PD symptoms before the age of 50, whilst at 75 years more than 90% of carriers show symptoms [718].

At the current time screening for *LRRK2* mutations in both affected and unaffected subjects remains a research rather than diagnostic tool. Much more data, and I believe a clear set of guidelines for testing and counseling, is required before it should come into routine clinical practice.

The known functional effect of these pathogenic *LRRK2* mutations is covered in more detail in chapter 7.

5.5.3 Non synonymous coding sequence variants

In this study I identified one heterozygous carrier for each of the putatively pathogenic missense mutations S1228T (c.3683G>C in exon 27) and M1869T (c.5606T>C in exon 38). Whilst the S1228T mutation was not identified in 68 age-matched UK controls, but was in her

unaffected sibling, one out of 75 age-matched UK controls was found to be a heterozygous carrier of the M1869T mutation (table 6.3).

5.5.3.1 The S1228T mutation

The proband carrying the S1228T mutation is from family 1 (subject III;1), her phenotype appeared typical of IPD and her symptoms responded well to Levodopa therapy. Her monozygotic twin sister (subject III:2) also carried the same mutation but was unaffected (see section D.5 and table 5.6).

There has only been one previous report of this mutation, in a sibling pair from a family in which segregation was consistent with an AD pattern. The phenotype in these subjects appears similar to that of the proband from our study. Both subjects from the previous study had onset of disease aged 49, one with tremor and the other with tremor and bradykinesia. The disease phenotype appeared typical for IPD with good response to Levodopa [294]. The authors did not find any carriers of this mutation in a screen of 337 IPD subjects and 1200 controls.

The c.3683G>C S1228T mutation is located in the LRR domain of LRRK2, one of the protein-protein interaction domains [274]. Therefore a mutation within this region could prevent accurate coordination of protein-protein interactions, such as those that may exist between its substrates, or for LRRK2 dimerisation.

From the demographic, clinical and epidemiological data available on these two subjects there is no clear explanation as to why the proband case should have developed PD whilst her twin sister did not. The two possible explanations for this finding are, first that this mutation has reduced penetrance. This would be in keeping with the reduced penetrance observed in other *LRRK2* mutations [283], [287], [281], [279], [281], [287], [721]. Second, the S1228T mutation

may not be pathogenic, or it may represent a risk factor for PD. To be certain of the true pathogenicity of this mutation co-segregation and/or functional evidence of a pathogenic effect of this mutation are required. The unaffected sister of the proband subject could of course be in the pre-symptomatic phase of PD, and performing DaTSCANs on both individuals could provide evidence of this, and thus provide further support for a pathogenic role for this mutation.

5.5.3.2 The M1869T mutation

The proband carrying the M1869T mutation is from family 3 (subject II:1). His phenotype generally appeared typical of IPD. His unaffected sister (subject II:2) did not carry the mutation (see section D.6 and table 5.6).

There have only been two previous reports of this mutation. One was in an affected member of a family in which disease segregation was consistent with an AD pattern. Similar to the proband from my study, the phenotype of this subject appeared typical for IPD, with disease onset aged 70. An affected sibling did not carry this mutation [293]. The second was of an apparently sporadic case of PD with onset aged 62. Her phenotype appeared to be typical of IPD, with good response to Levodopa [289]. The mutation was not found in 1000 and 278 controls in each of these studies [293], [289].

The c.5606T>C M1869T mutation is located in the COR domain of *LRRK2*. The R1441C mutation in the ROC domain has been reported to decrease GTP hydrolysis activity [663], [723] or increase GTP binding [691]. Furthermore, the Y1699C mutation in the COR domain also appears to increase GTP binding [691]. Therefore, a predicted consequence of the

mutations in this region of LRRK2 has been to increase the downstream kinase activity through perturbation of the GTPase domain.

In my study I also identified the M1869T mutation in an unaffected control subject. This individual is female, was 46 at the time of entry to the study and had no family history of PD. The findings of an affected sibling who did not carry the mutation and an unaffected control with the mutation argues against the M1869T mutation being pathogenic. However, as discussed before, phenocopies have been reported in *LRRK2*-related PD families [294], [290], [279], [282]. Furthermore, the unaffected individual was only aged 46 and may be at risk of developing PD later, this would be in keeping with the reduced penetrance observed in other *LRRK2* mutations [283], [287], [281], [279], [281], [287], [721].

To be certain of the true pathogenicity of this mutation co-segregation and/or functional evidence of a pathogenic effect of this mutation are required.

5.5.3.3 Other nonsynonymous coding sequence variants

There were nine other previously described nonsynonymous variants identified in this study (table 5.1), six of which reside in functional domains: I723V maps to the ANK domain; R1398H maps to the ROC domain; R1514Q, P1542S and S1647T map to the COR domain; and T2397M maps to the WD40 domain. For varying reasons, which are discussed below, these variations are not believed to be pathogenic. The homozygous variation 149G>A (p.R50H) was found in all probands, as in previous studies [293], [280], suggesting an error within the consensus genomic sequence. The heterozygous variation 356T>C (p.L119P) was found in one proband. This variation has been identified in patients at similar frequencies in

previous studies. Although it was not found in controls, there is no evidence to date for segregation with disease [27], [280], [666].

The variation 1653C>G (p.N551K) was found in 2 probands, the variation 2167A>G (p.I723V) in 4 probands, the variation 4193G>A (p.R1398H) in 3 probands, the variation 4934T>A (p.S1647T) in 29 probands, and the variation7190C>T (p.T2397M) in 22 probands. These variations have been identified previously in patients [293], [280], [26] and controls [666] at similar frequencies. There was no significant difference between genotype or allele frequencies in patients from our study and controls from the study by Paisan-Ruiz et al. (see appendix E).

The heterozygous variation 4541G>A (p.R1514Q) was found in one proband and three controls, and the heterozygous variation 4624C>T (p.P1542S) was found in three probands and four controls. These variations have been identified previously in patients [293], [280], [26] and controls [27], [280], [666] at similar frequencies. Again there was no significant difference between genotype or allele frequencies in patients from our study and controls from the study by Paisan-Ruiz et al. for the 4624C>T variation (see appendix E).

These variations are felt to be a neutral, disease unrelated changes.

5.5.4 Synonymous coding sequence variations

Nine synonymous coding sequence variations were identified in this study, including one variation not previously described 5412A>G (G1804G), found in one proband. His initial symptoms were micrographia and shuffling gait, which began at the age of 70 and he had good treatment response to Levodopa. However, he had had a rapid progression of his disease

and when assessed for this study, eight years after onset of disease, he had developed a severe dementia and was bed bound (Hoehn and Yahr stage 5).

The 5412A>G (G1804G) variation is in the COR domain, but is not predicated to affect function of the mature protein, or to affect splicing of *LRRK2* messenger RNA because it is not near the end of the exon, or likely to result in creation of a cryptic splice/donor site. I did not screen controls for this variation, and genetic material was not available on other family members to assess for segregation with disease.

Although six of the other synonymous variants are found in LRRK2 functional domains (table 5.2), it is unlikely that any of the nine variants are pathogenic because they are not predicted to affect function of the mature protein. Furthermore, eight of these variations have been identified previously in patients [293], [724] and controls [280], [666] at similar frequencies. There was no significant difference detected between the genotype or allele frequencies for any of these eight variants in patients from our study and controls from the study by Paisan-Ruiz et al. (see appendix E).

5.5.5 Intronic variations

Thirty-six intronic sequence variations were identified in this study, including thirteen novel variations (tables 5.3 and 5.4). The aim of the work in this chapter was to screen the coding sequence of *LRRK2*, the identification of intronic changes was as a consequence of the location of the primers in the intronic sequence. Therefore I did not generate control data for the novel changes and genetic material was not available to assess for segregation with disease. However, i*n silico* analysis (http://bioinfo.itb.cnr.it/oriel/spilce-view.html) showed

that none of the novel intronic variations appear to significantly modify intron/exon splice sites.

The 23 intronic variations which have previously been described were identified previously in patients and controls [280], [666] at similar frequencies. The homozygous variations IVS2-56G>A and IVS3+45T>C were found in all probands, as previously reported [280], suggesting an error within the consensus genomic sequence. There were nine intronic variantions detected with a MAF of 3% or higher, for which control data was available, and statistical analysis failed to provide evidence of an association with disease (see appendix E).

Although the 36 intronic variations are likely to be neutral, disease unrelated changes, control data for both the novel variations, and for those previously described variations for which control data was not available for analysis in this study, would be helpful to assess whether they associate with disease.

5.5.6 Olfaction

As a further aid to the clinical assessment, I performed olfactory testing on the proband cases. Olfactory dysfunction is found in 70-100% of PD patients [108], [109], [110] and is associated with neuronal loss and Lewy body deposition in the olfactory pathway [112]. Furthermore, olfactory dysfunction is markedly reduced in PD from the early stages (UPSIT mean scores of 20-21) [113], [108], [114], [115]. The data on the four subjects carrying the G2019S mutation, one subject carrying the R1441C mutation, one subject carrying the L2063STOP mutation and one subject carrying the T2397STOP mutation are discussed here.

The mean UPSIT-40 score for all seven subjects was 20.1 (SD 6.5). The mean score for the subjects with the G2019S mutation was as 24.8 (SD 4.1), whilst the scores for the subjects

with the R1441C, the L2063STOP and the T2397STOP mutations were 15, 15 and 12 respectively. The mean score is similar to a recent study of olfaction in PD in which the mean score in 18 UK subjects with IPD was 17.1, whilst in 27 age-matched controls the mean score was 27.6 [118].

The individual subject scores in this study can be compared to the normative values and percentiles for age and gender [482]. The individual UPSIT scores for the subjects with G2019S mutations ranged from 20-30 and were all either in the moderate or severe microsmia category. The individual scores for the subjects with the R1441C, L2063STOP and T2397STOP mutations were all within the anosmia category. Therefore all the subjects carrying these *LRRK2* mutations had at least moderately reduced olfactory function. The olfactory function in the two subjects with the novel frameshift mutations was more severely affected than the subjects with the G2019S mutation.

This data shows that the olfactory function is severely affected in subjects with PD who carry these *LRRK2* mutations and that the associated pathology appears to affect both the basal ganglia and olfactory pathway. The previous studies in which olfaction was assessed in subjects carrying *LRRK2* mutations demonstrated that most subjects exhibited moderate to severe loss of olfactory function, similar or slightly less than that seen in IPD [294], [279], [687], [725], [726], [667], [727], [728]. The postmortem data available on some of these subjects confimed Lewy body pathology in the olfactory pathways [726].

5.6 Future work

There are several further molecular genetic and functional studies that can be done to further both our knowledge on the novel frameshift mutations identified in this study and our understanding of the importance of *LRRK2* in fPD.

First, for the novel frameshift mutations, the identification and screening of other affected and unaffected family members will be crucial to clarify whether these mutations are indeed pathogenic. The further molecular genetic studies which can be used to determine the functional effects of these novel mutations, and our current knowledge of the functional effects of the G2019S and R1441C mutations, are covered in more detail in chapter 7. Although the L2063STOP mutation was screened for in the QSBB fPD cohort, it will also be important to screen for the two other novel mutations in this cohort. It would also be valuable to screen for all three novel mutations in other fPD cohorts to determine whether they are present in different fPD populations.

Second, given that distinct haplotypes for patients who carry the G2019S and R1441C mutations have been identified [673], [718] it would be valuable to assess the haplotype of these mutation carriers identified in my study.

Third, given the atypical clinical phenotype of the proband from family 32, further clinical investigations are warranted. A DaTSCAN could be performed in order to provide further supportive evidence for the clinical diagnoses, which should be abnormal in a subject with PD or parkinsonism [128], [129], [130].

Fourth, control samples could be evaluated for the novel synonymous coding sequence variation (G1804G) and the thirteen novel intronic sequence variations identified in this study, in order to determine whether they associate with disease.

Fifth, for the S1228T and M1869T mutations the identification and screening of other affected and unaffected family members will be crucial to determine whether these mutations are pathogenic. Performing DaTSCANs on both the proband who carries the S1228T mutation, and her unaffected sister who also carries the mutation, could provide further support for a pathogenic role for this mutation, as discussed in section 5.5.3.1

Sixth, analysis of copy number variation of *LRRK2*. As well as missense mutations, copy number variations in both *Alpha-synuclein* and *Parkin* have been shown to cause PD [251], [306], [307]. Copy number variation in *LRRK2* has only been studied twice, and no whole-gene deletions or duplications were detected [293], [666]. Multiple ligation-dependent probe amplification (MLPA) could be used to detect the presence or absence of copy number variation in *LRRK2* in my cohort of 46 fPD cases, to ensure that this is not another mechanism by which *LRRK2* could cause fPD. As discussed in section 4.3.2.3, MLPA is a well validated and high resolution method to detect copy number changes of up to 45 loci in one relatively simple, semi-quantitative PCR-based assay [618], [619], [620].

Seventh, recently the concept of digenic parkinsonism has been proposed. Dachsel et al. identified three patients simultaneously carrying mutations in *Parkin* and *LRRK2* [729]. Although compared to patients with a single *LRRK2* or *Parkin* mutations these patients did not present with earlier disease onset, or faster disease progression, and direct interaction of the two proteins was not observed in co-immunoprecipitation experiments, the authors proposed that potential genetic interactions could be concealed by the modulating effects of other genes [729]. Lesage et al. found the PD phenotype to be no more severe in two North African Arab patients who had both a heterozygous G2019S mutation and one or two *Parkin* mutations [669]. More recently Bras et al. identified a subject with a G2019S mutation and a

heterozygous duplication of *Parkin* exon 9, the subject had early onset of disease but no family history of PD [689].

Therefore it would be valuable to screen our fPD cohort for mutations within the other four genes for Mendelian PD for which there is strongest evidence: *Alpha-synuclein*, *Parkin*, *PINK1*, and *DJ-1*. It is most important to do this in the subjects who carry the pathogenic and novel *LRRK2* mutations in order to test the proposed link between mutations in *LRRK2* and other PD genes.

5.7 Conclusions

This is only the second comprehensive screen of *LRRK2* in a large cohort of UK fPD subjects. I detected three novel frameshift mutations in one proband subject each, which were associated with typical late-onset Levodopa responsive PD. This is the first report of frameshift mutations in the coding sequence of *LRRK2* and may indicate a novel pathogenic mechanism for *LRRK2*-related PD. The previously described pathogenic *LRRK2* mutations (G2019S and R1441C) were detected in 4 out of the 46 proband subjects screened (8.7%), the associated phenotype was in the most part typical of IPD, although one subject had a much more slowly progressive condition. This confirms the importance of these mutations in UK fPD and suggests an extended disease phenotype associated with the G2019S mutation. Taken together, my data suggests that *LRRK2* mutations are associated with 15% of fPD cases in this UK cohort.

6 Molecular Genetic Analysis of Eight Exons of the *LRRK2* Gene in a UK Sporadic PD Cohort

6.1 Introduction

The identification of the PARK8 gene *LRRK2* [25], [27] has led to renewed interest in the role that genetic factors may play in apparently sporadic PD (sPD). This is because the phenotype associated with *LRRK2* mutations resembles that of IPD and through the identification of *LRRK2* mutations in sPD. Indeed one mutation alone, G2019S in exon 41, is found in 1% to 2% of sPD cases [282], [283], [284], [285], [281], [282], [679], [730], [731], [675] and can be found at even higher frequencies in some populations, including Portuguese (6%) [286], Askenazi Jewish (13%) [670], and North African Arabs (41%) [669]. European and North American subjects appear to have two distinct haplotypes, suggesting two founders [669], [670], [281], [671], [672], [673].

There have been numerous studies of *LRRK2* in sPD over the last four years. However, in most studies the strategy has been to screen for the common known *LRRK2* mutations, and in some instances identified variants have not been screened for in a corresponding control cohort. This has led to a need for accurate data on the true frequency of *LRRK2* variations in sPD and controls, which will be addressed by work presented in this chapter.

6.1.1 LRRK2 variations in sporadic PD

In previous studies of *LRRK2* in sPD most investigators have screened for one or more of the five pathogenic mutations that have been shown to segregate with disease: R1441C, R1441G, Y1669C, G2019S and I2020T [18], [278], or focused on the exons in which these mutations

can be found. In some populations other variations have been found to be common, such as the G2385R mutation in the Asian population, which is found in up to 9% of patients surveyed [699], [697].

Screening for the G2019S mutation in different sPD populations has been the focus of much work since it was identified in 2005 [282], [283], [284], [285], [281]. The I2020T variant is also in exon 41 and some investigators have focused on screening for variations within this exon. Furthermore, as the R1441C and R1441G mutations are both found in exon 31, another strategy has been to just screen for mutations within these two 'hot spot' exons.

Although the results of different studies are not easily comparable, because of methodological considerations such as sample population, definition of fPD and sPD, and different genotyping techniques, I have outlined the findings of several of the more extensive screens of *LRRK2* performed in large cohorts of sPD in table 6.1.

Authors	Study population	Number *LRRK2* exons screened	Number of subjects	Number of controls	Results
Grimes et al. [732].	Canadian	7	230	129	No pathogenic mutations identified
Paisan-Ruiz et al. [666]	North American	51	272	275	(i) G2019S mutation in 4 PD subjects only (ii) 6 novel nonsynonymous variants in PD subjects only
Skipper et al. [690].	Singapore	29	160*	630	Two potentially pathogenic variants in PD subjects only : (i) R1067Q (ii) IVS33+6T>A**

Table 6.1 Summary of findings from three studies of *LRRK2* performed in sPD cohorts. The authors screened for both previously described and for novel variations in the relevant exons. *These subjects had a positive family history of PD, which was not compatible with AD inheritance, and had previously screened negative for the G2019S mutation. *LRRK2* variants identified in this initial screen were then screened for in 470 sPD patients. **Intronic variant IVS33+6T>A, decreases the consensus donor splice site sequence

The clinical phenotype of most *LRRK2* positive PD patients appears indistinguishable from that of typical IPD [27], [288], [283], [289], [290], [285], [625]. However, the age at onset is variable, ranging from 28 to 86 years in subjects with the G2019S mutation [285], [287], [288], [676], [282], [677], [289], [678], [290], [679], [279], [292], [286], [284], [281], [680], [293].

6.1.2 Denaturing high performance liquid chromatography (DHPLC)

There are several techniques which have been developed for mutation screening, in addition to sequencing, and these include single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and denaturing high performance liquid chromatography (DHPLC).

DHPLC was developed in 1995 as a high throughput method for detecting known and novel point mutations, deletions and insertions in PCR products [733], [734]. DNA sequence variations are detected on the basis of base pair mismatches between chromosomal fragments amplified by PCR. The PCR products are denatured and slowly re-annealed before analysis by DHPLC. This process results in the formation of homoduplexes and heteroduplexes, between the sense and antisense strands of either homoduplex, if a mutation is present (see figure 6.1). The heteroduplexes are thermally less stable than their corresponding homoduplexes.

Once re-annealed, the samples are loaded onto an alkylated stationary phase (DNA Sep™ Column) which is neutral and hydrophobic. A binding molecule triethylammonium acetate (TEAA) is used at this stage as the negatively charged DNA cannot bind by itself [493]. The DNA molecules are partially denatured on the column by elevated temperatures, typically 50-70°C, and an increasing gradient of the organic solvent acetonitrile. The duplexes are eluted from the column according to the degree of denaturation, the presence of base mismatches in the heteroduplexes causes them to denature more extensively than the corresponding homoduplexes. Therefore heteroduplexes are eluted first and are seen as one or more additional peaks in front of the homoduplexes, both of these fragments are detected by UV absorption and visualized as peaks on chromatograms (figure 6.1) [493].

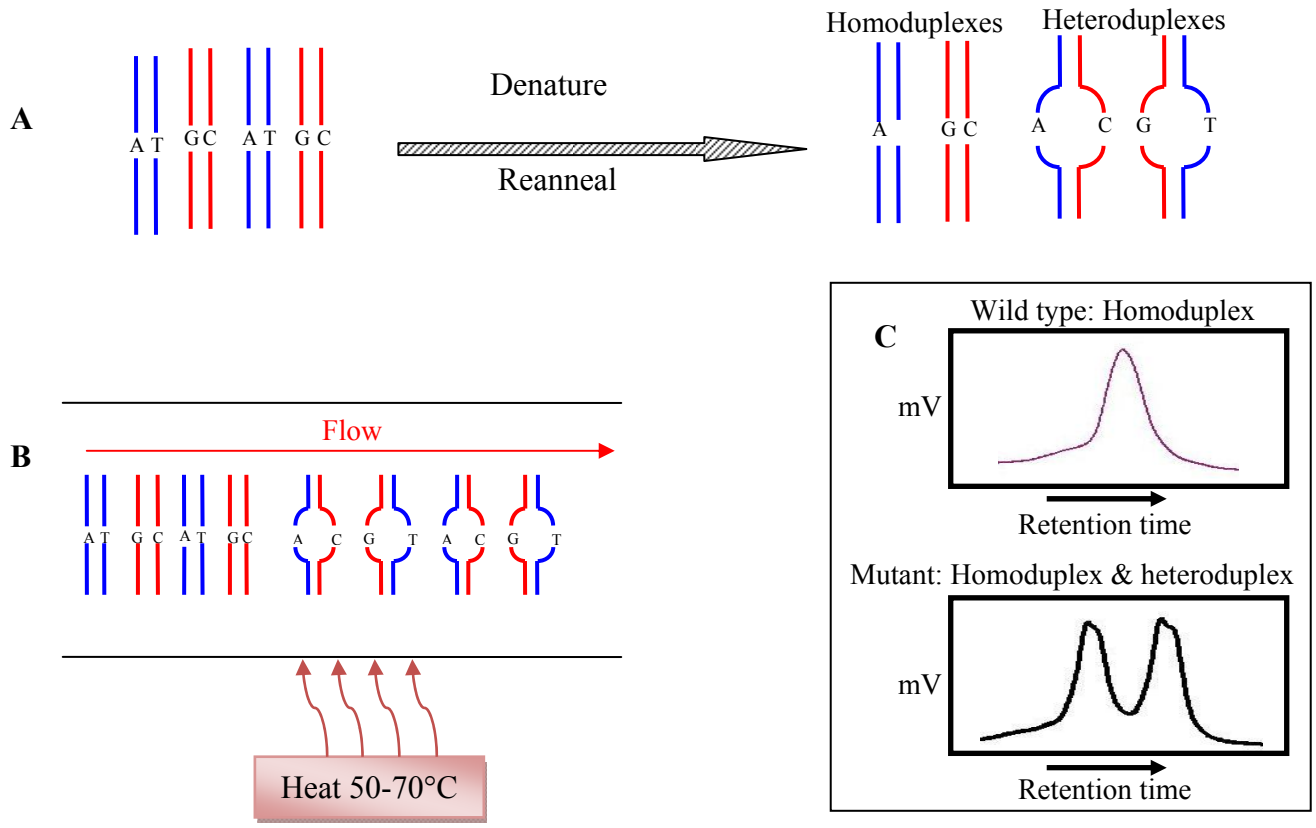


Figure 6.1 The principle of DHPLC. Panel A= PCR products denatured at high temperatures, slowly cooled to form homoduplexes and, if a mutation is present, heteroduplexes. Panel B= Samples loaded onto the column and flow through at preset temperature. Panel C= Chromatograms for wild type (homoduplex) and mutation containing (homoduplexes and heteroduplexes) samples.

DHPLC is a highly sensitive and specific technique, and over 100 genes have now been analysed using this method [488], [489], [490], [491], [492], [493].

6.1.3 Strategy for screening eight exons of LRRK2

The eight exons of *LRRK2* (24, 25, 31, 32, 38, 40, 41 and 42) which were screened in this part of the study on sPD were chosen for several reasons. First, mutations in seven of these exons have previously been described, suggesting that these may be mutational 'hot spots'. Second,

all eight exons are located within functional domains of *LRRK2*, suggesting that mutations within these regions may have important implications for the normal function of LRRK2. Third, mutations were identified in five of the eight exons in the fPD part of my study, suggesting that coding variation within these exons may be important in UK fPD, and hence may also be important in UK sPD (see figure 6.2).

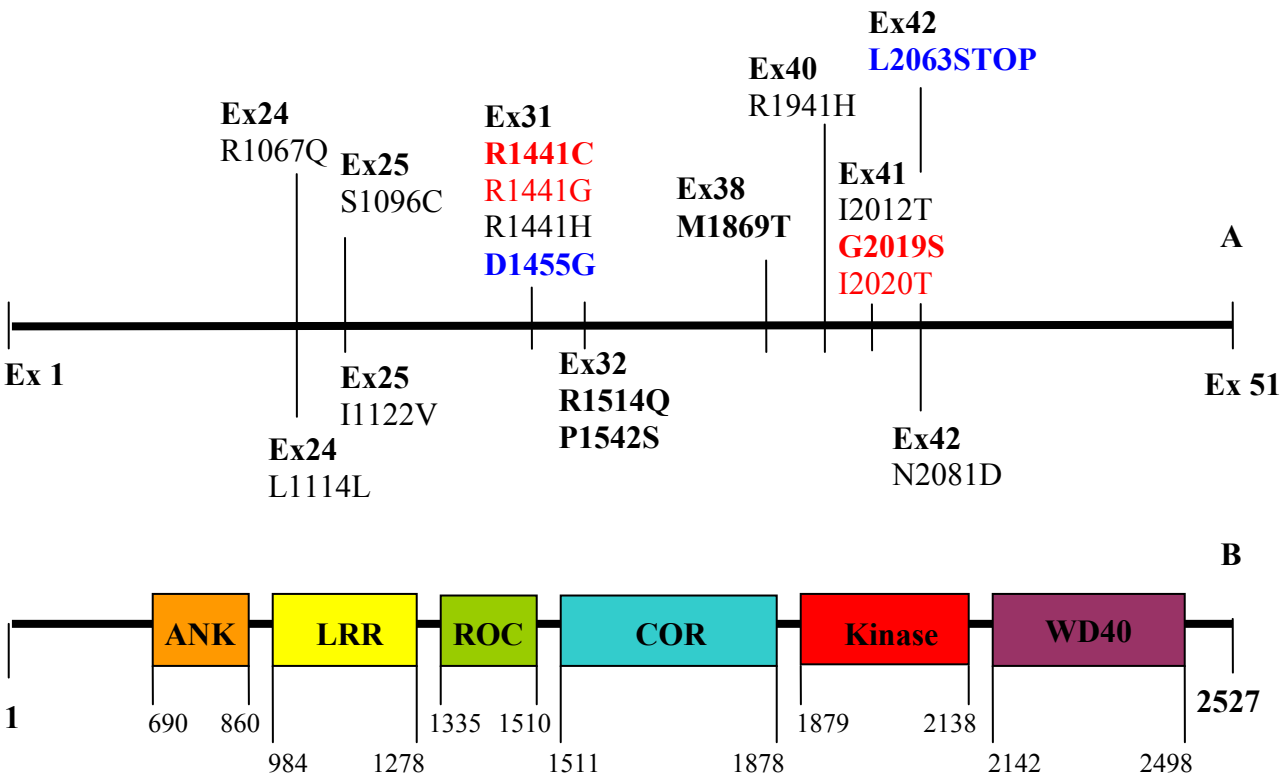


Figure 6.2 Schematic representation of LRRK2 protein domain structure and coding sequence variants identified in eight exons of *LRRK2*. Panel A= Schematic representation of *LRRK2* exons 1-51 with amino acid variants described to date. Putatively pathogenic amino acid substitutions are shown above, with those segregating with disease in red. Variants identified in my familial PD study are shown in bold with the novel variants in blue. Those variants believed to be polymorphisms are shown below. Corresponding exon numbers are shown in black. Corresponding exon numbers are shown in black. Panel B= schematic representation of LRRK2 protein structure from residue 1-2527, with estimated domain boundaries indicated by residue numbers beneath. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Adapted from Mata et al. 2006 and Nichols et al. 2007 [18], [26].

6.2 Aims of the chapter

The major aim of the work in this chapter was to screen for known and novel variation in eight exons of *LRRK2* in a large cohort of UK subjects with sPD and also in controls. The secondary aim was to evaluate the use of DHPLC as a high throughput screening tool for detecting *LRRK2* mutations.

6.3 Subjects and methods

6.3.1 Subjects

This chapter involved the screening of cohorts of sPD patients and controls for known and novel *LRRK2* variations in eight 'hot spot' exons selected as described above (section 6.1.3). The following cohorts of PD patients and controls were used: (i) PD GEN cohort of PD patients and controls; (ii) NDD cohort of PD patients and controls; (iii) PINE cohort of PD patients and controls; and (iv) Birmingham PD cohort of PD patients. These subjects had been recruited as described in section 2.1.2, the demographics of the patient cohorts can be found in section 3.5 and the demographics of the control cohorts can be found in appendix B.

6.3.2 Methods

6.3.2.1 DNA extraction and PCR

DNA was extracted from venous blood as described in section 2.3. Seven exons (including exon-intron boundaries) of the *LRRK2* gene (24, 25, 31, 32, 38, 40, and 42) were studied by PCR using primers and PCR temperatures as described in previous studies [283], [280]. Primers and reaction conditions are detailed in appendix C, each PCR was optimised as described in section 2.4.

In addition, specific primers were designed for screening for *LRRK2* variations within exon 41 by restriction fragment length polymorphism (RFLP) analysis or DHPLC as described below by using the Primer3 program (http://fokker.wimit.edu/primer3/input.htm). Primers selected and reaction conditions are detailed in appendix C. Each PCR was optimised as described in section 2.4.

6.3.2.2 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was used to screen for the G2019S mutation (c.6055G>A) in 110 patient samples from the Birmingham PD, NDD and PINE studies, as well as in 30 control samples from the PINE study. A restriction endonuclease Sfc I was used as described in section 2.6, the assay conditions can be found in appendix C.

6.3.2.3 Denaturing High Performance Liquid Chromatography (DHPLC)

DHPLC, as described in section 2.9, was used to screen for known and novel *LRRK2* variations in eight exons of *LRRK2,* in 357 patient and 90-180 control samples from the PD GEN study. In addition 80 patient and 60 control samples from the NDD and PINE studies were screened for mutations in exon 31. In order to detect homozygous mutations in exon 41 (see section 6.5.1.1) PCR products for exon 41 were mixed in a 1:1 ratio with known wild-type PCR products at the stage of 'duplexing', in order to generate homo and heteroduplexes. This work was all performed using the Transgenomic WAVE® system (Transgenomic Ltd.); column temperatures for analysis were determined by examination of PCR product melt profiles generated by WAVE navigator software.

Technical assistance for the work presented in this chapter was provided by Fenella Marley.

6.3.2.4 Sequencing

Direct sequencing was performed on any samples showing abnormalities on restriction digest or DHPLC. All sequencing was carried out using the dideoxy DNA sequencing method as described in section 2.7. The consequences of mutations on the amino acid sequence were predicted according to *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511). The effects on protein structure and function were predicted using the Protein Sequence Analysis Launcher (ProSAL)- (http://xray.bmc.uu.se/subnet/prosal.html), the Oriel Gene Mining Tools- (http://bioinfo.itb.cnr.it/oriel/spilce-view.html) and the Exonic splicing enhancers finder (ESEfinder)- (http://rulai.cshl.edu/tools/ESE2).

6.3.2.5 Statistical analysis

I performed statistical analysis using the SPSS for Windows release 15.0 (SPSS Inc, Chicago, Ill., USA). Observed genotypes were compared with the Fisher's exact test (two-tailed) and interpreted as significant if the distribution differed by $p<0.05$. Power calculations were made using a program developed by Heather Cordell in Microsoft Excel, and with the advice of a statistician, Dr Sayeed Haque, Department of Psychiatry, University of Birmingham.

6.4 Results

6.4.1 Genetic studies

In total I identified 15 heterozygous *LRRK2* sequence variations in this study, five novel variations and ten variations previously described in other studies. Seven variations were in the coding sequence, which lead to nonsynonymous amino acid substitutions, and eight were

intronic variations. The genotype frequencies did not deviate from those predicted by Hardy-Weinberg equilibrium (HWE) in either PD subjects or controls.

Of the known pathogenic *LRRK2* mutations, one heterozygous carrier of the exon 41 mutation G2019S was identified from the Birmingham PD study by RFLP, and two heterozygous carriers of the mutation were identified by DHPLC from the PD GEN collection (figures 6.3 and 6.4). One heterozygous carrier of the exon 31 mutation R1441C was identified by DHPLC from the PD GEN collection (table 6.2.and figure 6.4). Furthermore, five novel *LRRK2* variations were identified by DHPLC in samples from the PD GEN collection, of which one was a coding sequence variation**,** p.P2093P, which is predicted to alter the amino acid sequence of LRRK2 downstream of the Proline at 2093. Cross species alignment of P2093 reveals that this region is generally conserved (see figure 6.5). In addition, one intronic variation, IVS40+48C>T, was also found in the homozygous state. These results are summarised in tables 6.3-6.4. DHPLC chromatograms for all eight exons can be found in appendix F.

There were 2 *LRRK2* variantions detected with a minor allele frequency (MAF) of 3% or higher, c.4624C>T and IVS40+48C>T. The genotype frequencies did not deviate from those predicted by Hardy-Weinberg equilibrium (HWE). The genotype and allele frequency of these variations was tested against control data for evidence of association with disease. A similar genotype and allele frequency was observed in patients and controls for both of these variations, and statistical analysis failed to provide evidence of an association with disease (*p*-values of 0.624 and 0.327 respectively). However, this was not designed as an association study and the analysis must be interpreted with caution, given the relatively small number of cases and controls used, and the low minor allele frequencies of 0.03 and 0.125 for these

variations. With a minor allele frequency of 0.03, this study only had 35% power to detect a difference with an odds ratio of 1.8, whilst for a minor allele frequency of 0.125 the study had 75% power to detect a difference with an odds ratio of 1.8.

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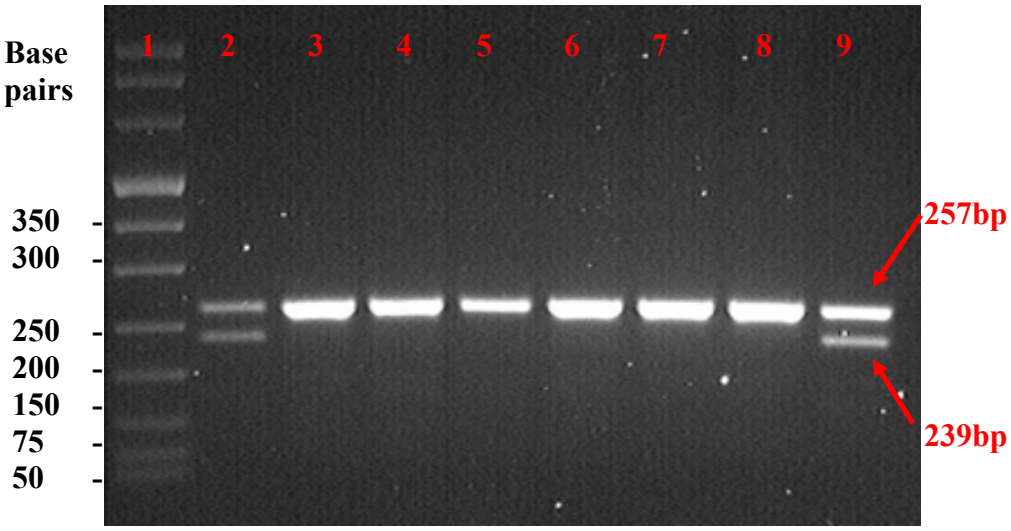


Figure 6.3 Agarose gel electrophoresis of exon 41 PCR products digested with Sfc I enzyme. The PCR products were digested with SfcI as described in section 2.6 and then run on a 2.5% agarose gel at 120V for 2 hours to ensure clear band separation. The digested PCR product was 257 base pairs in length in wild-type and 239 base pairs if the variant was present, as indicated by arrows. Lane 1 represents the sizing ladder run on the gel, a low molecular weight DNA ladder (New England Biolabs). Lane 2 represents a digested PCR product from a positive control for the heterozygous G2019S mutation. Lanes 3-8 represent digested PCR products from wild-type subjects and lane 9 represents a digested PCR product from a subject newly identified as a heterozygous carrier of the G2019S. Abbreviations: bp= base pairs.

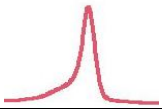
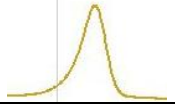
Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
31	59.5°C			c.4321C>T R1441C
	60.0°C			c.4321C>T R1441C
	61.5°C			c.4321C>T R1441C

Table 6.2 Screening for *LRRK2* variants in exon 31 by denaturing high performance liquid chromatography (DHPLC). PCR reactions were performed for eight LRRK2 exons as described in section 6.3.2.1 and then DHPLC used to screen for variants. This table demonstrates DHPLC chromatograms for exon 31, at three different column temperatures, for the *LRRK2* mutation R1441C from subjects who were known to be wild-type or heterozygous carriers of this mutation. The chromatograms for 60.0°C and 61.5°C show one peak for the subjects who were wild-type, representing homoduplexes only, and two peaks for the subjects carrying the variants, representing a mixture of homoduplexes and heteroduplexes. This technique allows discrimination between wild-type and mutants for this variation.

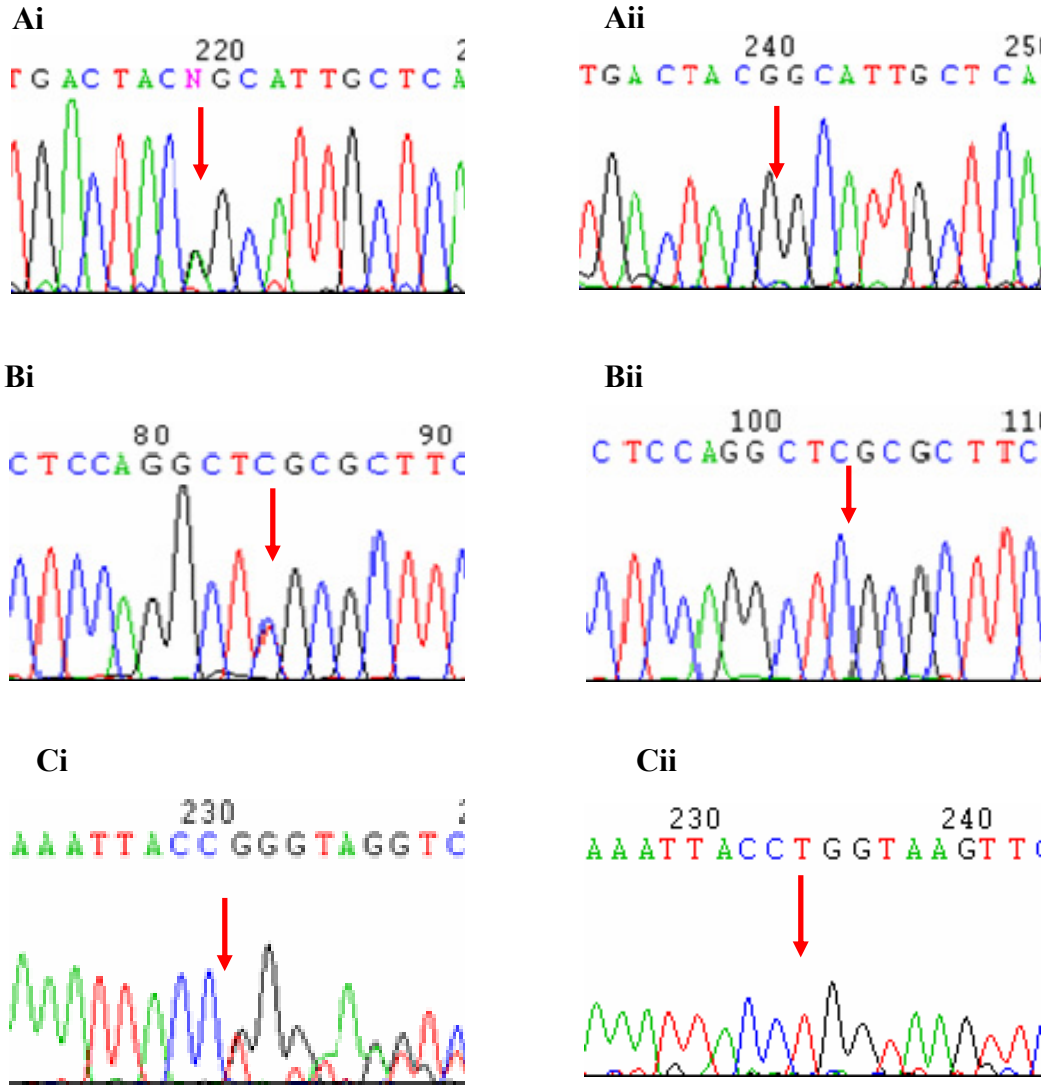


Figure 6.4 Mutant and wild-type chromatograms for pathogenic *LRRK2* mutations and novel non-synonymous *LRRK2* variant. Panels Ai and Aii- mutant and wild-type chromatograms for the c.6055G>A (G2019S) mutation in exon 41. Panels Bi and Bii- mutant and wild-type chromatograms for the c.4321C>T (R1441C) mutation in exon 31. Panels Ci and Cii- mutant and wild-type chromatograms for the c.6279delT (P2093P) variation in exon 42. Arrows indicate position of nucleotide variations.

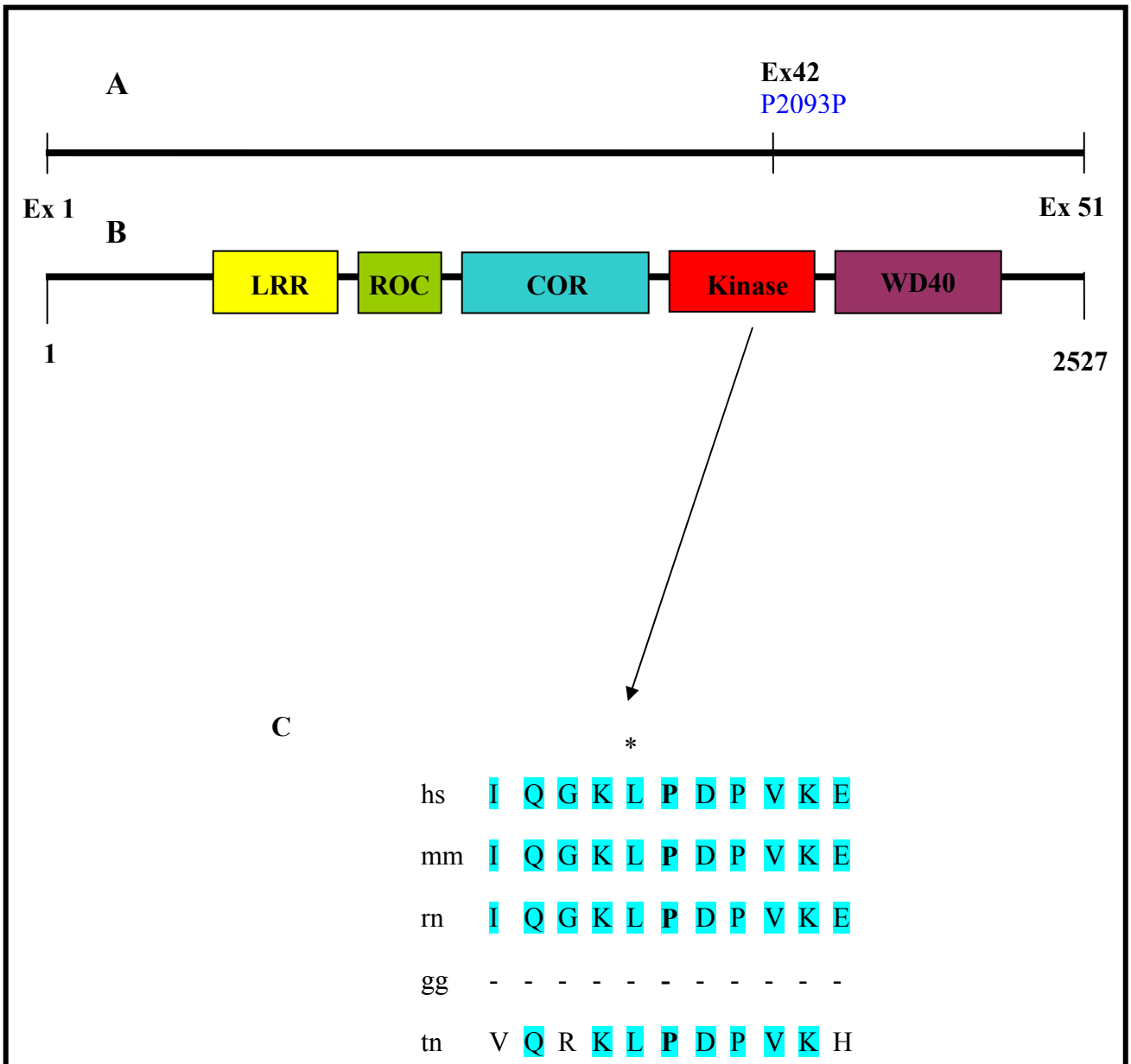


Figure 6.5 Schematic representation of LRRK2 protein domain structure, a novel *LRRK2* coding sequence variants identified in this study, and the cross species protein alignment of this variant. Panel A= Schematic representation of *LRRK2* exons 1-51 with three novel amino acid variant described in this study. Corresponding exon number is shown in black. Panel B= Schematic representation of LRRK2 protein structure from residue 1-2527. Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Panel C= Cross species alignment of LRRK2. Human (*Homo sapiens, hs*) LRRK2 protein AAV63975.1 aligned with other orthologs from Mouse (*Mus musculus, mm*) NP_080006; Rat (*Rattus norvegicus, rn*) XP_235581; Chicken (*Gallus gallus, gg*) XP_425418.1; and Tetraodon (*Tetraodon nigrovirdis, tn*) CAG05593.1. * Represents amino acid of interest in this novel variant. X Represents conserved amino acid across species. Adapted from Zimprich et al. 2004, Mata et al. 2006 and Nichols et al. 2007 [27], [18], [26].

Nucleotide Change	Exon	Genbank reference number	Predicted protein change	LRRK2 Domain	Number of sPD patients in which change identified (%)	Number of controls in which change identified (%)
c.4321C>T	31	rs33939927	p.R1441C	ROC	1/436 (0.2)	0/242 (0)
c.4624C>T	32	rs33958906	p.P1542S	COR	22/350 (6.3)	4/89 (4.5)
c.4541G>A	32	rs35507033	p.R1514Q	COR	8/350 (2.3)	3/89 (3.4)
c.5606T>C	38	rs35602796	p.M1869T	COR	0/314 (0)	1/75(1.3)
c.6055G>A	41	rs34637584	p.G2019S	Kinase	3/463 (0.6)	0/180 (0)
c.6241A>G	42	rs33995883	p.N2081D	Kinase	10/350 (2.9)	5/221 (2.3)
c.6279delT	**42**	**N/A**	**p.P2093P**	**Kinase**	**0/350 (0)**	**1/221 (0.5)**

Table 6.3 Seven *LRRK2* coding sequence variations which lead to non synonymous amino acid substitutions identified in this study of sPD with control data. Variant not previously described in the literature is shown in bold, pathogenic mutations are shown in red, other variants are known single nucleotide polymorphisms (SNPs).

Nucleotide Change	Intron	Genbank reference number	Number of sPD patients in which change identified (%)	Number of controls in which change identified (%)
IVS24+31T>C	**24**	**N/A**	**1/352 (0.3)**	**0/90 (0)**
IVS24+46G>T	**24**	**N/A**	**1/352 (0.3)**	**0/90 (0)**
IVS38-9A>G	37	rs4128646	2/314 (0.6)	0/75 (0)
IVS38+35G>A	38	rs1748434	15/314 (4.5)	0/75 (0)
IVS38+54T>C	**38**	**N/A**	**1/314 (0.3)**	**1/75 (1.3)**
IVS38+80A>	**38**	**N/A**	**1/314 (0.3)**	**0/75 (0)**
IVS40+34T>C	40	rs1744415	7/348 (2.0)	3/89 (3.4)
IVS40+48C>T	40	rs2404834	86/348 (24.7)	17/89 (19.1)

Table 6.4 Eight *LRRK2* intronic sequence variations identified in this study of sPD with control data. Novel variations are shown in bold, other variants are known single nucleotide polymorphisms (SNPs).

6.4.2 Clinical studies-subjects with known pathogenic and novel LRRK2 mutations

The clinical features of the four subjects who were identified as heterozygous carriers of pathogenic *LRRK2* mutations, which have previously been shown to segregate with disease, are given in detail in appendix D and summarised in table 6.5. In addition the demographics of the two controls, who were found to be heterozygous carriers of the putatively pathogenic M1869T mutation, and the novel p.P2093P variation, respectively, are also given.

ID & Gender	Age at evaluation	Age at disease onset	F/H PD	Initial symptoms	H&Y stage	MMSE	L-Dopa response	*LRRK2* mutation
PD237 M	71	70	No	Unilateral tremor, bradykinesia	N/A	N/A	Good	G2019S
PP0293 F	78	76	No	N/A	2.5	30	Good	G2019S
PP0360 M	61	60	No	N/A	1	28	Good	G2019S
PP0071 F	71	61	No	N/A	3	29	Good	R1441C
PC109 F	46	N/A	No	N/A	N/A	N/A	N/A	M1869T
PC157 F	49	N/A	No	N/A	N/A	N/A	N/A	P2093P

Table 6.5 Demographics and clinical features of the subjects and controls carrying pathogenic *LRRK2* mutations, and a novel *LRRK2* variation. Abbreviations: M= male; F= female; FH= family history; L-Dopa= levodopa; H&Y stage= Hoehn and Yahr stage; MMSE= mini mental state examination score.

6.5 Discussion

In this chapter eight 'hot spot' exons of *LRRK2* was screened for known and novel mutations in a large cohort of UK subjects with predominantly sPD. In total 15 heterozygous *LRRK2* sequence variations were identified. Of the known pathogenic *LRRK2* mutations, which have been shown to segregate with disease, three subjects were heterozygous carriers of the G2019S mutation (0.6%) and one subject was a heterozygous carrier of the R1441C mutation. Of the previously reported putatively pathogenic *LRRK2* mutations, for which disease segregation data is not available, one unaffected control individual was found to be a heterozygous carrier of the M1869T mutation. Furthermore, five *LRRK2* variations were identified which have not previously been described, one of which was a coding sequence variation (p.P2093P) identified in a control individual, which is predicted to alter the amino acid sequence of LRRK2 downstream of the Proline at 2093. All of these *LRRK2* variations are shown below in figure 6.6.

There have been very few previous studies in which extensive screens of *LRRK2* for known and novel mutations have been performed in large cohorts of subjects with sPD. The most comparable is by Grimes et al. who screened seven exons (19, 24, 25, 31, 35, 38 and 41) also by DHPLC for known and novel variations in 230 unselected Canadian PD patients and 129 controls [732]. In their cohort 63% of subjects were men, compared to 66% in the PD GEN cohort; 34% had a positive family history of PD compared to 15% in the PD GEN cohort and the average age of disease onset was 57.7 compared to 67.2 in the PD GEN cohort. They did not identify any of the known pathogenic mutations, but did find five novel and two known intronic sequence variants [732]. The only *LRRK2* variation identified in both studies, the intronic change IVS38+35G>A (rs17484342), was found at a minor allele frequency of 0.02 in both studies.

Paisan-Ruiz et al. screened the whole coding region of *LRRK2* in 272 subjects with PD from the USA, 24% of whom had a positive family history of the condition, and 275 neurologically normal controls. The authors identified 90 *LRRK2* variants (exonic and intronic). Four subjects with PD carried the G2019S mutation (1.5%), and six novel *LRRK2* nonsynonymous disease specific variants were also identified [666].

Skipper et al. performed a screen of 29 *LRRK2* exons (23-51) in 160 subjects with PD from Singapore. These subjects had a positive family history of PD, but it was not compatible with AD inheritance, and they had previously screened negative for the G2019S mutation. Any putative *LRRK2* variants identified in this initial screen were then screened for in 470 sPD patients. They found two potentially pathogenic variants, R1067Q and an intronic variant IVS33+6T>A, which decreases the consensus donor splice site sequence. Screening for these variants in the 470 sPD cases and 630 controls also from Singapore revealed no more R1067Q variants, but there were three PD subjects heterozygous for the IVS33+6T>A variant [690].

There is only one previous study in which a large cohort of UK subjects with sPD was screened for *LRRK2* mutations. This study only screened for mutations in exon 41 and found the heterozygous G2019S mutation in 1.6% of their cohort of 482 subjects [284].

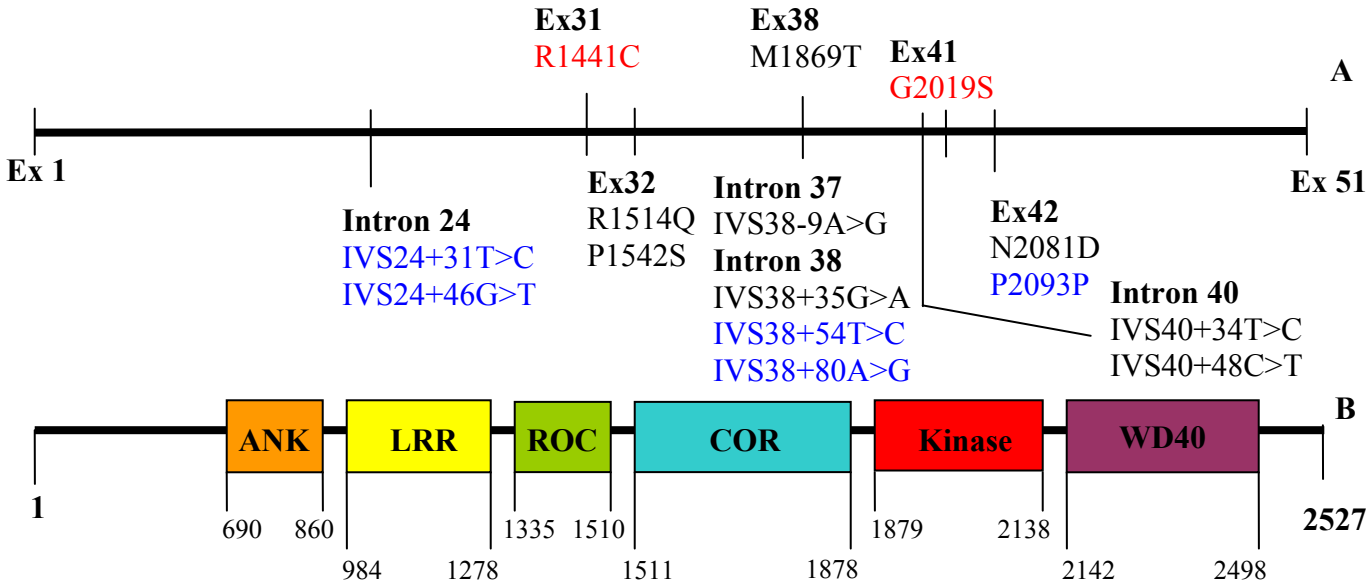


Figure 6.6 Schematic representation of LRRK2 domain structure and *LRRK2* sequence variants identified in this study of sPD. Panel A= Schematic representation of *LRRK2* exons 1-51 with sequence variants identified in this study. Putatively pathogenic amino acid substitutions are shown above, with those segregating with disease in red. Novel changes identified in this study are shown in blue. Variants believed to be polymorphisms are shown below. Corresponding exon numbers are shown in black. Panel B= schematic representation of LRRK2 protein structure from residue 1-2527, with estimated domain boundaries indicated by residue numbers beneath Abbreviations: ANK=ankyrin repeat region; COR=C terminal of Ras; Ex=exon; LRR=leucine-rich repeat domain; Roc=Ras of complex (GTPase); WD40= domain of 40-60 amino acids which terminate in a Trp-Asp (W-D) dipeptide. Adapted from Mata et al. 2006 and Nichols et al. 2007 [18], [26].

The different categories of *LRRK2* variations identified in our study are discussed below individually in terms of phenotype-genotype correlation, and for our understanding of the importance of genetic factors in apparently sPD. Unfortunately limited clinical data was available on subjects from the PD GEN study.

6.5.1 Previously described pathogenic mutations- G2019S and R1441C

The pathogenic roles of G2019S and R1441C have been established on the basis of absence in large number of control chromosomes, co-segregation with disease, cross species conservation and predicted crucial structural position of the amino acids involved [27], [283], [290], [281], [289], [703], [292]. Further evidence of support of a pathogenic role for these mutations comes from *in vitro* expression studies of several mutant LRRK2 proteins, including G2019S and R1441C, which have demonstrated toxicity in cultured cells [661], [457], [691], [664], [312]. Biochemical studies on cultured cells transfected to express LRRK2 have also demonstrated a modest (0.5- to 3-fold) but consistent increase in kinase activity for the G2019S mutation [429], [661], [691], [692]. The functional effects of *LRRK2* mutations are covered in more detail in chapter 7.

6.5.1.1 The G2019S mutation

The mean age at presentation of subjects carrying the G2019S mutation was 68.7 years (SD 8.1), with a range of 60-76. All three subjects had good response to Levodopa therapy, there was no evidence of major cognitive deficits and where information is available the phenotype appeared typical for IPD. This is in general agreement with previously reported findings, although in some studies a wider age of disease onset and more benign disease course has been reported [282], [284], [283], [677], [704], [678], [705], [682], [706], [675].

Interestingly the mean age of disease onset in our fPD subjects carrying the G2910S mutation was younger (54) than for our sPD subjects (68.7), although this difference was not statistically significant (p=0.06) and is probably due to small group sizes. The phenotype of all these subjects was for the most part typical of IPD. The reason for the younger age of onset is

unclear, although reporting bias must be considered as subjects with a strong family history of PD may recognise and report symptoms earlier in the disease course.

Subjects with fPD who are homozygous for the G2019S mutations have been reported previously [681], as outlined in section 5.1.3. Unlike in a previous study of *LRRK2* in sPD which used DHPLC as a screening technique [732] I specifically looked for this homozygous change. I achieved this by mixing PCR products for exon 41, of unknown genotype, with known wild-type PCR products for exon 41 in a 1:1 ratio at the stage of 'duplexing', in order to generate homo and heteroduplexes, as described in section 6.3.2.3. I did not detect the homozygous G2019S mutation in any of the subjects, confirming its rarity and suggesting that the homozygous mutation may only be of relevance in fPD.

The high prevalence of the G2019S mutation in sPD in many different populations, and its occasional occurrence in controls, is probably explained by its reduced penetrance, with estimates ranging from 15-17% at age 50, increasing to more than 50% by age 80, depending on cohort [669], [704], [721], [281], [675].

The majority of samples were screened for the G2019S mutation by DHPLC, as discussed below. However, data has also been included on some samples from the Birmingham PD, NDD and PINE studies which were screened by RFLP analysis as detailed in section 6.3.2.2. This was from preliminary screening work which I performed before I had access to the DHPLC machine.

6.5.1.2 The R1441C mutation

The subject carrying the R1441C mutation had onset of disease aged 61, with good Levodopa response and no evidence of major cognitive deficit, again in general agreement with previous

findings [27], [703] [293], [280], [718], [666]. The fPD subject from our study who carried the R1441C mutation had onset of disease aged 71 and her phenotype was again typical for IPD.

As discussed in section 5.5.2.2, the p.R1441 amino acid residue is the second most common location of pathogenic substitutions, although the majority of previously reported R1441C mutation carriers had fPD. Our study is the first reported identification of this mutation in a UK sPD cohort, confirming its importance in this population.

6.5.1.3 Summary

In my study of UK sPD, pathogenic *LRRK2* mutations were identified in 4 out of 467 (0.9%) subjects screened. The mean age of onset of symptoms of the subjects carrying the pathogenic *LRRK2* mutations was 66.8 (SD 7.6), with a range of 60-76, and the phenotype appeared typical for IPD. Our findings confirm the importance of *LRRK2* mutations in this population and raise the question as to whether screening for these pathogenic mutations in sPD should be considered in routine clinical practice. However, as discussed in section 5.5.2.3, there is as yet no therapy which is proven to be preventative or to alter the course of PD, and incomplete penetrance and phenocopies have also been reported in several *LRRK2*-positive families [283], [287], [281], [279], [282], [290], [294]. Therefore I believe that until a clear set of guidelines for testing and counselling has been established, screening for *LRRK2* mutations in sPD should remain a research tool.

6.5.2 Non synonymous coding sequence variations

6.5.2.1 The M1869T mutation

In this study one heterozygous carrier of the putatively pathogenic missense mutation, M1869T (c.5606T>C in exon 38), was identified in a control subject. This individual was 46 at the time of entry to the study and did not have a family history of PD (see table 6.5). As previously discussed in section 5.5.3.2, the M1869T mutation is located in the COR domain of LRRK2 and the predicted consequence of mutations in this region has been to influence the downstream kinase activity through perturbation of the GTPase domain [663], [723], [691].

As discussed in section 5.5.3.2 the M1869T mutation was identified in one proband subject from my fPD study. There are two previous reports of this mutation, one in fPD and one in sPD [293], [289]. The M1869T mutation has not previously been reported in an unaffected subject, indeed in the previous studies the variation was not found in 1000 and 278 controls respectively [293], [289]. The finding of the M1869T mutation in a control subject goes against a proposed pathogenic role for this mutation, as does a previous report that the proband case who carried the mutation had an affected sibling who did not carry the mutation [293]. However, the unaffected individual in my study who carried the mutation was only aged 46 and she may be at risk of developing PD later, in keeping with the age-related penetrance observed in other *LRRK2* mutations [283], [287], [281], [279], [281], [287], [721].

Therefore, the M1869T mutation may not be pathogenic, or it may represent a risk factor for PD. To be certain of the true pathogenicity of this mutation co-segregation with disease in a large pedigree and/or functional evidence of a pathogenic effect of the mutation are required.

6.5.2.2 The P2093P variation

One novel non-synonymous coding sequence variation was identified in this study, c.6279delT (p.P2093P) in exon 42, which was found in a single control subject. This individual was 49 at the time of entry to the study and did not have a family history of PD (see table 6.5).

The c.6279delT (p.P2093P) variation results in a shift in the reading frame, with a predicted alteration of the amino acid sequence subsequent to the Proline at position 2093 (which remains unaltered). The leucine at position 2093 is conserved across species (see figure 6.5) suggesting the importance of this region for proper function of LRRK2. Furthermore, this variation lies within the centre of the coding region for the kinase domain of LRRK2, and the alteration of the amino acid sequence in this location would be expected to affect protein function. Interestingly this novel variation is within the same exon as the novel c.6187delCTCTA (p.L2063STOP) mutation which I detected in my fPD cohort. There is evidence from biochemical studies on cultured cells transfected to express LRRK2 that the G2019S mutation, within exon 41 and hence also in the coding sequence for the kinase domain, causes a modest (0.5- to 3-fold) but consistent increase in kinase activity [429], [661], [691], [692]. However, the I2020T mutation, also within exon 41, has been shown to either modestly increase [428], [691], or decrease kinase activity [692].

The finding of the P2093P variation in a control subject only goes against a pathogenic role for this variation. This variation was also not found in a screen of exon 42 in my fPD cohort and has not been described before in fPD, although it would be valuable to screen for this variation in a larger cohort of subjects with sPD, such as I performed for the novel c.6187delCTCTA (p.L2063 STOP) variation identified in the fPD cohort (see section 5.4.1.2). However, the unaffected individual in my study who carried the mutation was only aged 49

and, as for the unaffected subject carrying the M1869T mutation discussed above, may be at risk of developing PD later, in keeping with the age-related penetrance observed in other *LRRK2* mutations [283], [287], [281], [279], [281], [287], [721].

Therefore, to determine whether the P2093P variation may be pathogenic, or even a risk factor for PD, co-segregation with disease in a large pedigree and subsequent functional evidence of a pathogenic effect for the variation are required.

6.5.2.3 Other nonsynonymous variants

There were three other known nonsynonymous variants identified in this study (table 6.3), all of which reside in functional domains but are not believed to be pathogenic. The heterozygous variants c.4541G>A (p.R1514Q), c.4624C>T (p.P1542S) and c.6241A>G (p.N2081D) were found at the same, or similar, frequencies in patients and controls. The R1514Q and P1542S variations were identified at the same frequencies in patients in both the sPD and fPD cohorts. Furthermore all three variations have previously been identified in patients [27], [293], [280], [26] and controls [665], [666] at similar frequencies.

6.5.3 Intronic variations

Eight intronic sequence variations were identified in this study, including four novel variations (see table 6.4). The aim of the work in this chapter was to screen the coding sequence of *LRRK2*, the identification of intronic changes was as a consequence of the location of the primers in the intronic sequence.

Two of the four known intronic variations (IVS38+35G>A and IVS40+48C>T) were also identified in our fPD cohort at the same and similar minor allele frequencies of 0.02 and 0.1 respectively. Indeed they have also previously been identified in a fPD cohort [280].

Although the intronic variations IVS38-9A>G and IVS38+35G>A were identified in patients only in my study, they have been identified previously in sPD patients and controls at similar frequencies [732], [666]. Intronic variations IVS40+34T>C and IVS40+48C>T were identified in both patients and controls at similar frequencies in the two groups. Indeed statistical analysis of the IVS40+48C>T variation failed to provide evidence of an association with disease (*p*-value of 0.327).

Each of the four novel variants IVS24+31T>C, IVS24+46G>T, IVS38+54T>C, and IVS38+80A>G were identified in one subject only, although only IVS38+54T>C was detected in the control cohort. *In silico* analysis (http://bioinfo.itb.cnr.it/oriel/spilce-view.html) of the novel intronic variations suggests that none of them appear to significantly modify intron/exon splice sites.

Therefore the eight intronic variations identified in this study are likely to be neutral, disease unrelated changes.

6.5.4 DHPLC as a method for mutation screening

The secondary aim of the work in this chapter was to evaluate the use of DHPLC as a high throughput screening tool for detecting *LRRK2* mutations. The analysis temperatures for each of the exons were determined through the use of the WAVE navigator software, and the availability of positive control samples from our fPD study for six of the exons. The two smaller exons (24 and 25) only required the use of two analysis temperatures, whilst the other

exons required three to four temperatures in order to ensure that the whole of the exon was screened by the technique (see appendix F). The chromatograms obtained from the mutant samples were easily distinguished from wild type profiles with both the 'by-eye' analysis (see appendix F) and the use of the Transgenomic 'mutation detection' software. Whilst the availability of positive controls is important in the optimization of DHPLC, I clearly demonstrated the ability of the technique to detect both variations for which I did not have positive controls (e.g. p.N2081D) and novel changes (e.g. IVS24+31T>C). The sensitivity of the technique is also demonstrated by its ability to detect intronic variations (see table 6.4) for which it was not specifically optimised. Furthermore the technique appeared highly specific, with only 12 DHPLC reactions demonstrating abnormal chromatograms, suggestive of a mutation, which were subsequently shown to be wild-type on sequencing.

There are several methods which I could have used in this part of the study, for example direct sequencing, single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE). Whilst direct sequencing is considered the gold standard in its ability to detect known and novel sequence variations, DHPLC does have several advantages, namely: high sample throughput, increased speed of analysis, reproducibility and cost effectiveness [493]. Whilst it is argued that DHPLC could miss some variations in comparison to sequencing, high levels of sensitivity ranging from 92-100% have been reported for fragments that are 198-732 base pairs long [488], [489], [492]. The exons analysed in this part of the study fell within this range. Further validation of DHPLC for this work comes from my fPD study in which I sequenced the eight exons studied here by DHPLC. I did not detect any *LRRK2* variations by sequencing which were not detected by DHPLC. Our group has a similar experience from studies of other genes with these techniques. Furthermore, even sequencing

will miss some types of mutations such as copy number variation, or mutations in the 5'or 3' untranslated or promoter regions if appropriate primer sets are not used. Therefore in such a large scale screening project as was undertaken in this chapter, the most sensitive but also practical and cost effective technique available must be used.

SSCP is based on the principle that changes in nucleic acid composition affect the secondary structure of single stranded DNA, therefore mutations which affect the mobility of the fragment can be detected by running products on polyacrylamide gels under non-denaturing conditions [735], [492]. Much like DHPLC this technique also requires optimisation, but the fragment size should ideally be small (150-200bp) and even at optimal sensitivity it can only detect 80-90% of base changes [736]. A form of SSCP has also now been developed for automated capillary electrophoresis, using fluorescence as the method of detection (F-SSCP), although this does require access to more expensive equipment [492].

In DGGE double stranded DNA fragments are separated according to the melting behaviour of the sequence. The PCR products are formed into heteroduplexes as in DHPLC and are then electrophoresed through a polyacrylamide gel containing a gradient of denaturant. The mutant or wild-type homoduplex and heteroduplex molecules are separated according to their different melting behaviours caused by differences in their sequence, or by mismatches present in the area of mutation. The homo and heteroduplexes denature at different times, which leads to separation on the gel and the formation of a unique banding pattern [737], [736]. Fragment size can be up to 1000bp and the addition of GC clamps to amplification primers can increase the sensitivity of this technique to approximately 95% [738], [736].

Therefore in comparison to these gel based methods DHPLC is highly automated and efficient, making it much less labour intensive and more suitable for large scale screening

projects, such as that undertaken here [491]. Furthermore the sensitivity of DHPLC is also more comparable to that of sequencing with a reported specificity of 100% [490], [493], [492]. One criticism of DHPLC is that it can miss homozygote changes. However of the known pathogenic mutations, homozygote changes have only been reported for the G2019S mutation [669], [681]. Therefore I screened for this homozygous change by 'spiking' all my PCR reactions for exon 41 with known 'wild-type' PCR product in order to generate homo and heteroduplexes. This is a well recognised technique to enable DHPLC to detect such changes [739], [493]. However as this is more time consuming, and homozygous mutations have not been described in other exons, I did not 'spike' my PCR products with 'wild-type' PCR product for the other exons.

Another potential limitation of the use of DHPLC in screening for *LRRK2* mutations is the high frequency of common polymorphisms in some exons, such as p.S1647T in exon 34 and p.T2397M in exon 49, and the finding of several different polymorphisms in the same amplicon, as identified in the fPD part of this study (see tables 5.1-5.4). This means that for some exons DHPLC would not be cost effective as too many samples would still need to be sequenced to confirm the presence of heterozygote variations identified on DHPLC. However for the majority of the remaining *LRRK2* exons this technique would clearly be suitable and indeed has been used in some previous studies of the gene [740], [671], [732].

6.6 Future work

There are several further molecular genetic and functional studies that can be done to further our understanding of the importance of *LRRK2* in sPD.

First, it would be valuable to extend my screen of *LRRK2* in sPD to include all 51 exons. I have demonstrated the effectiveness of DHPLC as a mutation screening technique, and screening the whole *LRRK2* coding sequence using this method would permit a complete comparison between the *LRRK2* variation frequency in UK fPD and sPD. However, some exons may not be suitable for DHPLC, as discussed above, and may need to be screened by direct sequencing. The remaining exons could be screened by DHPLC, although as I do not have access to positive controls for all the exons I would need to generate these in order to optimise the DHPLC reactions. One way to generate mutations would be via site directed mutagenesis, a technique which has been used successfully in DHPLC method validation in the past [741].

Second, as discussed in section 5.6, analysis of copy number variation of *LRRK2*. Copy number variation in *LRRK2* has only been studied once in sPD, and no whole-gene deletions or duplications were detected [666]. Multiple ligation-dependent probe amplification (MLPA) could be used to detect the presence or absence of copy number variation in *LRRK2* in my sPD cases, to ensure that this is not another mechanism by which *LRRK2* could cause sPD. As discussed in section 4.3.2.3, MLPA is a well validated and high resolution method to detect copy number changes of up to 45 loci in one relatively simple, semi-quantitative PCR-based assay [618], [619], [620].

Third, in order to confirm the findings of my extensive study of *LRRK2* in sPD, and those of the previous two similar studies in North American cohorts [732], [666] it would be valuable to repeat this work on other sPD cohorts around the world. This would for two reasons: (i) to determine whether the novel c.6279delT (p.P2093P) variation described in this study is found in subjects with sPD in other populations. (ii) It would enable us to determine whether there

are any other *LRRK2* variations in sPD, not present in the existing study cohorts, which may be important in different populations.

Fourth, as discussed in section 5.6, given that distinct haplotypes for patients who carry the G2019S and R1441C mutations have been identified [673], [718], [280], [719] it would be valuable to assess the haplotype of these mutation carriers identified in our study.

Fifth, further molecular genetic studies are needed to better understand the functional effects of both the pathogenic *LRRK2* mutations and the novel coding sequence variation identified in this study. Our current knowledge of the functional effects of the G2019S and R1441C mutations, and the further molecular genetic studies which can be used to determine the functional effects of these mutations, and the novel *LRRK2* mutations which I detected in my study of fPD, are covered in more detail in chapter 7.

Sixth, as discussed in section 5.6, the concept of digenic parkinsonism has recently been proposed. Several authors have identified subjects with *LRRK2* and *Parkin* mutations. Although associated phenotypes have not been more severe, there may be potential genetic interactions [729], [669], [689]. Therefore it would be valuable to screen the sPD subjects found to have pathogenic *LRRK2* mutations for mutations within the other four genes for Mendelian PD for which there is strongest evidence: *Alpha-synuclein*, *Parkin*, *PINK1*, and *DJ-1*.

6.7 Conclusions

This is the most extensive screen for known and novel *LRRK2* mutations performed in a large cohort of UK subjects with sPD and is the first use of the PD GEN cohort. Four subjects (0.9%) were found to carry known pathogenic mutations, all of whom had late-onset

Levodopa responsive PD. Otherwise, screening of the other 'hot spot' exons failed to reveal any of the other known pathogenic or novel coding sequence variations within this patient cohort. I also demonstrated that DHPLC is an effective method for high throughput mutation detection in the *LRRK2* gene.

The work presented here confirms the importance of the G2019S and R1441C mutations in UK sPD, and suggests that in the future screening for these mutations may become indicated in the clinical setting, with appropriate pre-test genetic counselling.

7 Functional Analyses of Two Novel *LRRK2* Mutations

7.1 Introduction

In chapter 5 data was presented from my comprehensive molecular genetic analysis of the coding sequence of *LRRK2* in proband cases from 46 families with three or more members affected by PD, compatible with AD inheritance. The most striking finding from this study was the identification of three novel coding sequence variations which are predicted to result in premature termination codons: D1455G in exon 31, L2063STOP in exon 42 and T2397STOP in exon 49.

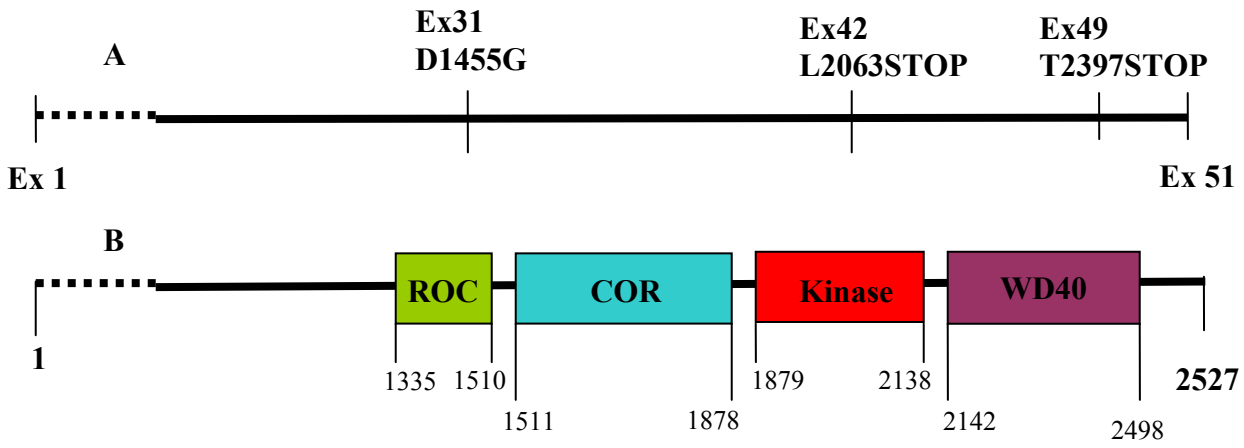


Figure 7.1 Schematic representation of LRRK2 domain structure and three novel frameshift mutations identified in familial PD study. Panel A= Schematic representation of LRRK2 exons 1-51, with location of the three novel mutations indicated, along with corresponding exon numbers above. Panel B= Schematic representation of LRRK2 protein structure, from residue 1 to 2527, the estimated domain boundaries are indicated by residue numbers beneath. Abbreviations: Ex=exon; Roc=Ras of complex (GTPase); COR=C terminal of Ras; WD40= cytoskeleton assembly domain. Adapted from Mata et al. 2006 and Nichols et al. 2007 [18], [26].

To my knowledge, these are the first *LRRK2* frameshift mutations to have been described. The introduction to this chapter gives an overview of some of the functional research that has been performed on *LRRK2* to date, focusing on the current understanding of how pathogenic *LRRK2* mutations are believed to lead to the development of PD. The results of the functional analyses performed on two of these novel *LRRK2* mutations are then presented and discussed in the context of existing data.

7.1.1 Structure and function of LRRK2

Initial reports using RT-PCR and Northern blot analysis showed that LRRK2 mRNA is expressed in various regions of the neuroaxis, including whole brain, spinal cord, substantia nigra and striatum [27], [25]. Subsequent studies have confirmed that *LRRK2* mRNA is localised to a variety of neuronal populations in human and rodent brains [662], [742], [743], [744], [23], [745], [746], [747]. Furthermore all published studies have shown that LRRK2 protein is highly expressed in the areas of the CNS receiving a dopaminergic input, such as caudate-putamen and frontal cortex [662], [742], [743], [744], [746], [656], [663].

More recent reports of *LRRK2* mRNA expression in the SN of both rodents and humans have revealed contradictory results. Investigators used a variety of in situ hybridization and RT-PCR techniques, with some studies showing low levels or even absence [743], [744], [746], whilst others have shown clearly detectable levels [745], [747], [742]. The different results obtained may be as a result of biological variability or be due to methodological considerations. Furthermore, some recent immunofluorescence analyses have revealed LRRK2 protein in dopaminergic neurons of the SN [662], [743], [23].

LRRK2 is predominantly a cytoplasmic protein and is associated with cellular membrane structures such as mitochondria, Golgi apparatus, endoplasmic reticulum and cytoskeleton [429], [428], [661], [656], [662], [312]. Data from cultured mouse primary neurons, suggests that both wild-type and mutant LRRK2 associates with lipid rafts [748]. This may be important as lipid rafts are known to play important roles in cellular functions such as signal transduction, membrane trafficking and cytoskeletal organization [749]. Intriguingly, both Parkin and α-synuclein have also been shown to associate with lipid rafts [750], [751], [752]. A recent report has also suggested a regulatory role for LRRK2 in synaptic vesicle endocytosis [753].

LRRK2 acts as a serine/threonine kinase that can undergo autophosphorylation [429], [457], [691]. *In vitro* studies have shown that LRRK2 can phosphorylate the generic kinase substrate myelin basic protein [429]. Recently, other likely more physiological substrates such as ezrin, radixin and moesin (the ERM protein family) have been identified [692]. These closely related proteins are crucial components that provide a regulated linkage between membrane proteins and the cortical cytoskeleton, just below the plasma membrane, and also participate in signal-transduction pathways [754]. Collapsin response mediator protein (CRMP-2) has also been identified as a potential LRRK2 substrate [692]. CRMP-2 is of interest because it is involved in regulation of growth cones and microtubule dynamics [755] and it has been shown to interact with Numb, an endocytosis-related protein that controls cell number in neurogenesis [756], [755]. Furthermore CRMP-2 is expressed in areas active in neurogenesis such as the olfactory bulb [757], [758] and phosphorylated CRMP-2 has also been found in neurofibrillary tangles in Alzheimer's disease [759], [760].

Several studies have now shown that through its ROC domain LRRK2 can bind GTP [663], [457], [691], [723], [761] although the evidence in support of the ability of LRRK2 to hydrolyse GTP is more mixed. Studies using cultured cells have shown a low level, or even an absence, of GTPase activity [663], [723], [761]. However, epitope-tagged LRRK2 which was produced and purified from transgenic mouse brain expressing human LRRK2, demonstrated robust GTPase activity compared to Rac1 [663]. These observations may be due to methodological differences between the studies and/or the presence of an LRRK2-specific GTPase-activating protein in mouse brains. GTP binding certainly stimulates LRRK2 kinase activity and GTP/GDP binding appears to be required for this activity as specific artificial mutations that disrupt the GTP/GDP binding site abolish the kinase activity [457], [691], [761]. The presence of the protein-protein interaction domains (ankyrin, LRR and WD40) suggests that LRRK2, in addition to protein kinase and GTPase activities, may also serve as a scaffold to a multiprotein complex.

The physiological function of LRRK2 is not yet established. However, there is evidence from *in vitro* as well as *in vivo* over-expression and mutagenesis experiments to suggest that the protein is likely to regulate neurite maintenance and neuronal survival. MacLeod et al. used a rodent model in which they generated a mouse/human chimera. Over-expression of PD-associated mutant *LRRK2* alleles (G2019S and I2020T) in the kinase domain led to reduced neurite complexity, the formation of tau-positive inclusions, lysosomal abnormalities and apoptotic cell death [664].

More recently a *Drosophila* model for *LRRK2*-PD was described, in which the investigators generated transgenic *Drosophila* expressing either wild-type human LRRK2 or LRRK2-G2019S [762]. Expression of wild-type LRRK2 or LRRK2-G2019S in neurons produced

adult-onset selective loss of dopaminergic neurons, locomotor dysfunction, and early mortality. The expression of mutant LRRK2-G2019S was reported to cause a more severe phenotype than expression of equivalent levels of wild-type LRRK2 [762]. We await reports on the phenotype and pathology associated with knockout of *LRRK2* in rodent models, which may provide us with important clues as its function.

7.1.2 Effects of the known pathogenic LRRK2 mutations

There have been previous studies of the five mutations which are believed to be definitely pathogenic: R1441C, R1441G, Y1669C, G2019S and I2020T [18], [278]. *In vitro* expression of several mutant LRRK2 proteins (R1441C, Y1669C, G2019S and I2020T) causes toxicity in cultured cells [661], [457], [691], [664], [312]. Some of the LRRK2 protein variants result in the formation of aggregates within cultured COS7 cells (transformed African Green Monkey kidney fibroblast cells) [661], suggesting that perhaps these mutations can cause misfolding of LRRK2. An active kinase domain was also shown to be required for the toxic effect of mutant LRRK2 expressed in these cells. This was demonstrated by mutating three residues within the kinase domain to create a 'Kinase-dead' construct, COS-7 cells were then transfected with either full length LRRK2 harboring separate pathogenic mutations, or the 'kinase dead' protein containing the same mutations. There were fewer aggregates noted within the cells transfected with the 'kinase dead' version of the protein [661].

Biochemical studies on cultured cells transfected to express LRRK2 show a modest (0.5- to 3-fold) but consistent increase in kinase activity for the G2019S mutation [429], [661], [691], [692]. Given the location of residue G2019 within the conserved Mg^{2+}-binding motif of the kinase domain it is not surprising that this mutation has an effect on kinase activity. However,

since the residue is universally conserved in kinases one might expect the opposite effect i.e. the mutation to impair kinase activity.

The effect of the other *LRRK2* mutations on kinase activity is more controversial. Some studies have shown the R1441C mutation to increase kinase activity [429] [691], whilst others have reported no significant change [661], [692]. The I2020T mutation has been shown to either modestly increase [428], [691] or decrease kinase activity [692]. The Y1699C mutation has been shown to have either no effect [661], [692], or to cause an increase in kinase activity [691]. A modest increase in kinase activity was reported for the R1441G mutant [691], but this was not observed in another study [692]. The putatively pathogenic mutations G2385R and I1371V do not appear to affect kinase activity [691], whilst the I2012T variant appears to decrease kinase activity [691], [692].

One of the main reasons for these apparently conflicting reports may be due to variation in methodological or analytical techniques between the different studies. For example, use of autophosphorylation rather than phosphorylation of myelin basic protein or moesin as the kinase substrate, how the kinase was expressed or purified with different tags, or whether full-length or truncated protein was expressed may all contribute to some of the conflicting results. It is also possible that changes in kinase activity alone may not be the only, nor even the key, noxious effect of *LRRK2* mutations.

The R1441C and R1441G mutations in the ROC domain have been reported to decrease GTP hydrolysis activity [663],[723] or increase GTP binding [691]. Furthermore, the I1371V and Y1699C mutations located outside the ROC domain, also seem to increase GTP binding [691]. Therefore, a predicted consequence of mutations in this region of LRRK2 would be that they influence the downstream kinase activity through perturbation of the GTPase domain. Indeed

recent information on the crystal structure of the Roc domain confirms that LRRK2 acts as a dimmer, with R1441 and I1371 involved in stabilising dimer formation, hence mutations at this position could result in decreased GTPase activity [763], [764].

7.1.3 Nonsense LRRK2 mutations

The importance of the three novel mutations identified in my study is further highlighted by the fact that there are only three previous reports of nonsense mutations in *LRRK*2. None of these mutations were reported with any associated functional data. Two point mutations, the first one a known SNP, that are predicted to result in truncated proteins have also been described c.5175C>T (p.R1725STOP) in exon 36 [293] and c.5620G>T (p.E1874STOP) in exon 38 [695]. Both truncated proteins would lack the entire kinase and WD40 domains. No clinical details are available for the first of these variations, but the second was identified in a Taiwanese subject with sPD. The authors speculated that it could be a disease-causing do novo mutation or an inherited mutation displaying reduced penetrance [695]. No relatives were available for clinical assessment or genotyping.

Recently a 4 base pair deletion in the splice donor site of exon 19 (IVS20+4delGTAA) was reported in a subject with fPD [665]. Assuming all other *LRRK2* exons splice at full length *in vivo*, this deletion would shift the reading frame and correspond to a resultant tuncated protein of 771 amino acids. The authors speculated that the mutant mRNA is likely to be degraded by a process known as nonsense-mediated decay (NMD) [665].

NMD is a normal cellular mechanism highly conserved across all eukaryotes examined to date. It reduces the abundance of transcripts containing a premature termination codon (PTC) to approximately 5-25% of the normal (PTC-free) level. If translated these mRNAs would

produce truncated proteins, possibly with dominant-negative or deleterious gain-of-function activities [494], [495], [15], [496]. In general only PTCs located more than 50-55 nucleotides upstream of a splicing-generated exon-exon junction within mRNA elicit NMD [765], see figure 7.2.

The core NMD machinery is comprised of several factors within a large complex of proteins that is deposited on mRNAs at exon-exon junctions during RNA splicing in the nucleus, the so called exon-junction complex (EJC). This consists of three *trans*-acting factors called up-frameshift (UPF) proteins. One of these proteins, UPF1, is a group I helicase family member recruited to mRNAs upon recognition of stop codons by the translation apparatus. Rapid decay of PTC-bearing mRNAs is triggered when UPF1 is allowed to interact with the two other UPF proteins, UPF2 and UPF3. Other components of the EJC include those in its tetramer core (eIF4AIII, MLN51, and the Y14/MAGOH heterodimer), which also participate in NMD. In addition, NMD requires factors that regulate UPF1 phosphorylation. The Suppressor with Morphogenetic effect on Genitalia-1 (SMG-1) protein phosphorylates UPF1, whereas SMG-5, SMG-6, and SMG-7 promote UPF1 dephosphorylation. The requirement for all four of these SMG factors implies that a cycle of UPF1 phosphorylation and dephosphorylation drives NMD [15], [496].

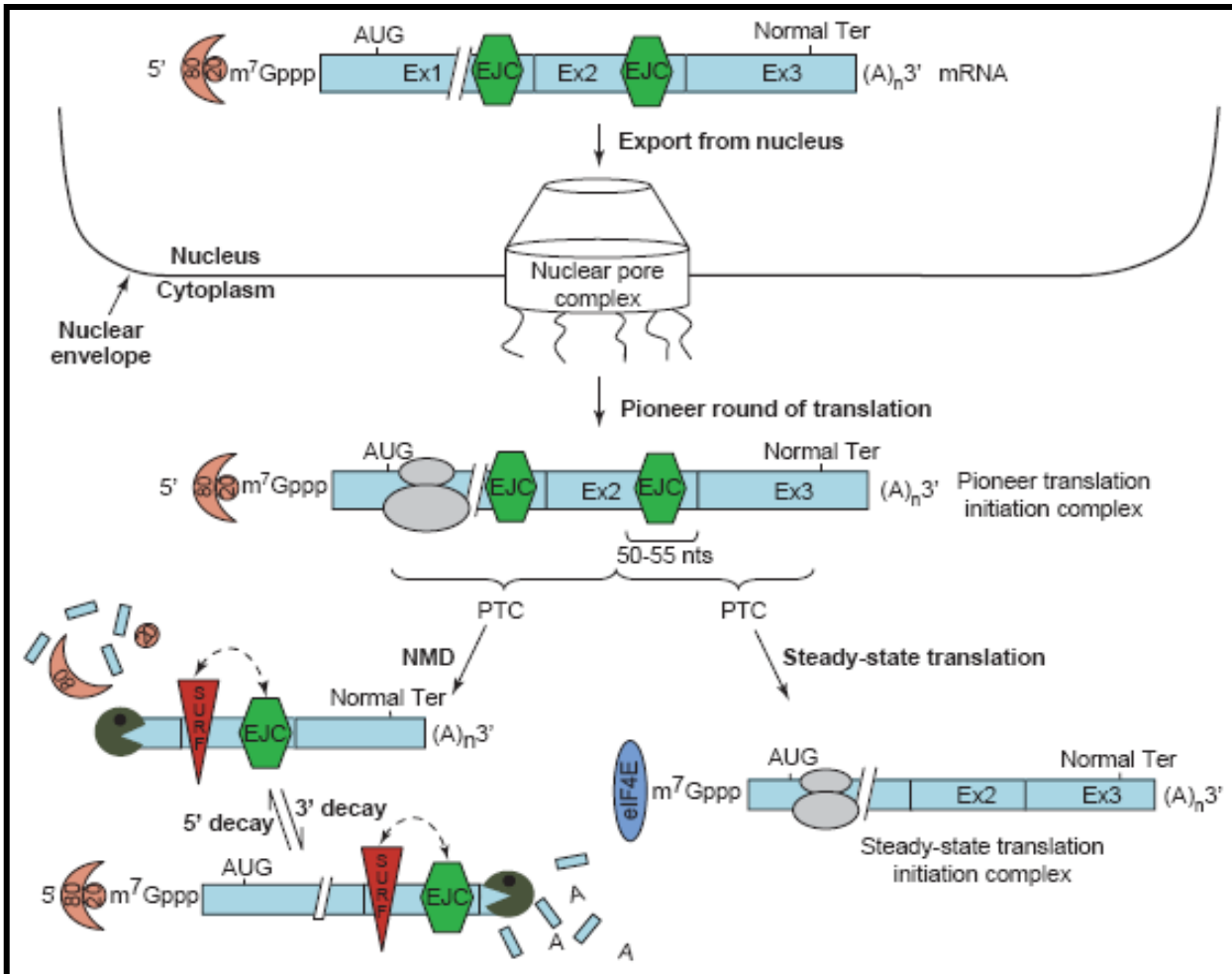


Figure 7.2 The NMD rule. Nonsense codons located more than 50–55-nucleotides (nts) upstream of a splicing generated exon–exon junction within mRNA generally elicit NMD. Removal of introns and ligation of resulting exons (Ex) by splicing results in the deposition of an exon junction complex (EJC) approx. 20–25-nucleotides upstream of each exon–exon junction. Many mRNAs undergo the first ('pioneer') round of translation during the process of export, but can be translated by cytoplasmic ribosomes (light grey subunits). The SURF complex interacts with EJC and triggers NMD. NMD involves mRNA decay from one or both of the 5' and 3' ends (dark grey pacmen symbolize degradative activities). If PTCs reside less than 50–55 nucleotides upstream of the final exon–exon junction or downstream of this junction), then NMD will not occur, and the mRNA is remodeled to the steady-state translation initiation complex. Taken from Kuzmiak & Maquat 2006 [15].

Providing evidence of this process in *LRRK2*-PD would be important and clearly distinct from the kinase over-activity models proposed to date for mutant LRRK2.

7.2 Aims of the chapter

The first aim of the work in this chapter was to generate primary lymphoblast and fibroblast cell lines from subjects carrying two novel *LRRK2* frameshift mutations, L2063STOP and T2397STOP, identified in this study.

The second aim of the work in this chapter was to investigate the effect of these two novel *LRRK2* frameshift mutations on RNA expression.

The third aim of the work in this chapter was to assess and optimize three commercially available LRRK2 antibodies, and subsequently use these antibodies to investigate the effects of the two novel *LRRK2* frameshift mutations on protein expression.

7.3 Methods

The three proband subjects carrying the novel frameshift *LRRK2* mutations were asked if they were willing to donate blood and skin samples for further research analyses. Only the proband subjects from families 12 and 38, who carried the L2063STOP and T2397STOP respectively, were willing to participate in these further studies.

Technical assistance for the work presented in this chapter was provided by Dr Thomas Lambert.

7.3.1 Primary cell lines

The primary lymphoblast and fibroblast cell lines were kindly generated by Professor Malcolm Taylor in the Institute for Cancer Studies, at the University of Birmingham. Control lymphoblast and fibroblast cell lines were obtained as gifts from Dr Andrew Filer in the department of Rheumatology, University of Birmingham. Control fibroblast line 1 (CF1) was

from a female subject aged 62, with a 20 year history of sero-positive Rheumatoid arthritis. Control fibroblast line 2 (CF2) was from a female subject aged 59 also with a 20 year history of sero-positive Rheumatoid arthritis. Control lymphoblast line 1 (CL1) was from a male subject aged 30 with no significant past medical history. Control lymphoblast 2 (CL2) was from a male subject aged 32 with no significant past medical history. These cell lines were subsequently cultured as described in section 2.10.

7.3.2 DNA extraction

DNA was extracted from fibroblast and lymphocyte cell lines by the use of the DNeasy® kit (Qiagen) as described in section 2.10.3.1

7.3.3 RNA extraction and generation of cDNA

RNA was extracted from fibroblast and lymphocyte cell lines by the use of the RNeasy® mini kit (Qiagen) and treated to remove any contaminant DNA with the DNA-*free*™ kit (Applied Biosystems) as described in section 2.10.3.2. The cDNA was subsequently generated from RNA with the High capacity cDNA archive kit, using MultiScribe™ Reverse Transcriptase (Applied Biosystems) and an RT-PCR reaction as described in section 2.10.3.3.

7.3.4 PCR fragment analysis and NMD assay

I designed two primer pairs specific for the cDNA sequence of *LRRK2* in order to investigate the effect of the L2063STOP and T2397STOP mutations on RNA expression, and to sequence the cDNA in this region. They were designed by using the Primer3 program (http://fokker.wimit.edu/primer3/input.htm) and spanned at least one non-coding region of

LRRK2, to ensure that cDNA rather than any contaminant DNA was amplified. Each PCR was optimised as described in section 2.4. The primers selected and reaction conditions are listed in appendix C.

I performed semi-quantitative PCR fragment analysis on these products using a fluorescent-labeled DNA length marker and GeneScan® software (Applied Biosystems), as described in section 2.8, This can be used to assess the relative proportion of wild-type and mutant mRNA [485], [486], [487]. The PCR has three phases- exponential, linear and plateau (figure 7.3). In the exponential phase the amount of amplified product increases exponentially as reagents are not limited. The linear phase is characterised by a linear increase in product as PCR reagents become limited and the PCR eventually reaches the plateau phase when the amount of product will not change because some reagents become depleted [7]. To ensure that PCR product abundance lay within the linear phase of PCR amplification, when the ratio of allele-specific products still accurately reflects the relative abundance of target molecules, I selected a PCR cycle length of 25 for both reactions. This was on the basis of PCRs I carried out for both mutations at cycle lengths of 20, 25 and 31 in order to quantify PCR products by gel electrophoresis. This data suggested that at 25 cycles both PCRs are within their linear phases.

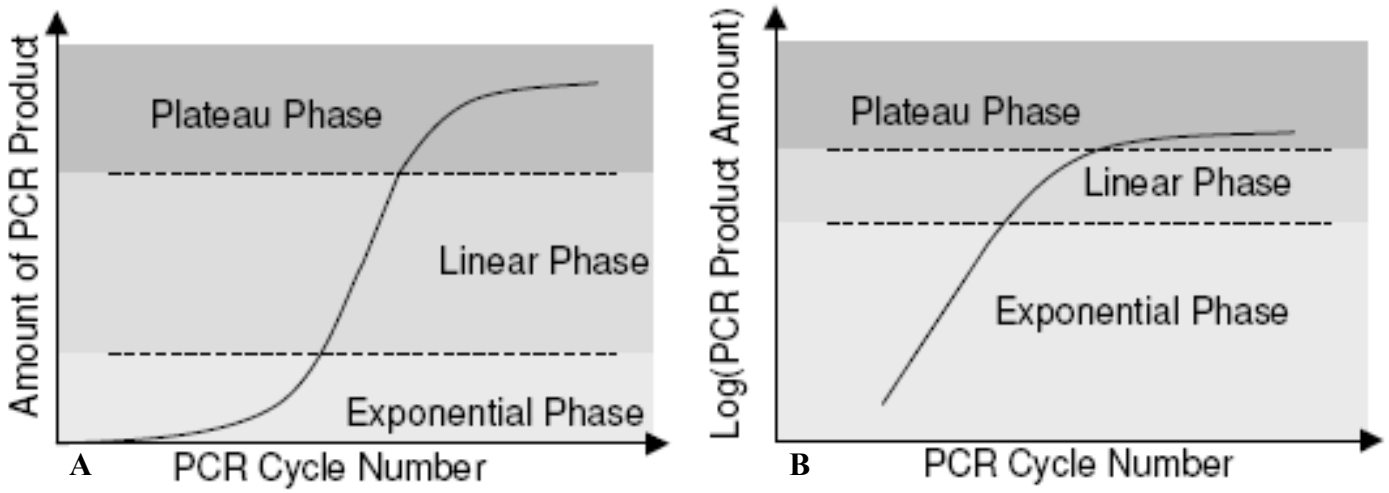


Figure 7.3 Theoretical plot of PCR cycle number against PCR product amount.
Panel A: Three phases can be observed for PCRs: exponential phase, linear phase and plateau phase. Panel B: Theoretical plot of PCR cycle number against logarithm PCR product amount. Taken from Yuan et al. 2006 [7].

The NMD assay is described in section 2.11. Fibroblast cell lines from subjects carrying the L2063STOP and T2397STOP mutations, and two control cell lines (CF1 and CF2), were incubated in triplicate with and without 500μM cycloheximide (CHX) (Sigma), a potent inhibitor of NMD. All 4 cell lines were at passage number 8. At baseline, 2 and 8 hours cells from 3 CHX-treated flasks and 3 untreated flasks for each subject were harvested for RNA extraction. cDNA was subsequently generated from each RNA sample (section 2.10.3.3) for analysis of the relative proportions of wild-type and mutant mRNA before and after this exposure.

7.3.5 Sequencing

All sequencing of DNA and cDNA was carried out using the automated Sanger (dideoxy) DNA sequencing sequencing method as described in section 2.7.

7.3.6 Protein extraction

For the purpose of protein extraction, lymphoblast and fibroblast cells were harvested using Cytobuster™ protein extraction reagent (Promega) with a protease inhibitor cocktail (Sigma), chilled to 4°C. The total protein concentration in each sample was quantified using the Biorad *DC* protein assay (Biorad) according to the manufacturer's instructions. In order to prepare lysates for western blotting, proteins within the samples were denatured by the addition of Novex sodium dodecyl sulphate (SDS) sample buffer (Invitrogen) containing 20mM of the reducing agent dithiothreitol (DTT) (Sigma) and boiled for 5 minutes (section 2.12.1).

7.3.7 Western blotting

I prepared polyacrylamide gels with an 8% resolving gel for separating the protein samples, gel electrophoresis and transfer (sections 2.12.2-2.12.4). Membranes were incubated with one of three primary LRRK2 antibodies NB300-267, NB300-268 or NB110-58771 (Novus Biologicals). All three are rabbit polyclonal antibodies, and the secondary antibody used was a goat anti-rabbit HRP-conjugated antibody (Dako).

In order to quantify the amount of LRRK2 protein detected in patient and control samples, the membranes were subsequently stripped of the LRRK2 probes using Restore™ Plus western blot stripping buffer (Thermo Scientific), according to the manufacturer's instructions. Briefly, the blot was washed three times for 10 minutes each in PBS-tween, then stripping buffer was

added to cover the blot. This was incubated at room temperature for 15 minutes, then removed and the membrane washed three times for 10 minutes each in PBS-tween. The membrane was then 'blocked' as previously described for 1hr at room temperature, prior to being re-probed for a 106kDa loading control protein, Nucleolin. This was detected using a mouse monoclonal antibody to Nucleolin (abcam) and a goat anti-mouse secondary antibody (Dako). The antibody dilution factors are listed below in table 7.1. Antibody application and signal detection are described in sections 2.12.5-2.12.6.

In order to quantify the amount of LRRK2 protein detected in patient and control samples band strength estimation, with comparison to the Nucleolin loading control, was performed with reflectance densitometry, using the Genescan (SynGene) gel documentation system and Genetools software (SynGene).

Primary antibodies	Dilution factor (in 5% dried milk powder/PBS-tween)
LRRK2 (NB267)-rabbit (Novus Biologicals)	1:100
LRRK2 (NB268)-rabbit (Novus Biologicals)	1:500
LRRK2 (NB58771)-rabbit (Novus Biologicals)	1:500
Nucleolin (4E2)- mouse (abcam)	1:5000
Secondary antibodies	**Dilution factor (in 5% dried milk powder/PBS-tween)**
Goat anti-rabbit (Dako)	1:2000
Goat anti-mouse (Dako)	1:5000

Table 7.1 Antibody dilutions used in western blots.

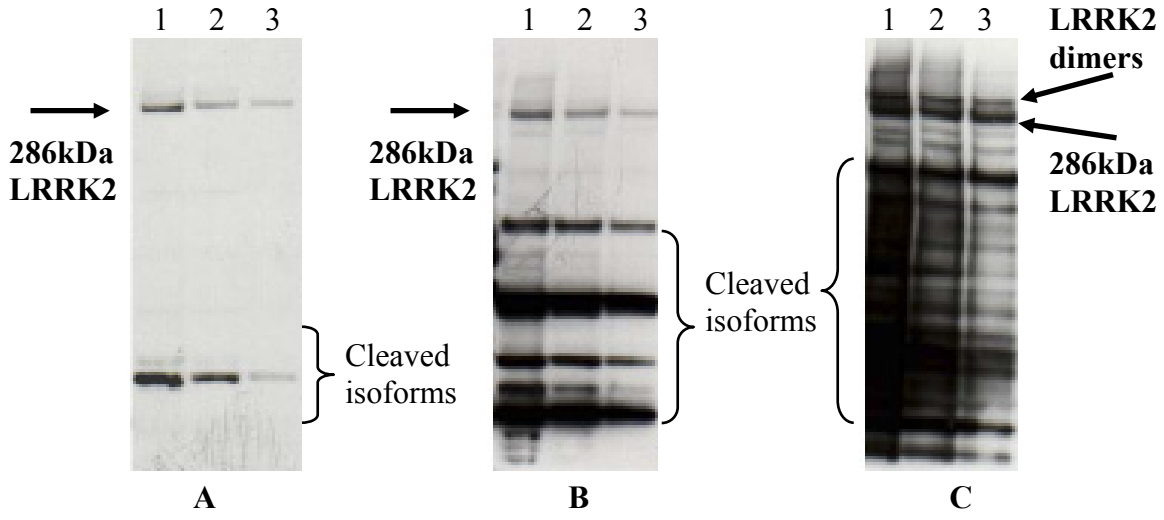


Figure 7.4 Example of immunoblotting of human lymphoblast lysates with commercially available LRRK2 antibodies. Panel A= Blotting with NB58771 at dilution factor 1:10000, Panel B=Blotting with NB267 at dilution factor 1:500, and Panel C=Blotting with NB268 at dilution factor 1:500. Lanes 1, 2 and 3 were loaded with decreasing amounts of protein: 50µg, 25µg and 12.5µg, from human lymphoblast lysates from a single control subject. All recognized a major 286 kDa LRRK2 human protein band, as indicated by the arrows. Additional bands were detected, the larger bands were thought to represent LRRK2 dimers, as indicated by the arrow, whilst those at 100, 170 and 200 kDa were thought to be N-terminal cleaved isoforms. Taken from Melrose et al. 2007 [23].

7.3.8 Statistical analysis

I performed statistical analysis using SPSS for Windows release 15.0 (SPSS Inc, Chicago, Ill., USA). Continuous variables were first tested for normal distribution using Kolmogorov-Smirnov test, and homogeneity of variance was tested using the Levene's statistic. As they showed departures from normality, they were subsequently investigated using a Mann Whitney U Test. A p value <0.05 was considered significant.

7.4 Results

7.4.1 Cell lines and DNA sequencing

Lymphoblast and fibroblast cell lines were successfully generated from the proband subject from family 12 who carried the L2063STOP variation. A fibroblast cell line was successfully generated from the proband subject from family 38 who carried the T2397STOP variation, but we were unable to successfully grow the lymphoblast cell line.

DNA was extracted from cultures of the lymphoblast and fibroblast cell lines. Direct sequencing of exons 42 and 49 of *LRRK2* confirmed the presence of the c.6187delCTCTA (p.L2063STOP) and c.7187insGT (p.T2397STOP) variations in the proband subject from family 12 and in the proband subject from family 38 respectively. Sequencing results from control cell lines revealed wild-type sequence only.

7.4.2 Fragment analysis, sequencing and NMD assay

The cDNA samples were generated using mRNA extracted from the lymphoblast and fibroblast cell lines from the proband subject carrying the c.6187delCTCTA (p.L2063STOP) variation, the fibroblast cell line from the proband subject carrying the c.7187insGT

(p.T2397STOP) variation and both fibroblast and lymphoblast cell lines from control subjects. Sequencing of the cDNA confirmed the presence of the c.6187delCTCTA and c.7187insGT variations in the proband subjects and wild-type sequence in the control subjects. Fragment analysis using Genescan ®, indicated that in cell lines from the two proband subjects wild-type mRNA was clearly present whilst the mutant mRNA was present at a much lower level (figure 7.5). In the control cell lines only wild-type mRNA was detected. The area under each peak represents the amount of either wild-type or mutant mRNA, which was calculated automatically by Genemapper 3.5^{TM} software (Applied Biosystems).

In the NMD assay the fibroblast cell lines from the proband subjects carrying the c.6187delCTCTA and c.7187insGT variations, and control cell lines, were incubated with 500μM CHX. There was a selective and significant increase noted in the level of mutant mRNA for both variants (P=0.05), at both 2 and 8 hours incubation, compared to the fibroblast cell lines from these proband subjects which had been incubated without CHX (figures 7.6 and 7.7 and table 7.2). There appeared to be no effect from incubation with CHX on the level of wild-type mRNA in the control subjects.

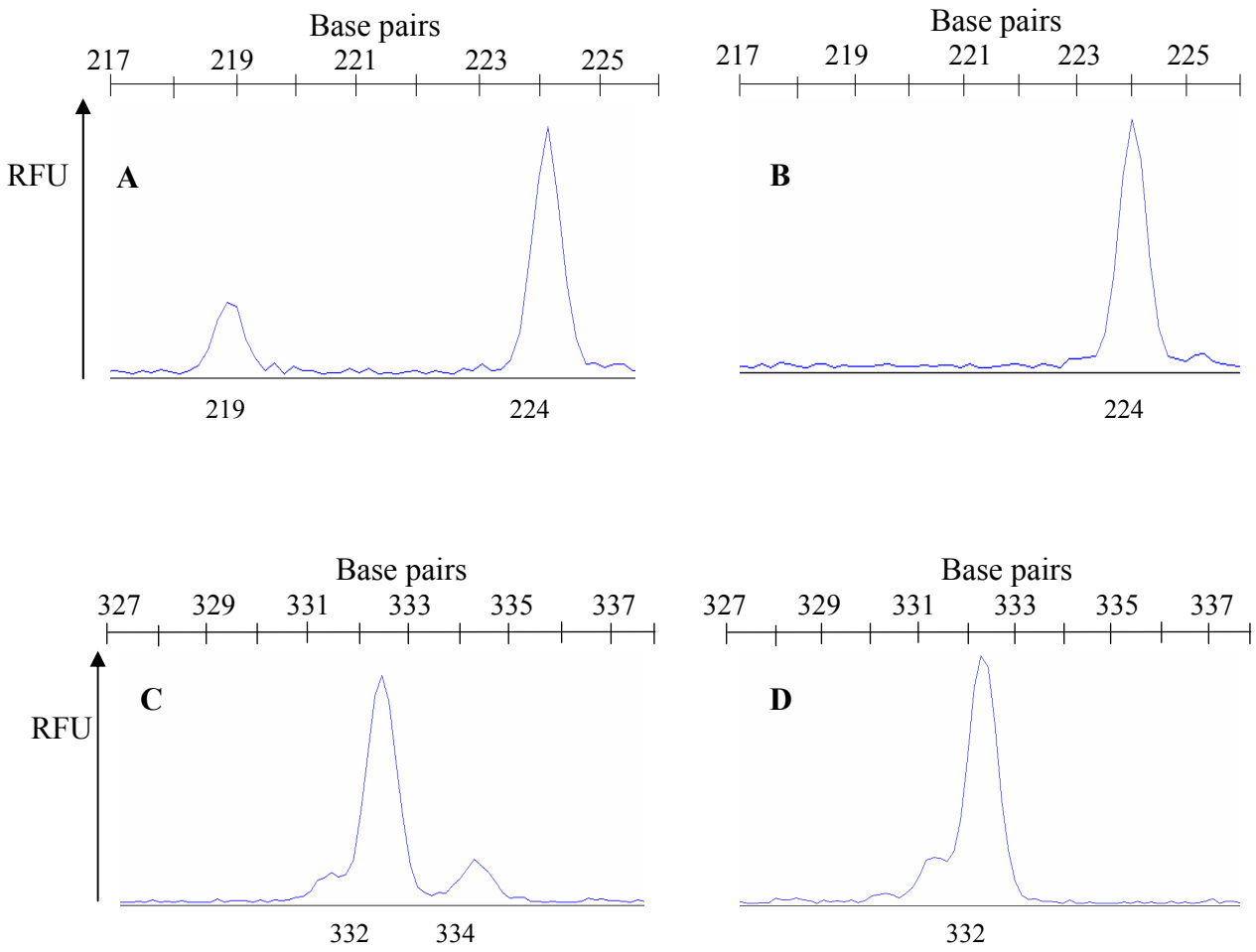


Figure 7.5 Fragment analysis of PCR products using Genescan®, electrophoretograms showing relative wild-type and mutant mRNA levels in fibroblast cell lines from proband and control subjects. Panels A and B show electrophoretograms of amplified fluorescent-labeled PCR products from cDNA template for the c.6187delCTCTA variation. Panel A represents cDNA from subject carrying the variation and panel B represents cDNA from a control subject. The wild-type PCR product is 224 base pairs and the mutant PCR product is 219 base pairs in size. Mutant mRNA is present at a lower level than wild-type in proband subject and absent in the control subject. Panels C and D show electrophoretograms of amplified fluorescent-labeled PCR products from cDNA template for the c.7187insGT variation. Panel C represents cDNA from subject carrying the variation and panel D represents cDNA from a control subject. The wild-type PCR product is 332 base pairs and the mutant PCR product is 334 base pairs in size. Mutant mRNA is present at a lower level than wild-type in proband subject and absent in the control subject. The y-axis represents the intensity of fluorescence, expressed as relative fluorescence units (RFU).

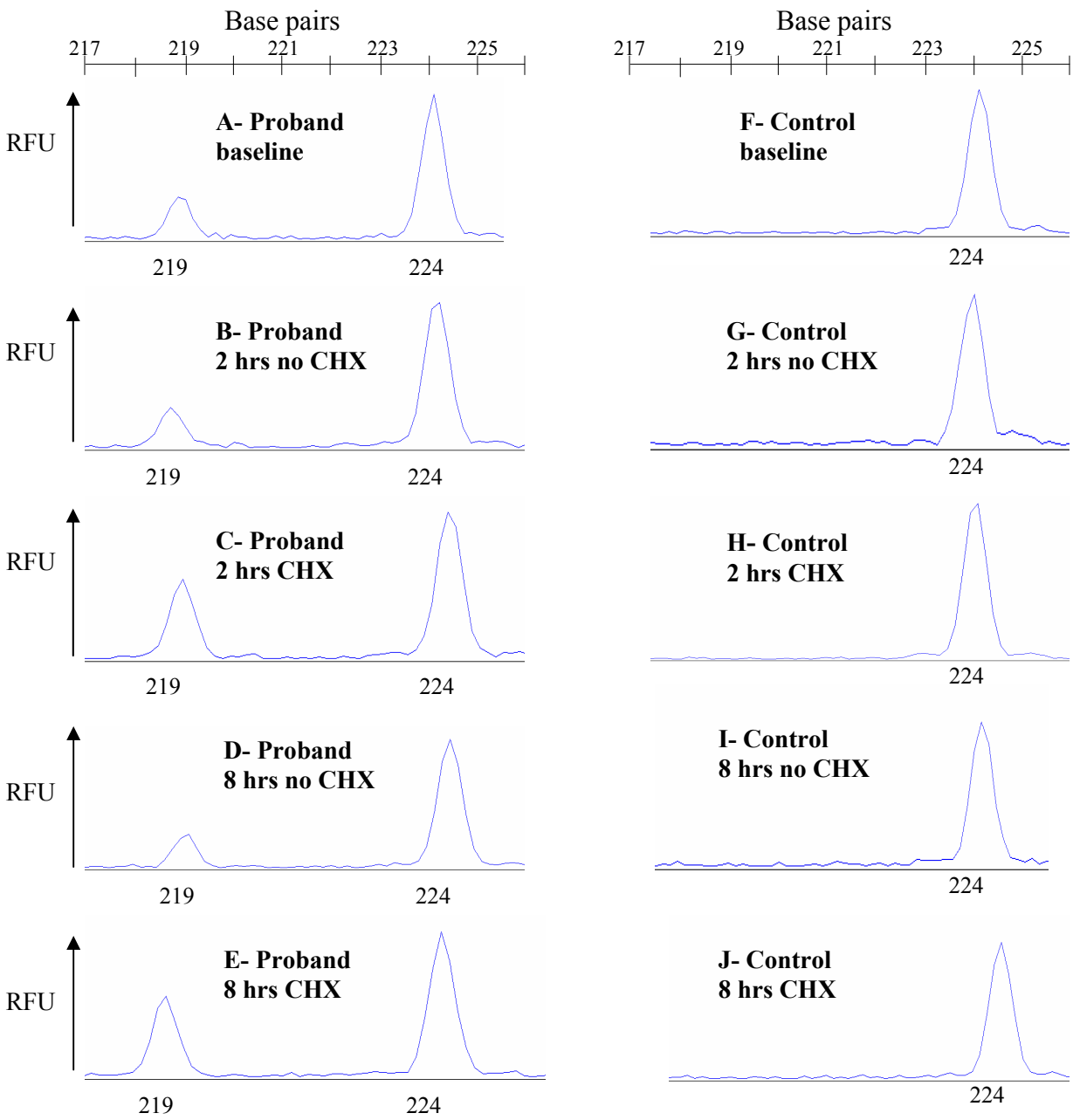


Figure 7.6 Fragment analysis of PCR products using Genescan®, electrophoretograms showing effect on mutant mRNA expression for the c.6187delCTCTA variation after incubation with cycloheximide (CHX). Panels A to E show electrophoretograms of amplified fluorescent-labelled PCR products from cDNA template for proband subject carrying the c.6187delCTCTA variation. The panels depict mRNA levels from baseline to 8 hrs of incubation with or without CHX. Panels F to J show electrophoretograms of amplified fluorescent-labelled PCR products from cDNA template for a control subject. The panels depict mRNA levels from baseline to 8 hrs of incubation with or without CHX. The wild-type PCR product is 224 base pairs and the mutant PCR product is 219 base pairs in size. At baseline the mutant mRNA is present at a lower level than wild-type in proband subject and absent in the control subject. Incubation with CHX for 2 and 8 hours both result in a selective increase in the level of mutant mRNA in the proband subject compared to incubation without CHX. Incubation with CHX appears to have no effect on level of wild-type mRNA in the control subject. The y-axis represents the intensity of fluorescence, expressed as relative fluorescence units (RFU).

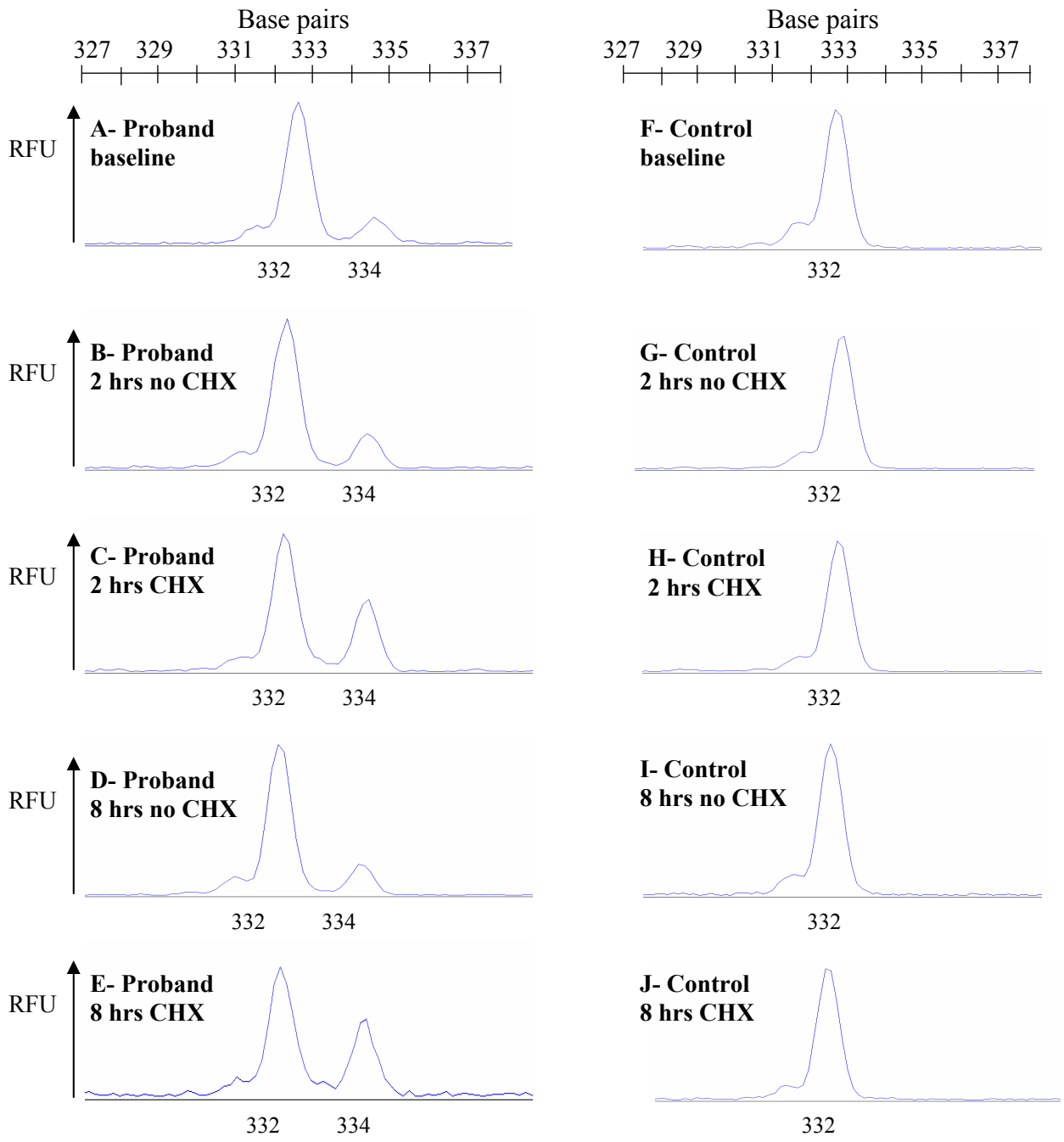


Figure 7.7 Fragment analysis of PCR products using Genescan®, electrophoretograms showing effect on mutant mRNA expression for the c.7187insGT variation after incubation with cycloheximide (CHX). Panels A to E show electrophoretograms of amplified fluorescent-labelled PCR products from cDNA template for proband subject carrying the c.7187insGT variation. The panels depict mRNA levels from baseline to 8 hrs of incubation with or without CHX. Panels F to J show electrophoretograms of amplified fluorescent-labelled PCR products from cDNA template for a control subject. The panels depict mRNA levels from baseline to 8 hrs of incubation with or without CHX. The wild-type PCR product is 332 base pairs and the mutant PCR product is 334 base pairs in size. At baseline the mutant mRNA is present at a lower level than wild-type in proband subject and absent in the control subject. Incubation with CHX for 2 and 8 hours both result in a selective increase in the level of mutant mRNA in the proband subject compared to incubation without CHX. Incubation with CHX appears to have no effect on level of wild-type mRNA in the control subject. The y-axis represents the intensity of fluorescence, expressed as relative fluorescence units (RFU).

Time after treatment with CHX	Mutant mRNA peak as % of wild-type mRNA peak			
	L2063STOP mutation		T2397STOP mutation	
	CHX	No CHX	CHX	No CHX
0 hours		24.3		21.1
		31.7		18.5
		26.1		23.6
2 hours	52.5	23.1	47.2	24.8
	50.1	27.4	41.8	26.1
	36.5	28.9	37.3	20.5
8 hours	48.4	17.1	47.9	22.1
	53.0	24.6	51.7	19.4
	45.4	21.8	46.8	23.1

Table 7.2 Summary of NMD assay in fibroblast cell lines from two subjects with *LRRK2* mutations. The mutant mRNA peak is expressed as percentage of wild-type mRNA peak. Percentages are given for the assay in which cells were incubated for up to 8 hours with cycloheximide (CHX) and without cycloheximide (No CHX).

7.4.3 Optimisation of LRRK2 antibodies and western blotting

I was unable to produce consistently reliable results on western blotting with protein samples from either lymphoblast or fibroblast cell lines, with the three commercially available LRRK2 antibodies used. This was despite using similar protein concentrations and conditions as described in the literature for these antibodies [23], [766]. Some of the problems encountered in using these LRRK2 antibodies included: detection of small bands only with antibodies NB267 and NB268 (figure 7.8), and high background signal due to non-specific antibody binding for NB268 (figure 7.9). Larger bands were also detected with the NB268 antibody (figure 7.9), which may represent LRRK2 dimers.

The data which was obtained from the lymphoblast line for the subject carrying the c.6187delCTCTA (p.L2063STOP) variation with all three antibodies suggests that the full size protein (286kDa) is produced in this subject (figures 7.8-7.10). Furthermore, there was no evidence of a smaller mutant protein, which I would predict to be 240kDa (figures 7.8-7.10). Using Nucleolin as a protein loading control, comparison of the amount of LRRK2 detected in proband and control lymphoblast lysates was made. The results in figure 7.10 appear to suggest that there is more LRRK2 detected in proband than control lysates, but this was not a consistent finding, and provisional densitometry data suggests that the relative amount of full size LRRK2 detected is similar between the patient and control lines.

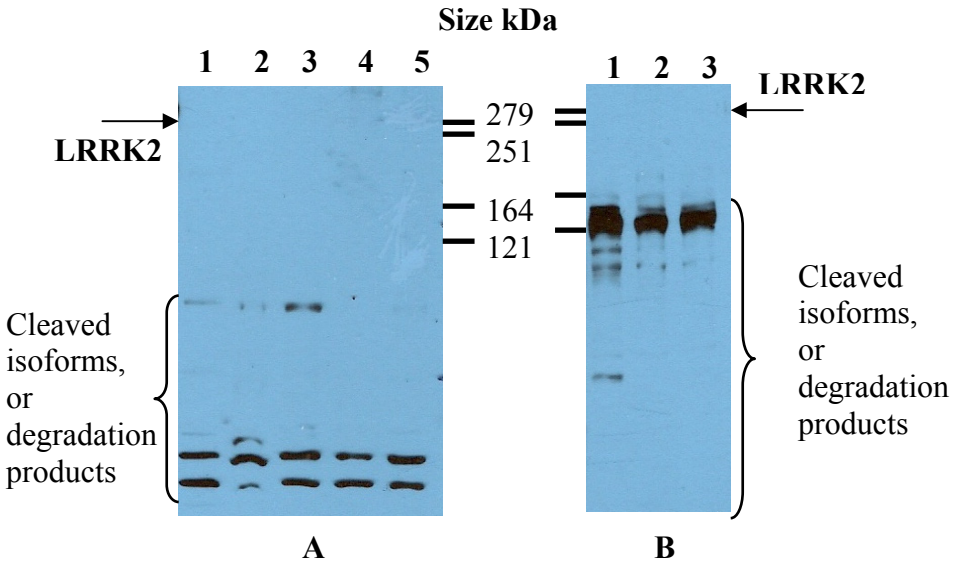


Figure 7.8 Immunoblotting with commercial LRRK2 antibodies of human lymphoblast lysates from the proband subject carrying the c.6187delCTCTA variation and controls (a). Panel A= antibody NB267. Lanes 1, 2 and 5 were loaded with 20µg of protein from the proband subject and lanes 3 and 4 were loaded with 20µg of protein from a control subject. Only small bands were detected, which may represent cleaved isoforms or degradation products of LRRK2. Panel B= antibody NB268. Lanes 1 and 3 were loaded with 20µg of protein from a control subject and lane 2 was loaded with 20µg of protein from the proband subject. The position of the anticipated LRRK2 band (286kDa) is indicated by the arrow. Only small bands were detected, which may represent cleaved isoforms or degradation products of LRRK2. Molecular mass markers for each blot are represented on the side of each panel.

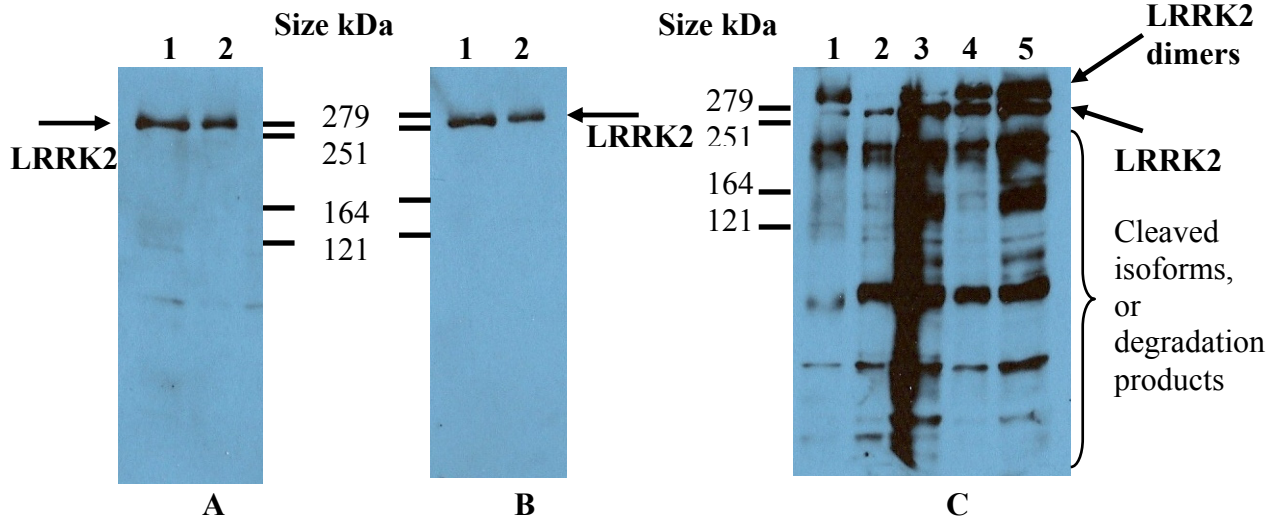


Figure 7.9 Immunoblotting with commercial LRRK2 antibodies of human lymphoblast lysates from the proband subject carrying the c.6187delCTCTA variation and controls (b). Panel A= antibody NB267. Lanes 1 and 2 were loaded with 20µg and 10µg of protein from the proband subject. A band of the correct predicted size of 286kDa for LRRK2 was detected, as indicated by the arrow, with no smaller bands. Panel B= antibody NB58771, lanes 1 and 2 were loaded with 20µg and 10µg of protein from the proband subject. A band of the correct predicted size of 286kDa for LRRK2 was detected, as indicated by the arrow, with no smaller bands. Panel C= antibody NB268, lanes 1, 4 and 5 were loaded with 5µg, 10µg and 20µg of protein from the proband subject and lanes 2 and 3 were loaded with 10µg and 20µg of protein from the control subject. A band of the correct predicted size of 286kDa for LRRK2 was detected, as indicated by the arrow. A larger band, possibly representing LRRK2 dimers, as indicated by the arrow, was also noted for the proband samples but not the controls. High background as a result of non-specific antibody binding, and smaller bands, which may represent cleaved isoforms or degradation products of LRRK2, are also noted. A ladder of known molecular mass markers for each blot is represented on the side of each panel.

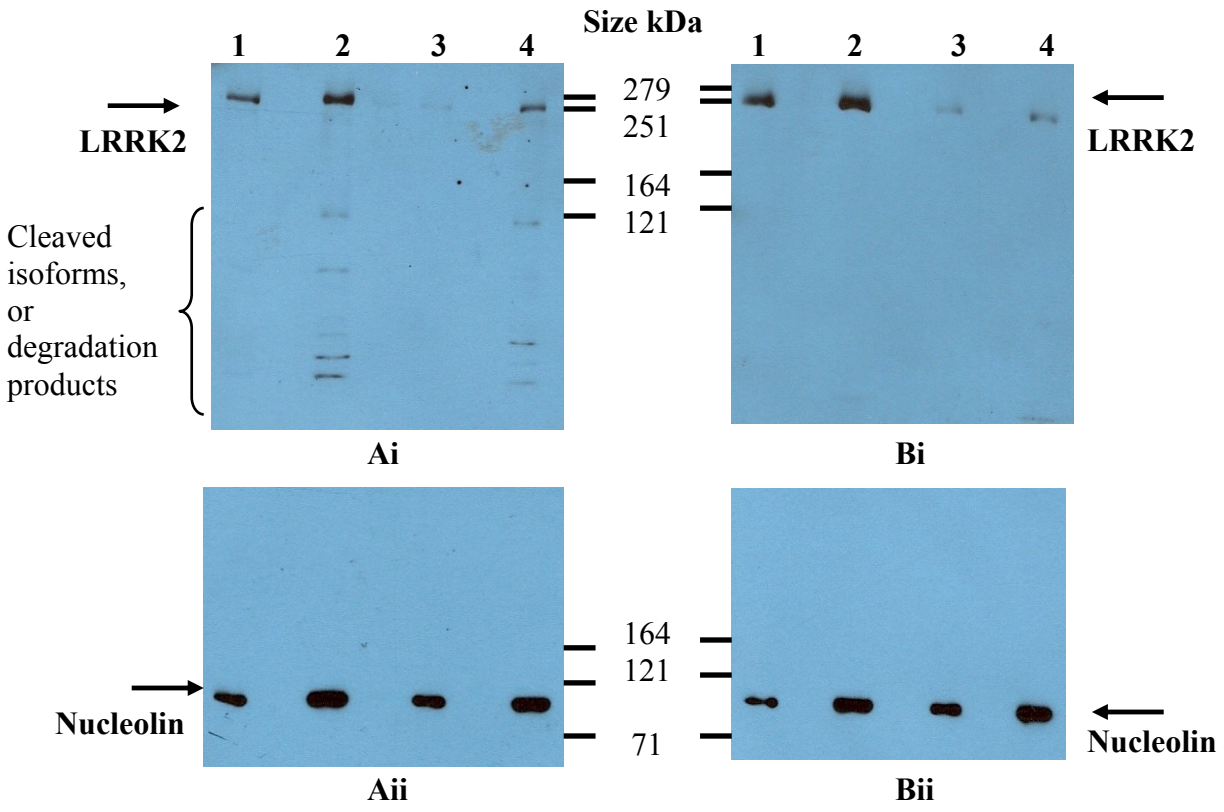


Figure 7.10 Immunoblotting with commercial LRRK2 antibodies of human lymphoblast lysates from the proband subject carrying the c.6187delCTCTA variation and controls (c). Panel Ai= antibody NB267. Lanes 1 and 2 were loaded with 10µg and 20µg of protein from the proband subject. Lanes 3 and 4 were loaded with 10µg and 20µg of protein from a control subject. A band of the correct predicted size of 286kDa for LRRK2 was detected, as indicated by the arrow, as were smaller bands, which may represent cleaved isoforms or degradation products of LRRK2. Panel Aii= membrane Ai stripped of LRRK2 probes and re-probed with Nucleolin antibody. A band of the correct predicted size of 106kDa for Nucleolin was detected, as indicated by the arrow, and the band strengths appear consistent with amount of protein loaded. Using the Nucleolin bands as protein loading controls, there appears to be less LRRK2 detected in the control lanes than the proband. Panel Bi= antibody NB58771. Lanes 1 and 2 were loaded with 10µg and 20µg of protein from the proband subject. Lanes 3 and 4 were loaded with 10µg and 20µg of protein from a control subject. A band of the correct predicted size of 286kDa for LRRK2 was detected, as indicated by the arrow. Panel Bii= membrane Bi stripped of LRRK2 probes and re-probed with Nucleolin antibody. A band of the correct predicted size of 106kDa for Nucleolin was detected, as indicated by the arrow, and the band strengths appear consistent with amount of protein loaded. Using the Nucleolin bands as protein loading controls, there appears to be less LRRK2 detected in the control lanes than the proband. A ladder of known molecular mass markers for each blot is represented on the side of each panel.

7.5 Discussion

7.5.1 Cell lines

Whilst we were able to generate fibroblast cell lines for both patients, we were unable to successfully generate a lymphoblast cell line from the proband subject of family 38 who carried the T2397STOP variation. There are several possible reasons for this. First, the blood sample from which lymphocytes were extracted was taken approximately 18 hours before extraction and second, the relatively older age of this subject. Over many years of experience of creating lymphoblast cell lines, Professor Taylor has noted a clear trend that lines are more successfully generated in younger patients, and when the lymphocytes are extracted as soon as possible after the blood sample has been taken (Malcolm Taylor, personal communication). Alternatively this may simply represent a failure of the technique in this individual case.

7.5.2 PCR fragment analysis and NMD assay

The major finding from the PCR fragment analysis studies performed on cDNA samples, was that for both variations wild-type mRNA was clearly present in the cell lines, whilst the mutant mRNA was present at a much lower level compared to wild type- 26% for the L2063STOP mutation, and 21% for the T2397STOP mutation (figure 7.5 and table 7.2). The PCR fragment analysis technique used is semi-quantitative, permitting intra-sample comparison of amounts of wild-type and mutant mRNA. These techniques are valid for this type of study and have been used before in similar work [498], [499], [502].

I proposed that the mutant mRNA may be degraded by NMD, and in order to confirm this, patient and control fibroblast cell lines were incubated with CHX for up to 8 hours. The use of CHX treatment to inhibit NMD is a well recognized technique and has been used in several

different types of cultured cell lines such as fibroblasts and lymphoblasts [498], [499], [500], [501], [502], [503]. Numerous protocols have been detailed, with varying concentrations of cycloheximide, types and numbers of cells, as well as incubation times ranging from 4 to 48 hours [498], [499], [500], [501], [502], [503]. The protocol I used was based upon that used by Baker et al. (2006) and also recommended by a collaborator Dr Henry Holden.

My data showed a selective and significant increase in the level of mutant mRNA for both variations after incubation with CHX, compared to the patient cell lines which had been incubated without CHX (figures 7.6 and 7.7 and table 7.2). Control cell lines were also incubated with CHX, which appeared not to have any effect on wild-type mRNA levels. However, the Genescan® fragment analysis technique is only semi-quantitative, and to be able to fully quantify the effects on mRNA expression a qRT-PCR (real time PCR) technique needs to be used, as discussed later. This data provides the first evidence that NMD may indeed be an important mechanism in *LRRK2* PD. There is only one previous report of a *LRRK2* variation in which the authors speculated that mutant mRNA is likely to undergo NMD [665]. The process has been demonstrated previously in PINK-related PD using a similar technique [499].

NMD is a normal cellular mechanism which reduces the abundance of potentially deleterious transcripts containing a premature termination codon (PTC) to approximately 5-25% of the normal (PTC-free) level, and thus reduces synthesis of the encoded truncated protein [494], [495], [15], [496]. In general only PTCs located more than 50-55 nucleotides upstream of a splicing-generated exon-exon junction within mRNA elicit NMD [765], see figure 7.2. Both of the novel frameshift *LRRK2* mutations which I have studied introduce PTCs which fit these criteria.

I hypothesise that these two newly identified *LRRK2* frameshift mutations cause disease by creating functional null alleles, with the mutant mRNAs being degraded by NMD. The overall result is a loss of functional LRRK2 due to haploinsufficiency. Although the degradation of the mutant allele is not 100% complete, and it cannot be excluded that low amounts of truncated protein lead to a dominant-negative or gain-of-function mechanism, this is an apparently novel pathogenic mechanism in *LRRK2* PD.

The mechanism that has previously been proposed for pathogenic *LRRK2* mutations is kinase over-activity, particularly the G2019S and R1441C mutations for which there is most evidence. Biochemical studies on cultured cells transfected to express LRRK2 show up to a 3-fold increase in kinase activity for the G2019S mutation [429], [661], [691], [692]. The R1441C mutation in the ROC domain has been reported to affect GTP hydrolysis and binding [663], [723], [691]. A predicted consequence of mutations in this region would be that they influence the downstream kinase activity through perturbation of the GTPase domain. However the putatively pathogenic mutations G2385R and I1371V do not appear to affect kinase activity [691], whilst the I2012T variant appears to decrease kinase activity [691], [692].

Some of the previously conflicting data on the effect of pathogenic *LRRK2* mutations on kinase activity may be explained by variations in methodological or analytical techniques between different studies. However, my findings for the two novel frameshift mutations, which suggest a possible loss of functional LRRK2 due to haploinsufficiency, provide further evidence that kinase over-activity alone may not be the only pathological effect of *LRRK2* mutations.

7.5.3 Optimisation of LRRK2 antibodies and western blotting

Due to the lack of replicable data I am cautious in making definitive conclusions about the work performed in this section of the study. However my data supports the findings from my mRNA studies, in that for the subject carrying the c.6187delCTCTA (p.L2063STOP) variation a smaller mutant protein does not appear to be produced. These findings may be explained by the process of NMD of the mutant mRNA, as I have already proposed, or may be a result of degradation of an unstable small mutant protein. Either of these explanations would support my theory of haploinsufficiency rather than toxic gain of function as a pathogenic mechanism for *LRRK2*–related PD.

There are two important caveats to this conclusion. First, my own data also suggests that the amount of full size LRRK2 protein appears consistent between both the patient and control cell lines. Second, whilst I did not detect a smaller toxic protein in my studies, it may indeed be present *in vivo* and my experimental technique was not sensitive enough to detect it. This was despite me using LRRK2 antibodies and protocols which had previously been used successfully in published studies and recommended personally by other researchers [766], [23], (Vincenzo Bonifati, personal communication), (Heather Melrose, personal communication).

The complexity of performing work on the LRRK2 protein, and in particular optimising LRRK2 antibodies, is highlighted by two recent studies. Biskup et al. evaluated 21 commercially available antibodies. Just over half detected over-expressed human LRRK2 protein in cell lines, and only four (including NB267 and NB268) had sufficient specificity to detect endogenous LRRK2 in human brain lysate samples [766]. The authors also commented that whilst they were successful in visualizing LRRK2 protein by western blotting in fresh frozen brain tissue, post mortem delay of only a few hours rendered the technique

unsuccessful in most cases. They speculated that this may be due to a high rate of degradation or processes that otherwise render LRRK2 inaccessible to detection by immunoblotting. They did not however observe significant decay in LRRK2 protein within protein lysate when stored at -20 or -80°C, or dramatic loss of LRRK2 due to freeze/thaw cycles [766].

Melrose et al. compared immunohistochemical findings for six LRRK2 antibodies (two 'in house' and four commercially available), including NB267, NB268 and NB58771 used in my study. Antibody NB58771 was originally developed by Melrose et al. as one of their 'in house' antibodies [23]. As in my study, the investigators used human lymphoblast lysates and found that all the antibodies recognized a major 286 kDa LRRK2 human protein band (figure 7.4). They also detected additional protein bands with several antibodies. The larger bands were thought to represent LRRK2 dimers [23], as previously reported [428]. The smaller bands were thought to represent truncated LRRK2 proteins [23]. At least three N-terminal cleaved isoforms of LRRK2 (100, 170 and 200 kDa) have previously been identified by immunoprecipitation and mass spectrometry [767]. Indeed, the manufacturer (Novus Biologicals) claims that many of these additional bands can be blocked by using the control pepitde appropriate for each LRRK2 antibody, suggesting that they are indeed degradation products (http://novusbio.com/data_sheet/index/NB300-268). Some of these additional bands may also represent non-specific cross-reactivity with other proteins. As in my study, Melrose et al. also found that the NB267 and NB268 antibodies displayed the greatest number of additional bands, whereas their in-house antibodies, including NB58771 which I also used, displayed the least number of additional bands [23].

The western blots of lymphoblast lysates from the subject carrying the c.6187delCTCTA (p.L2063STOP) variation for the three LRRK2 antibodies used in the current study (figures

7.8-7.10) closely resemble those published by Melrose et al. (figure 7.4). The clearest band for LRRK2, from both my work and that from Melrose et al., can be seen using NB58771. This was also confirmed directly to me by the investigators (Heather Melrose, personal communication). However, as the recognition sites of the NB58771 and NB268 antibodies are located between amino acids 2500-2527 (figure 7.11), I was reliant on NB267 to detect any truncated protein produced in association with the c.6187delCTCTA (p.L2063STOP) variation.

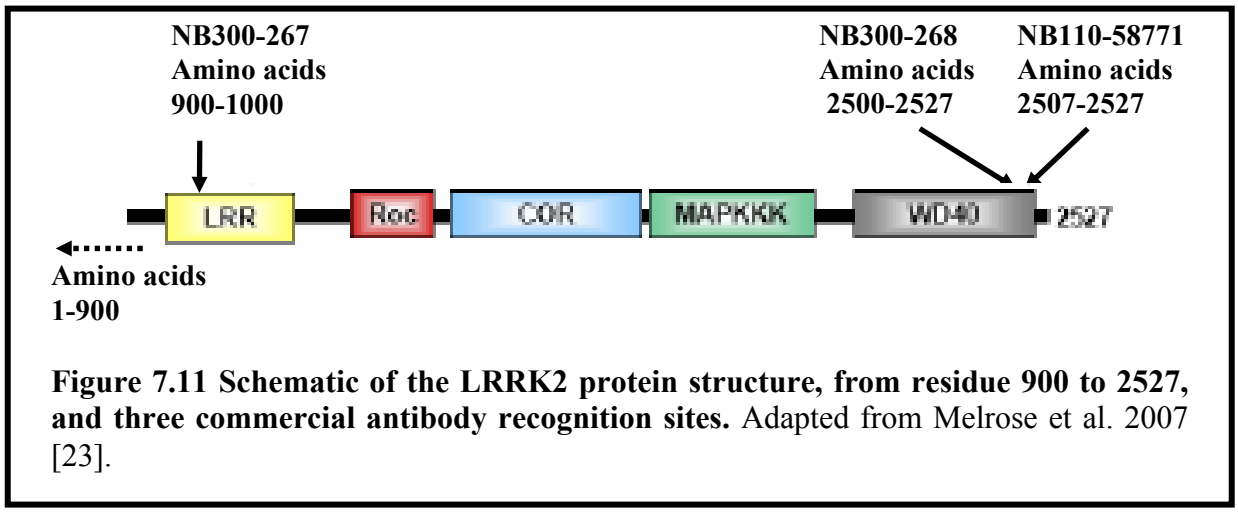


Figure 7.11 Schematic of the LRRK2 protein structure, from residue 900 to 2527, and three commercial antibody recognition sites. Adapted from Melrose et al. 2007 [23].

The lack of consistent data with the three antibodies used in my study may be for several reasons. Firstly, as outlined in both of the studies discussed above, working with such a large protein as LRRK2 is technically challenging in many aspects, especially in preventing protein degradation. In addition, there may have been inconsistencies between different batches of antibodies used in my study and those in previously published work. With particular regard to determining whether a truncated protein is produced with the c.6187delCTCTA

(p.L2063STOP) variation, I was hindered in not being able to use the most reliable antibody available.

7.6 Future work

There are several further molecular genetic and functional studies that could be performed to further both our knowledge on the effects of the three novel frameshift mutations and our understanding of *LRRK2* PD in general.

First, re-bleeding the proband subject from family 38, who carried the T2397STOP variation, would allow us another attempt to grow a lymphoblast line from this subject. Having lymphoblast cell lines on both of the subjects carrying the novel *LRRK2* variations would allow me to confirm the findings of the fragment analysis, sequencing and NMD assay obtained from the fibroblast cell lines.

Second, for the NMD assay, it can be argued that RNA obtained from cultured cells does not represent a true baseline level. It has been shown that RNA expression levels can be affected by lymphoblastic differentiation (for example the somatostatin receptor expression has been demonstrated to increase) [768], [769], [770]. Therefore, re-bleeding both subjects carrying the novel *LRRK2* variations, and extracting RNA from lymphocytes before they are immortalized, would allow me to confirm the baseline findings for this assay.

Third, in order to fully quantify the amount of mRNA between samples a qRT-PCR (real time PCR) technique, with use of an internal standard, is required. This has been used before in some previous similar studies of NMD [771], [498], [772]. Real-time PCR detects the accumulation of products during the reaction, and is accurately able to exploit the fact that the quantity of PCR products in exponential phase is in proportion to the quantity of initial

template [773], [774]. I was unable to set up this technique due to time constraints, but its use would also allow confirmation of the findings from my PCR fragment analysis studies.

Fourth, in order to confirm my findings from the mRNA studies at the protein level further work needs to be performed. The experimental design of the protein studies with the three previously used antibodies could be re-optimised in order to produce more consistent and reliable data. This could be achieved using epitope-V5 tagged LRRK2 transfected into HEK293T cells as positive controls, such as used in a previous study [23]. Furthermore, fibroblast cell lysates could be used in addition to the lymphoblast cell lysates in order to confirm the results in a second cell line. In addition other commercial LRRK2 antibodies could be investigated and optimised within the experimental system in order to determine if they produce more consistent and reliable data.

The development of novel LRRK2 antibodies using other potential antigenic sites within the protein would be another strategy. I have in fact already tried this in conjunction with a collaborator, Dr David Ramsden, Department of Medicine, University of Birmingham. We synthesised a peptide corresponding to amino acids 329 to 349 of LRRK2, as this region appeared most promising in terms of antigenicity. This is within a protein interaction domain, armadillo repeats (residues 180-660), named after the β-catenin-like Armadillo protein of *Drosophila* [18]. However, the antigenic response of sheep to our LRRK2 peptide was poor, despite re-immunisation. As well as trying other peptide sequences a variant of this peptide could be synthesized in which random amino acids are inserted near the N-terminus in order to increase its antigenicity.

Fifth, in order to further explore the effect of the novel variations on LRRK2 function, it would be extremely valuable to investigate how they influence the kinase activity of LRRK2.

Using similar approaches to those described in the introduction *in vitro* expression models of these mutant LRRK2 proteins could be generated. These models would allow me to perform a number of assessments, most notably kinase assays, but the effects on cell toxicity, aggregation, neurite outgrowth and branching could also be determined.

7.7 Conclusions

In this chapter I investigated the effects of two novel *LRRK2* frameshift mutations on RNA and protein expression, and the data presented here provides the first evidence that NMD is occurring in *LRRK2* PD. I thus suggest that in *LRRK2* PD one must consider other disease-causing mechanisms aside from the 'kinase over-activity' paradigm that has been previously proposed by others.

8 General Discussion

8.1 Introduction

The broad aims of this project were to recruit a large well characterised cohort of subjects with autosomal dominantly inherited Parkinson's disease (PD) from around the United Kingdom (UK); to assess the contribution of coding sequence variations within the *LRRK2* gene in these subjects with familial PD (fPD) and also in large cohort of UK subjects with sporadic PD (sPD); and to provide initial functional characterisation of any novel mutations identified.

Proband subjects were successfully recruited from families with three or more members affected by PD and known pathogenic *LRRK2* mutations were identified in both the familial and sporadic groups at frequencies predicated from previous work. In addition, three novel coding sequence variations were identified in the fPD group, all of which are predicted to cause a shift in the reading frame, and result in premature termination codons. For two of these variations my functional data suggests that nonsense mediated decay (NMD) of the truncated transcript has occurred, perhaps leading to haploinsufficiency, a novel pathogenic mechanism for *LRRK2*-related PD.

As part of this project I also performed a clinical and molecular genetic study of a unique kindred in which affected members display a variety of phenotypes, ranging from PD to Dopa-responsive dystonia (DRD). I predict that variable expression of a novel missense mutation identified within the *GCH1* gene may result in this wide range of observed phenotypes.

In this chapter I will discuss these findings particularly with regard to our understanding of *LRRK2*-related PD and Dopa-responsive dystonia (DRD), but also with regard to some of the

broader concepts in this field of research, such as gene-environment interactions and the development of neuro-protective therapies.

8.2 Familial PD

As discussed in chapter 3, a cohort of 46 UK subjects with PD from families in which there were three or more members affected by PD and where segregation of disease in the family appeared to follow an autosomal dominant pattern were recruited. Importantly the similarity of the demographic and clinical characteristics of this cohort to those of fPD cohorts previously described in the literature (section 3.6 and table 3.12) makes the results of my molecular genetic studies generalisable. Whilst taking into consideration that this was not a population-based study designed specifically to investigate environmental influences in PD, the data from my epidemiological survey indicates: head injury and cerebral infection do not appear to be significant risk factors for PD in this cohort; a history of toxin exposure, rural living and well water consumption was demonstrated in around 20% of all participants, suggesting that if these risk factors are important in the aetiology of PD they would only appear to be so in up to a quarter of cases.

8.2.1 LRRK2 in UK familial PD

One of the major criticisms of previous molecular genetic studies into *LRRK2* has been that they have not examined the whole coding sequence of the gene, and hence may have missed important sequence variations. Indeed only one previous comprehensive screen of *LRRK2* in a large cohort of UK subjects with fPD has been performed [279]. As discussed in section 5.5, in screening of the entire predicted coding region of *LRRK2* I identified 61 sequence

variations, of which 16 were in protein coding regions and are predicted to lead to nonsynonymous amino acid substitutions (see table 5.1). Four probands (8.7%) were found to be heterozygous carriers of one of the previously described *LRRK2* mutations for which there is data to suggest pathogenicity, a figure which corresponds to those quoted in previous reports [279], [280], [293], [665], [26], [775]. Three of these probands carried the G2019S mutation and one carried the R1441C mutation. The mean age of onset of symptoms of these probands was 58.6 (SD 8.2), with a range of 49-71. Three probands displayed phenotypes typical for IPD, whilst one proband carrying a G2019S mutation had a more slowly progressive disease. This atypical phenotype is unusual in association with the G2019S mutation, although there are some reports in the literature of younger disease onset and slower disease progression [282], [776], [667]. On olfactory testing all four of these subjects also displayed microsmia, or anosmia, which again fits with data from previous reports [294], [279, 777], [667]. Therefore the findings from my study confirm the importance of these two pathogenic mutations in UK fPD.

Another important observation is that in this study I found no *LRRK2* variations which increased the risk of developing fPD. This is in contrast to two variations found in Asian populations which appear to be true risk variants for sporadic PD, the first G2385R has been associated with a two-fold risk increase in Taiwanese [293], [695], [697], [698], Japanese [699], and Chinese [700], [701], [702] populations [778]. This variation does however appear to be restricted to certain Asian populations only, and has not been found in other Caucasian populations tested to date [779], [294], [279], [280]. The R1628P variation has also recently been identified as a risk allele [780], [781] within Asian PD populations and was not identified in my study.

8.2.2 Novel LRRK2 mutations

Three novel heterozygous variations c.4364delAT (D1455G), c.6187delCTCTA (p.L2063STOP), and c.7187insGT (p.T2397STOP) were identified in one proband case each. This highlights the importance of performing a comprehensive screen of the whole coding sequence of the gene when studying fPD. All three variations result in a shift in the reading frame, with the introduction of a premature termination codon. As discussed in section 5.5.1 this is the first time that frameshift mutations in the coding sequence of *LRRK2* have been described. The phenotype observed in all three novel variations described here appears to be typical for IPD, as has been observed in previous studies of *LRRK2* positive PD patients [27], [288], [283], [289], [290], [285], [625]. These three novel mutations appear to be rare familial mutations, as I did not find them in an extensive screen of sPD chromosomes.

Most *LRRK2* mutations described to date are missense mutations and the working hypothesis of the mechanism of pathogenicity has been through a dominant negative effect. The identification of these three novel frameshift mutations suggests that at least for these variations pathogenicity may be through a novel mechanism for *LRRK2,* of haploinsufficiency. Indeed, as discussed in section 7.5.2, the results from my nonsense mediated decay (NMD) assay suggest that the c.6187delCTCTA (p.L2063STOP) and c.7187insGT (p.T2397STOP) frameshift mutations may cause disease by creating functional null alleles, with the mutant mRNAs being degraded by NMD, which results in loss of functional LRRK2 (haploinsufficiency). This is an apparently novel pathogenic mechanism in *LRRK2* PD and is in contrast to the previously proposed kinase over-activity theory for the pathogenic *LRRK2* mutations [429], [661], [691], [692].

8.3 Sporadic PD

This part of the project involved the study of four cohorts of patients from around the UK, the majority of whom had apparently sporadic PD (sPD). When studying sPD one of the major aims is to ensure that the study cohort is as representative of a 'community-based' IPD cohort as possible. This is in order to ensure that when genetic analyses are performed, any variations which may be identified are as relevant as possible to the 'typical' IPD seen by general practitioners, physicians and neurologists rather than the more 'atypical' cases that might be seen in specialist movement disorder clinics. The demographics of the 357 subjects from the PD GEN cohort were the most representative of a 'community-based' sample of IPD, and this cohort was therefore used for molecular genetic analyses of sPD providing us with generalisable data.

8.3.1 LRRK2 in UK sporadic PD

Eight 'hot spot' exons of *LRRK2* were screened for known and novel mutations in subjects from the PD GEN cohort by denaturing high performance liquid chromatography (DHPLC) and at total of 15 heterozygous sequence variations were identified (see figure 6.6). Four subjects (0.9%) were found to be heterozygous carriers of known pathogenic mutations, previously shown to segregate with disease, of which three carried the G2019S mutation and one the R1441C mutation. This corresponds to figures previously reported in the literature [282], [283], [284], [285], [281], [282]. All four of these subjects had typical late-onset levodopa-responsive PD, again corresponding to previous reports [288], [283], [289], [290], [285], [681], [667]. There were no novel sequence variations identified in protein coding regions that are predicted to lead to nonsynonymous amino acid substitutions.

This is the most extensive screen for known and novel *LRRK2* mutations performed in a large cohort of UK subjects with sPD and is the first use of the PD GEN cohort. Furthermore this project also demonstrated that DHPLC is an effective method for high throughput mutation detection in the *LRRK2* gene, and could be used to complete further screening of this gene. My data confirms both the importance of the G2019S and R1441C mutations in UK sPD, as well as the proposed aetiological link between the fPD and sPD.

8.4 Kindred with variable phenotypes and a novel GCH1 mutation

The clinical diagnosis in affected subjects from this unique family with phenotypes ranging from PD to DRD was challenging for over 20 years because of the atypical nature of the symptoms and signs. Furthermore, olfaction was moderately impaired in affected subjects, but with no clear correlation with phenotype. My identification of a novel heterozygote *GCH1* missense mutation, c.A5G (p.E2G), in affected subjects only, has at last provided an explanation for the diverse clinical presentations observed in this family. The association of this novel *GCH1* mutation with such a wide range of phenotypes has also furthered our understanding of the way late onset DRD can present. Intriguingly, in this family both normal and abnormal functional neuro-imaging was noted in association with the same *GCH1* mutation.

As well as the considerations for DRD, the association of this novel *GCH1* mutation with a form of late onset familial parkinsonian is perhaps of even greater interest as it suggests a potential role for the *GCH1* gene in the much more prevalent condition of PD. I will consider this subject again in section 8.6 below.

8.5 Implications for pathogenesis of PD and neuro-protection

Since the c.6055G>A (G2019S) mutation in exon 41 was first identified in 2005 in both fPD and sPD [281], [282], [283], [284], [285] elucidating the function of the LRRK2 protein, and how its dysfunction leads to neurodegeneration, has been the focus of much work. Indeed given the location of this mutation within the kinase domain most investigators have focused on the effects of on kinase function. As discussed within section 7.1 there is now some evidence for a modest increase in kinase activity for the G2019S mutation [429], [661], [691], [692], [782], although the effect of the other pathogenic mutations is more controversial [429], [691], [661], [692]. Another of the 'PD genes' PINK1 also has serine/threonine kinase activity, with evidence that loss of function leads to a complex cellular phenotype including defects in mitochondrial morphology, increased sensitivity to cellular stressors and reduction in subsets of dopaminergic neurons [227], [329], [330], [331], [332], [333], [334], [335], [336]. Hence we continue to investigate the role of the kinases in the pathogenesis of fPD, and ask whether kinase inhibitors could be therapeutic options for the future.

We should, however, be cautious in our interpretation of previous results as the true substrates for LRRK2 remain to be determined, and hence the model systems used may be inappropriate. The identification of the three novel *LRRK2* mutations in my study also raises some important questions about this theory of toxic gain of function related to kinase activity. The evidence from my *in vitro* studies of the c.7187insGT (p.T2397STOP) and c.6187delCTCTA (p.L2063STOP) mutations suggests that they may act by creating functional null alleles, resulting in loss of LRRK2 function. Clearly further investigation of the mechanism of these mutations is required, but taken together with the previous reports, that some pathogenic *LRRK2* mutations may not increase kinase activity [661], [692], I am cautious about focusing solely on kinase over-activity as the pathogenic mechanism. There are also two important

reports that describe abolishing LRRK2 kinase function, with the finding that the toxicity of all LRRK2 mutants was diminshed, even those that did not show evidence of kinase over-activity [661], [457]. It may be that at least some pathogenic mutations cause cell death by altering some other feature of LRRK2 biology, but still require intact (basal) kinase function. A unifying explanation may be that LRRK2 is a signaling molecule, with kinase activity as a key part of the signaling process, such that when the kinase activity is affected (increased or decreased) the molecule is unable to function correctly [783], [784], [782].

As outlined in the Introduction, PD is a complex condition and despite the identification of a number of 'PD genes' it is still believed that exposure to environmental toxins may play a role in the pathogenesis of the condition. Even in the case of *LRRK2*-related PD the finding of an age-dependent penetrance for the G2019S mutation [281], [287], [721] and the identification of the G2385R and R1628P risk alleles within several Asian populations [293], [695], [697], [698], [699], [700], [701], [702], [778], [780], [781] raises the age-old question as to whether there may be a potentially important role for environmental factors.

Determining what is environmental, what is genetic and the interaction between the two has always been difficult to prove. Many observed interactions have proved difficult to reproduce, not just in PD, but for many human diseases [785]. Whilst further studies are clearly required to investigate this issue, previous attempts including the Geoparkinson study and a *Drosophila* parkinsonian model have not as yet yielded much useful information [153], [786]. One way in which this may be overcome is to ensure that prospective studies of disease include a sample biobank (usually a DNA collection) and good quality environmental exposure data on both subjects and controls. The PD GEN study is a good example of this (www.pdgen.bham.ac.uk). DNA samples from this study were used here, and I have also been involved in recruitment to

the study. In the case of *LRRK2*-PD it will also be crucial to identify physiological substrates for the protein, which may then give us clues as to which environmental toxins to investigate.

8.6 Future directions and experimental studies

I have already outlined the future experimental work required to expand upon the data generated by this study in the discussion to each of chapters 4 to 7. It is also important to consider future studies in general terms, in order to develop a better understanding of the direction in which research into the genetics of PD will move over the next few years.

With regard to *LRRK2*-related PD, it is clear from the work presented in this thesis that in future a comprehensive screen of the whole coding sequence of the gene in every fPD cohort studied will be crucial in order not to miss potentially important variations. Furthermore, it will be extremely valuable to determine whether the three novel missense mutations identified in this study are found in other fPD, and indeed sPD, cohorts from various populations around the world. In order to further elucidate the functional effects of these mutations it will be vital to show whether there is any expression of the truncated proteins. Developing *in vitro* expression models of these mutant LRRK2 proteins, alongside wild-type protein, will be important in order to better predict their downstream effects. This will specifically allow us to use GTPase and Kinase activity assays, as well as permitting us to model other potentially pathogenic mechanisms for *LRRK2,* such as apoptosis. In order for us to understand how dysfunction of LRRK2 causes PD it will also be crucial for us to develop a much better understanding of the normal function and physiological substrates for LRRK2. Finally, I believe that it will also be important for us to assess copy number variation in *LRRK2,* for both fPD and sPD, as it has previously been shown to cause PD in *Alpha-synuclein* and *Parkin*-

related disease [251], [306], [307]. To date it has only been studied twice in *LRRK2,* and although no whole-gene deletions or duplications were detected [293], [666] it may still be another mechanism by which *LRRK2* could cause PD.

As outlined in sections 1.5.2 and 1.5.3 of the Introduction, we are increasingly aware of interactions between the 'PD genes' which may be important within the already complex pathogenic process of PD (see figure 1.6). Indeed, the concept of digenic parkinsonism has been gaining increasing support, with several recent reports of subjects simultaneously harboring mutations in *Parkin* and *LRRK2* [729], [669], [689]. There is also a report of digenism of *SCA2* and *LRRK2* mutations, with earlier disease onset [787]. Given that identical *LRRK2* mutations in the same kindred can result in different pathological findings, a modulating effect by other genetic variations is an interesting concept [788]. Furthermore, there are an increasing number of novel interactions being proposed, such as the observed interaction of the Roc domain of LRRK2 with microtubules [789]. This is especially interesting given the potentially important role for microtubule-associated protein tau (MAPT) in the pathogenesis of PD (outlined in section 1.5.2.8 of the Introduction).

My own data may also be of importance in relation to investigation of these potential interactions, with the discovery of a putative role for *GCH1* in PD. This still needs further investigation. In the first instance we need to screen for *GCH1* mutations in cohorts of subjects with fPD and also sPD to confirm if there is an association with PD. As discussed in section 4.5.6, an assessment of the functional effects of the mutation will also be important to give us information regarding the pathogenic mechanisms involved.

Re-evaluating the pathogenic process in PD in terms of metabolic pathways involved may also give us clues as to how and which genes may be interacting. For example, as discussed in

section 1.5 of the Introduction, there is now increasing evidence that defects in the breakdown of glucosecerebroside to ceramide and glucose may be linked to PD, with mutations in *alpha-synuclein*, *GBA*, *ATP13A2* and possibly even *LRRK2* implicated in this process [790]. Metabolomic profiling of plasma, Cerebrospinal fluid (CSF), or urine from subjects with PD may give insight into this currently under researched area.

I believe that the concept of digenic parkinsonism may be potentially crucial, and hence an area to focus on over the next few years. In addition to screening for variations in multiple genes, it will be necessary to develop both *in vitro* and *in vivo* models of potential interactions between the various proteins and pathways involved.

Finally, whilst I have used a candidate gene approach in this study, it is important to highlight that another increasingly popular method by which we can investigate genetic factors not just in PD, but also for a number of other complex diseases, is through genome wide association studies [297]. The idea behind the strategy is to comprehensively test common variations for association to disease [791]. Such studies have become feasible through the availability of large marker panels, as well as cost-effective and high through-put genotyping technologies. Importantly such studies are also able to detect interactions between genetic loci, as well as assessing whether small individual effects might contribute more substantially to disease risk through nonadditive interactions among loci (known as epistasis). This is all dependent upon having access to good quality statistical analytical strategies or programs, which are now readily available [791], [792]. Interestingly, there is also now some data from such studies to suggest that the poor reproducibility of many genetic association studies, well recognised in many fields of genetic research, may not only be due to statistical fluctuations, but could also reflect the role of gene-gene interactions, with these effects varying across different

populations tested and re-tested [791], [792]. Genome wide analysis also requires DNA to be available on a well phenotyped cohort of subjects, and hence the fPD and PD GEN cohorts used in this thesis are ideal for this type of study. Therefore, in collaboration with colleagues in Wales (Morris et al.), we are participating in a genome-wide association study (GWAS) of young onset PD, and plan to use these samples again in future similar projects. Of course the genome wide association studies to date have been based on the common disease, common variant hypothesis. The single-nucleotide polymorphisms (SNPs) tested in this screening process are all fairly common. If PD genetic susceptibility is due to a series of rare genetic variants, these will not be detected using the current GWAS approaches.

8.7 Implications for clinical practice

Whilst most of the work presented in this thesis has important implications for research, there are some findings that that may influence our clinical practice. Our study of the kindred with variable phenotypes and a novel *GCH1* mutation reinforces the view that DRD can have a very wide range of clinical presentations, and that we should consider the diagnosis even in the most atypical cases. The functional neuro-imaging data also serves to remind us of its value in difficult diagnostic cases, but also that test/re-test variability can occur with any of our imaging modalities.

The finding that may have most influence on our current clinical practice is the confirmation of the importance of the G2019S mutation in UK sPD. My use of the PD GEN cohort for this study also adds further generalisability to this finding. There are now several diagnostic laboratories which offer testing for the G2019S mutation, and it is something which we can order from clinic. This raises the issue as to whether we should screen for this mutation in

patients with apparently idiopathic PD. My view is that at present we should probably not offer screening for the G2019S mutation to all sPD individuals. The clinical features and response to treatment of patients with PD who carry the G2019S mutation are indistinguishable from those of subjects with idiopathic PD. A positive test result would not alter the way that we manage their disease. A positive test may result in requests from unaffected relatives to be tested, and at the current time there is no therapy which is proven to be preventative or to alter the course of the condition. In addition, the mutation is known to demonstrate incomplete penetrance [283], [287], [281], [279], [721], leading to major difficulties in defining clinical prognosis. Caution must be taken when screening for *LRRK2* mutations in a clinical setting in both affected and unaffected subjects, and I suggest that a clear set of guidelines and framework for testing and counselling is required before it should come into routine clinical practice.

8.8 Summary and Conclusions

In this thesis I have presented the data from a molecular genetic study of the 'PD gene' *LRRK2* in fPD and sPD in the UK, and also from a clinical, imaging and molecular genetic study of an individual kindred with variable phenotypes and a novel *GCH1* mutation. The broad aims presented in section 1.6 of the introduction have been met.

The similarity of *LRRK2*-related PD to idiopathic PD, and the identification of pathogenic mutations in both fPD and sPD, has stimulated much research effort since 2004, including this project. However, progress has been hampered by few comprehensive screens of the gene, particularly in UK populations, and a limited understanding of the effect of the pathogenic

mutations, although a mechanism of kinase over-activity has been proposed [429], [661], [691], [692], [782].

The work on fPD presented in this thesis confirms the importance of *LRRK2* mutations in UK subjects. Through a comprehensive screen of the gene in fPD I also identified three novel heterozygous frameshift mutations. For two of these mutations, c.6187delCTCTA (p.L2063STOP) and c.7187insGT (p.T2397STOP), my data suggests a novel pathogenic mechanism in *LRRK2*-PD, nonsense mediated decay, possibly leading to haploinsufficiency. Whilst requiring further investigation, these findings may be of crucial importance for our understanding of how LRRK2 dysfunction can result in the development of PD, and also for future development of neuro-protective therapies.

The work on sPD presented in this thesis demonstrated that the PD GEN collection is a valuable resource for use in this type of molecular genetic study, with similar demographics to previously used cohorts, hence providing data which is generalisable. Furthermore, this part of the study confirmed the importance of *LRRK2* mutations in subjects with sPD from the UK, which may have implications for our clinical practice. I was also able to confirm that DHPLC is a valuable high-throughput screening method to detect *LRRK2* mutations.

The clinical, imaging and molecular genetic study of an individual kindred with variable phenotypes and a novel *GCH1* mutation, presented in this thesis, has after 20 years provided an explanation for the diverse clinical presentations observed in this family. The association of this *GCH1* mutation with a wide range of phenotypes furthers both our understanding of late onset DRD, and suggests a potential role for the *GCH1* gene in the pathogenesis of PD.

Appendix A

The information which I have provided in this appendix relates to the clinical assessment of subjects who participated in the familial Parkinson's disease (fPD) study as detailed in section 2.1. I have provided a copy of the UPDRS III (Unified Parkinson's Disease Rating Scale, Part III) [6] which I used to obtain a disease severity score in each PD subject in their 'on state' (figure A.1). In addition I have provided copies of the epidemiological questionnaire, based on the PD GEN questionnaire (http://www.pdgen.bham.ac.uk/docs/PG%20GEN%20Questionnaire.pdf), and the Folstein MMSE (Mini Mental State Examination) [19] which I completed for each PD subject as an assessment of environmental exposures and cognitive function (figures A.2 and A.3).

The Unified Parkinson's Disease Rating Scale, Part III (Motor examination)

Speech
0 = Normal.
1 = Slight loss of expression, diction and/or volume.
2 = Monotone, slurred but understandable; moderately impaired.
3 = Marked impairment, difficult to understand.
4 = Unintelligible.

Facial Expression
0 = Normal.
1 = Minimal hypomimia, could be normal "Poker Face".
2 = Slight but definitely abnormal diminution of facial expression
3 = Moderate hypomimia; lips parted some of the time.
4 = Masked or fixed facies with severe or complete loss of facial expression; lips parted 1/4 inch or more.

Tremor at rest
0 = Absent.
1 = Slight; present with action.
2 = Moderate in amplitude, present with action.
3 = Moderate in amplitude with posture holding as well as action.
4 = Marked in amplitude; interferes with feeding.

Action or Postural Tremor of hands
(Judged on passive movement of major joints with subject relaxed in sitting position. Cogwheeling to be ignored)
0 = Absent.
1 = Slight or detectable only when activated by mirror or other movements.
2 = Mild to moderate.
3 = Marked, but full range of motion easily achieved.
4 = Severe, range of motion achieved with difficulty.

Rigidity
(Patient taps thumb with index finger in rapid succession)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

Finger Taps
(Patient opens and closes hands in rapid succesion.)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

Hand Movements
(Pronation-supination movements of hands, vertically and horizontally,
with as large an amplitude as possible, both hands simultaneously)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

Rapid Alternating Movements of Hands
(Patient taps heel on the ground in rapid succession picking up entire leg. Amplitude should be at least 3 inches)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

Leg Agility
(Patient attempts to rise from a straight-backed chair, with arms folded across chest)
0 = Normal.
1 = Slow; or may need more than one attempt.
2 = Pushes self up from arms of seat.
3 = Tends to fall back and may have to try more than one time, but can get up without help.
4 = Unable to arise without help.

Arising from Chair
0 = Normal erect.
1 = Not quite erect, slightly stooped posture; could be normal for older person.
2 = Moderately stooped posture, definitely abnormal; can be slightly leaning to one side.
3 = Severely stooped posture with kyphosis; can be moderately leaning to one side.
4 = Marked flexion with extreme abnormality of posture.

Posture
0 = Normal.
1 = Walks slowly, may shuffle with short steps, but no festination (hastening steps) or propulsion.
2 = Walks with difficulty, but requires little or no assistance; may have some festination, short steps, or propulsion.
3 = Severe disturbance of gait, requiring assistance.
4 = Cannot walk at all, even with assistance.

Gait
0 = Normal.
1 = Walks slowly, may shuffle with short steps, but no festination (hastening steps) or propulsion.
2 = Walks with difficulty, but requires little or no assistance; may have some festination, short steps, or propulsion.
3 = Severe disturbance of gait, requiring assistance.
4 = Cannot walk at all, even with assistance.

Postural Stability
(Response to sudden, strong posterior displacement produced by pull on shoulders while patient erect with eyes open and feet slightly apart. Patient is prepared)
0 = Normal.
1 = Retropulsion, but recovers unaided.
2 = Absence of postural response; would fall if not caught by examiner.
3 = Very unstable, tends to lose balance spontaneously.
4 = Unable to stand without assistance.

Body Bradykinesia and Hypokinesia
(Combining slowness, hesitancy, decreased armswing, small amplitude, and poverty of movement in general)
0 = None.
1 = Minimal slowness, giving movement a deliberate character; could be normal for some persons. Possibly reduced amplitude.
2 = Mild degree of slowness and poverty of movement which is definitely abnormal. Alternatively, some reduced amplitude.
3 = Moderate slowness, poverty or small amplitude of movement.
4 = Marked slowness, poverty or small amplitude of movement.

Figure A.1 The UPDRS III (Unified Parkinson's Disease Rating Scale, Part III). Adapted from http://www.mdvu.org/library/ratingscales/pd/updrs.pdf and Fahn & Elton 1987 [6].

The Mini-Mental State Examination

Subject:_____Examiner:_____
Date:_____

Maximum Score

Orientation

5	( )	What is the (year) (season) (date) (day) (month)?
5	( )	Where are we (state) (country) (town) (hospital) (floor)?

Registration

3	( )	Name 3 objects: 1 second to say each. Then ask the patient to name all 3 after you have said them. Give 1 point for each correct answer. Then repeat them until he/she learns all 3. Count number of trials and record. Trials _____

Attention and Calculation

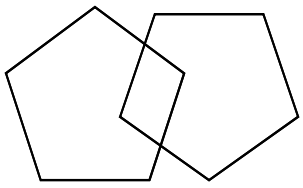
5	( )	Serial 7's. 1 point for each correct answer. Stop after 5 answers. Alternatively spell "world" backward.

Recall

3	( )	Ask for the 3 objects repeated above. Give 1 point for each correct answer.

Language

2	( )	Name a pencil and watch.
1	( )	Repeat the following "No ifs, ands, or buts"
3	( )	Follow a 3-stage command: 'Take a paper in your hand, fold it in half, and put it on the floor.'
1	( )	Read and obey the following: **CLOSE YOUR EYES**
1	( )	Write a sentence.
1	( )	Copy the design shown.



_____ Total Score

Figure A.2 Mini-Mental State Examination (MMSE). Adapted from *Try This*:® The Hartford Institute for Geriatric Nursing, Division of Nursing, New York University www.hartfordign.org. and Folstein et al. 1975 [19].

Familial PD Epidemiology Questionnaire

Subject: **Examiner:**

Date:

Ethnic origin

Asian (Indian origin) ☐	Asian (Pakistani origin) ☐	Asian (Chinese origin) ☐
Asian (Other) ☐	Black (Caribbean origin) ☐	Black (African origin) ☐
Black (Other) ☐	White ☐	Other ☐

In which country were they born? _____

Employment

Present employment (or last type of employment if retired) _____
How long have they been in this job? years & months _____
What other jobs have they had? List the main six (with dates)
(1) _____ (2) _____
(3) _____ (4) _____
(5) _____ (6) _____

Have they ever been admitted to hospital due to intoxication by chemical compounds? **Yes / No**
If YES, *what was the name of the chemical?* _____

Have they worked in their employment with: Hydrocarbons (e.g. paints, dry cleaning industry)? **Yes / No**
If YES, *what was the name of the chemical?* _____

Have they worked in their employment with: Pesticides or insecticides? **Yes / No**
If YES, *what was the name of the chemical*? _____

How long did they work with hydrocarbons/pesticides? ___ years

Environment

Have they lived most of their life in: countryside? ☐ town/city? ☐ mixture of the two? ☐

Have they drunk water from a well for more than 6 months of their life at any time? **Yes / No**
Please specify

_____

Smoking Habits

Do they smoke **NOW** (includes all forms of tobacco)? **Yes / No**
*If **YES,** in what year did they start smoking?*

_____

How many cigarettes (or the equivalent) on average do they smoke daily?
e.g. per day Cigarettes per day _____

*If **NO**, have they smoked in the past?* **Yes / No**

In what year did they start smoking? In what year did they stop smoking?

_____

How many cigarettes (or the equivalent) on average did they smoke daily?
e.g. per day Cigarettes per day _____

Coffee/Tea Consumption

How many cups of **coffee** on average have they drunk each day over the last 10 years?

None—— One—— Two—— Three—— Four—— Five—— Six or more ——

How many cups of **tea** on average have they drunk each day over the last 10 years?

None—— One—— Two—— Three—— Four—— Five—— Six or more ——

Parkinson's Disease

Is there, or has there been, anyone in their family with Parkinson's disease? **Yes / No**
If Yes, please list their names and relationship (e.g. brother, mother, etc):

Name	Relationship
_____	_____
_____	_____
_____	_____

Alzheimer's Disease

Has anyone in their family had a problem with memory loss, such as Alzheimer's disease? **Yes / No**
If Yes, please list their names and relationship (e.g. brother, mother, etc):

Name	Relationship
_____	_____
_____	_____
_____	_____

Other Neurological Conditions

Have they ever had a serious head injury that put them in a coma? **Yes / No**
Have they ever had meningitis (i.e. infection of the lining of the brain)? **Yes / No**
Have they ever had encephalitis (i.e. severe infection of the brain tissue) **Yes / No**

Figure A.3 Familial PD Epidemiology Questionnaire. Based on the PD GEN questionnaire (http://www.pdgen.bham.ac.uk/docs/PG%20GEN%20Questionnaire.pdf).

Appendix B

The information which I have provided in this appendix relates to the Queen Square PD (QSPD) patient and control collection and the control cohorts used in the molecular genetic studies performed in chapters 5 and 6 (sections 5.3.1 and 6.3.1).

I used 776 patient samples and 31 control samples from the QSPD collection in screening for the c.6187delCTCTA (p.L2063 STOP) variation in exon 42 of *LRRK2* (table 5.5). As listed in table B.1 the 776 patient samples are made up of a mixture of fPD (inheritance in the family followed an AD pattern); young onset PD (YOPD) where onset of disease was before the age of 45 years, but there was no family history of PD; sPD; and samples from the Queen Square Brain Bank for Neurological Diseases with pathologically proven PD (no family history of PD).

In table B.2 I have listed the demographics of the control samples which I used in the molecular genetic studies performed on fPD and sPD in chapters 5 and 6 of this project. These samples were from the PD GEN cohort, Birmingham MND collection, Birmingham Neurodegenerative Disorders DNA Bank (NDD) and PINE cohort. All were predominantly Caucasian (95%-100%).

	QSPD DNA collection					
	fPD (AD)	**YOPD (sporadic)**	**sPD**	**Brain Bank**	**Total number patients**	**Controls**
Number of samples screened	80	84	293	319	776	31

Table B.1 PD Patient and control samples from the QSPD collection used in screening for the c.6187delCTCTA (p.L2063 STOP) variation in exon 42 of *LRRK2*. The 776 patient samples are made up of a mixture of fPD samples (inheritance in the family followed an AD pattern); young onset PD (YOPD) where onset of disease was before the age of 45 years, but there was no family history of PD; sPD; and samples from the Queen Square Brain Bank for Neurological Diseases With pathologically proven PD (no family history of PD).

Control collection	**Male (%)**	**Female (%)**	**Mean age (range)**
PD GEN	56 (25)	165 (75)	64.8 (32-86)
MND	47 (35)	57 (65)	57.0 (20-89)
NDD	17 (36)	30 (64)	69.2 (55-85)
PINE	33 (62)	20 (38)	77.4 (56-93)

Table B.2 Demographics of control samples used in molecular genetic studies of fPD and sPD in chapters 5 and 6. Control cohorts: PD GEN cohort, Birmingham MND collection, Birmingham Neurodegenerative Disorders DNA Bank (NDD), and PINE cohort.

Appendix C

The information which I have provided in this appendix relates to the molecular genetic analyses (PCRs and restriction fragment length polymorphism (RFLP) analyses) I performed in chapters 5, 6 and 7 of this project.

Each PCR was optimised as covered in section 2.4, the constituents of the standard reaction mixture were as listed in table 2.1 and the standard cycling conditions were as provided in figure 2.3. In the tables below I have listed the PCR primer sequences, annealing temperatures and magnesium chloride ($MgCl_2$) concentrations used. In some instances the $MgCl_2$ concentration was adjusted from the constituents listed in table 2.1, and this is indicated below. The PCRs for the DHPLC study in sPD (results presented in chapter 6) were performed using Accuzyme PCR reagents (Bioline) as described below.

C.1 Comprehensive screen of LRRK2 in familial PD

In my study of fPD the whole coding sequence and exon-intron boundaries of the *LRRK2* gene were studied (chapter 5) using primers and PCR temperatures as described in previous studies [283], [280]. These primers, along with annealing temperatures and $MgCl_2$ concentrations are listed below in table C.1

Exon	Primer Sequences	Annealing Temperatures	$MgCl_2$ Concentration (mM)
1F 1R	5'-CCCCTGCCGGTTCCCTGAG-3' 5'-GGACAAAATTTGCAAATGTAAGG-3'	60°C	1mM
2F 2R	5'-AGAATTTCAGGAAGGTCTTCAC-3' 5'-AACATTGCCAGTAAAACGTCTCC-3'	58°C	1.5mM
3F 3R	5'-AAGTGAGATAAGCATCTATTCC-3' 5'-GCCTTGGATTGCAAACACAGTG-3'	58°C	2mM
4F 4R	5'-CAGGGAATTAAATACAATGAGAG-3' 5'-GCTACCCTAATCCTGATCTTC-3'	58°C	1.5mM
5F 5R	5'-CAAACCATTCACAGTCTTCATG-3' 5'-TGACAGACAACTCCCACTTC-3'	58°C	1.5mM
6F 6R	5'-AATCTCCGTAGCTTGTTTTCTC-3' 5'-TTCAGTATAATGTCAGTGAATG-3'	58°C	1.5mM
7F 7R	5'-TTATGCTGCCATCTATTTACAG-3' 5'-TCAAAGTATGTCAATATGCTAC-3'	58°C	1.5mM
8F 8R	5'-TTGCTCAATCACTTCCATTGTC-3' 5'-CATTGAATGCTTCCATCTGTAC-3'	58°C	1.5mM
9-10F 9-10R	5'-GACTTAGAGTTGGTCAAACTG-3' 5'-ACAATAAAAGTTACGGTTAAGG-3'	58°C	1.5mM
11F 11R	5'-CTCTTGTAAGTGGAGGTGGC-3' 5'-AGTTTACACATAGAAGTCCGG -3'	58°C	1.5mM
12F 12R	5'-ATGCTTTCCTGTAAATTTGGAC-3' 5'-GAAATATTGATATTCTACCTGG-3'	56°C	2.5mM
13F 13R	5'-ATATTGGTTCTGCCCTCCTG-3' 5'-AATCTAAGTGACTCTTCTGCATC-3'	58°C	2mM
14F 14R	5'-GACAATTTCTAGAAAGTAACAG-3' 5'-TGTCTCTAACCACATGACTTCC-3'	58°C	1.5mM
15F 15R	5'-TACAATGCCTGGCACAGAAC-3' 5'-AAAGACATCCAGTCACCAGC-3'	58°C	1.5mM
16F 16R	5'-GTATCCAGATGACCAAGGTC-3' 5'-GTGGGAATTACACAAACTGC-3'	58°C	2mM
17F 17R	5'-TAAATACTTTAAAGCACCAACCC-3' 5'-CAAAACACTTGCAACAGAGG-3'	58°C	1mM
18F 18R	5'-AAAAGGATCAACAGGTACAGTG-3' 5'-AGATACACAATGGCAGGGCTC-3'	60°C	1.5mM
19F 19R	5'-GTTTGATTTGCCAGTCTCC-3' 5'-TCAAACTGGCATGAATAACCAC-3'	58°C	1.5mM
20F 20R	5'-CAATACGTAAGAACTTTGGTCC-3' 5'-GGTCAGGTTTTTGTCTTGGG-3'	58°C	2.5mM
21F 21R	5'-AAAGTGAAAAACCAACATGGC-3' 5'-TTTCATACATCAGGGAAATCC-3'	58°C	1.5mM
22F 22R	5'-ATTTGAGCACTGAACTGGAG-3' 5'-GGAAATTCAACCAAACACTGC-3'	58°C	2mM
23F 23R	5'-GCCTGATTGCTAGGAGGTGC-3' 5'-GGGGACTTATCACCCAGTG -3'	58°C	1.5mM
24F 24R	5'-GTGTGTAAGGCAGAAATATTAG-3' 5'-CTCATTGCTATAAAAATGTCAGC -3'	58°C	1.5mM
25F 25R	5'-CCTCTTTGATGCTGTTCTTTG-3' 5'-TAAAGGGTCCATATATGACTC-3'	58°C	1.5mM
26F 26R	5'-TGGAAGTATTAAGGCTATTACC -3' 5'-TATATGACTAAATCGAAATCATG -3'	58°C	2.5mM
27F	5'-ATGCTAGTTTTGACGTTACAC-3'	58°C	1.5mM

Exon	Primer Sequences	Annealing Temperatures	$MgCl_2$ Concentration (mM)
27R	5'-GCTTCTAGTTTCATGAAATTGG-3'	58°C	1.5mM
28F	5'-CTGTTGAAAGGTTGTGCAAGC-3'	58°C	2.5mM
28R	5'-AATAGTAACAATATATGTCCATC-3'		
29F	5'-GTGTGACATGTAAAAGAACTC-3'	58°C	2mM
29R	5'-AATTCCATACAGTCTACCAGG-3'		
30F	5'-CCTAGTAAAAACCCAGAATAG-3'	58°C	1.5mM
30R	5'-AGACTGAAGCAATTGTTTGCC-3'		
31F	5'-TCTGAAGTCTGCTAGTTTCTC-3'	58°C	2.5mM
31R	5'-TCTGACATTTCTAGGCAGTTG-3'		
32F	5'-AACTGTTAGCACTGAATTTGC-3'	58°C	2.5mM
32R	5'-AGTGGCCTATTAAAGAACCG-3'		
33F	5'-AAAATGAGGAAGTTGGACTAG-3'	58°C	1.5mM
33R	5'-TGCTCTGGGCGCCATCTG -3'		
34F	5'-TTCTGACTACTTTCACTGAGC-3'	58°C	2mM
34R	5'-AATACATCTATTATAGACTACAG-3'		
35F	5'-ACCTTCATTGACTTTAAGCAG-3'	58°C	2.5mM
35R	5'-GCCATCTCCCTAATTTCTCT-3'		
36F	5'-GACTCATATCAGTAACAACCC-3'	58°C	1.5mM
36R	5'-TCAACACCTTGTCTAAACCTC -3'		
37F	5'-TTGTAGAAAAGGTAAGGAAATC-3'	56°C	2mM
37R	5'-ATTTTTCAACTCATCCCTTATGG-3'		
38F	5'-CCAAGTAGATAGATACTAAGTG-3'	58°C	2mM
38R	5'-CATCCTCATCTTGATCCTAGTC-3'		
39F	5'-CAATGAAACAAGTAGGTCAGG-3'	58°C	1.5mM
39R	5'-GTATGCAAAATAAGAGTTCCAG-3'		
40F	5'-CCATGTTCAGCCTGTTGATGC-3'	58°C	1.5mM
40R	5'-CACAGTGTTACTGGGAAGTG-3'		
41F	5'-TTTAAGGGACAAAGTGAGCAC-3'	58°C	2.5mM
41R	5'-ACTCTGTTTTCCTTTTGACTC-3'		
42F	5'-ACAGCCTGGTTTAGAACATC-3'	58°C	2mM
42R	5'-AAGGAATTAAGCATACAACTAC-5'		
43F	5'-TTCTTTGCAATGTCTGGACC-3'	58°C	2mM
43R	5'-GAGCATGTGAGGTTTTGGC-3'		
44F	5'-CAGAGCTATAACACTTCAGTC-3'	58°C	1.5mM
44R	5'-GTTCTAACAGAAGGCTAATTG-3'		
45F	5'-GAAAGCAAAAAGAGTTATGTTG-3'	58°C	2mM
45R	5'-TATGCAAACCAATGAAAATAGG-3'		
46F	5'-AATTTATAGTTGAGAAGTCTATC-3'	58°C	2.5mM
46R	5'-GCTCTCAGGGAGTTTATAATC-3'		
47F	5'-TAATCATTTAAGATGGAAAGTAG-3'	58°C	2.5mM
47R	5'-CTTCCTTATGAATTATCAACAG-3'		
48F	5'-TATAAGGTTGTATTACACGTAG-3'	58°C	2mM
48R	5'-AAAATTCTCTATTCAGAGGCAG-5'		
49F	5'-CATAATGGTGGTGGTGTCATG-3'	58°C	1.5mM
49R	5'-CTGTGTGACCCTCCAAGACC-3'		

Exon	Primer Sequences	Annealing Temperatures	$MgCl_2$ Concentration (mM)
50F 50R	5'-CCAAGGTATTTGTGTCTTAAAC-3' 5'-AAAATTCCTGATTATACCATATG-3'	58°C	2mM
51F 51R	5'-TAAAAATACATGAGCCAAACTG-3' 5'-AAAAATGTGAGTACCCTTTCC-3'	58°C	1.5mM

Table C.1 PCR primer sequences, annealing temperatures and $MgCl_2$ concentration for amplification of LRRK2 exons 1-51. I studied the whole coding sequence and exon-intron boundaries of the *LRRK2* gene in the fPD cohort by PCR (see chapter 5) using primers and PCR temperatures as described in previous studies [283], [280]. Abbreviations: F= forward primer; R= reverse primer; $MgCl_2$= magnesium chloride.

C.2 Screening for *LRRK2* variants in sPD by DHPLC

In my study of sPD (results presented in chapter 6) the PCRs for exons 24, 25, 31, 32, 38, 40, 41 and 42 of *LRRK2,* which were subsequently analysed by DHPLC, were performed using Accuzyme PCR reagents (Bioline). This was because the Accuzyme high fidelity Taq polymerase can utilise the detergent-free reaction constituents required for loading onto denaturing high performance liquid chromatography (DHPLC) columns. The PCR primers, reaction constituents and cycling conditions are listed below. The same concentration of $MgCl_2$ (1.5mM) and PCR cycling conditions were used to amplify all eight exons of *LRRK2* in this part of the study (table C.2-C.3 and figure C.1).

Primers	Annealing Temperature
24DF 5'-GTGTGTAAGGCAGAAATATTAG-3' 24DR 5'-CTCATTGCTATAAAAATGTCAGC -3'	58°C
25DF 5'-CCTCTTTGATGCTGTTCTTTG-3' 25DR 5'-TAAAGGGTCCATATATGACTC-3'	58°C
31DF 5'-TCTGAAGTCTGCTAGTTTCTC-3' 31DR 5'-TCTGACATTTCTAGGCAGTTG-3'	58°C
32DF 5'-AACTGTTAGCACTGAATTTGC-3' 32DR 5'-AGTGGCCTATTAAAGAACCG-3'	58°C
38DF 5'-CCAAGTAGATAGATACTAAGTG-3' 38DR 5'-CATCCTCATCTTGATCCTAGTC-3'	58°C
40DF 5'-CCATGTTCAGCCTGTTGATGC-3' 40DR 5'-CACAGTGTTACTGGGAAGTG-3'	58°C
41DF 5'-AGGGACAAAGTGAGCACAGAA-3' 41DR 5'-GAGGTCAGTGGTTATCCATCCT -3'	58°C
42DF 5'-ACAGCCTGGTTTAGAACATC-3' 42DR 5'-AAGGAATTAAGCATACAACTAC-5'	58°C

Table C.2 Primer sequences and annealing temperatures for amplification of eight exons of *LRRK2* by PCR. I studied eight exons (24,25,31,32,38,40,41 and 42) of the *LRRK2* gene in the sPD cohort by PCR and subsequent DHPLC analysis (see chapter 6). Accuzyme PCR reagents (Bioline Ltd) were used which are detergent free, making them ideal for DHPLC analysis. Abbreviations: F= forward primer; R= reverse primer.

Reagent	Stock concentration	Final concentration
Water (distilled and autoclaved)	N/A	N/A
10x Buffer (Bioline)	10x	1x
dNTP mix (Fermentas)	2mM	120µM
$MgCl_2$ (Bioline)	50mM	1.5mM
Forward primer (AltaBioscience) / (Eurogentec)	10pmole/µl	0.5pmole/µl
Reverse primer (Altabioscience) / (Eurogentec)	10pmole/µl	0.5pmole/µl
Accuzyme Taq (Bioline)	2.5u/µl	0.05u/µl
DNA	100ng/µl	5ng/µl

Table C.3 Constituents of a PCR master mix for exons 24, 25, 31, 32, 38, 40, 41 and 42.

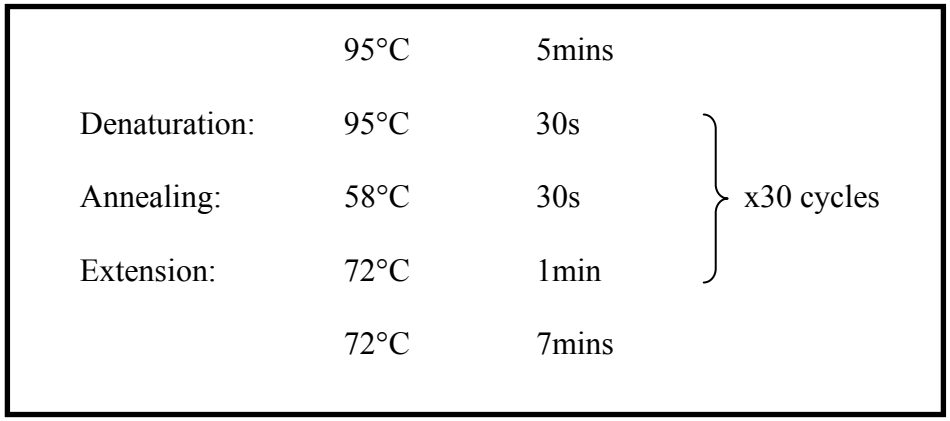


Figure C.1 PCR cycling conditions for exons 24, 25, 31, 32, 38, 40, 41 and 42.

C.3 Screening for LRRK2 variations within exon 41 by restriction fragment length polymorphism (RFLP) analysis.

Specific PCR primers were designed for screening for *LRRK2* variations within exon 41 by RFLP analysis. These primers, along with annealing temperatures and $MgCl_2$ concentrations, are listed below in table C.4. Each PCR was optimised as covered in section 2.4, the constituents of the reaction mixture were as listed in table 2.1 and the standard cycling conditions were as provided in figure 2.3.

Primers	Annealing Temperature	$MgCl_2$ Concentration
41PF 5'- GAGCACAGAATTTTGATGCTTG -3'	55°C	2mM
41PR 5'- TTTTATCCCCATTCCACAGCAGTAC-3'		

Table C.4 PCR primer sequences, annealing temperature and $MgCl_2$ concentration for amplification of *LRRK2* exon 41, for subsequent RFLP analysis. Abbreviations: F= forward primer; R= reverse primer; $MgCl_2$= magnesium chloride.

C.4 Screening for novel *LRRK2* variation within exon 42 by agarose gel electrophoresis.

Specific PCR primers were designed to screen for the novel c.6187delCTCTA (p.L2063 STOP) mutation in exon 42 of *LRRK2,* which was identified during my fPD study (see section 5.3.2.4). The PCR products were screened for the mutation by gel electrophoresis with a 3% high resolution RESponse^{TM} Research agarose (Geneflow) gel, as described in section 2.5.1. These primers, along with annealing temperatures and $MgCl_2$ concentrations, are listed below in table C.5. Each PCR was optimised as covered in section 2.4, the constituents of the reaction mixture were as listed in table 2.1 and the standard cycling conditions were as provided in figure 2.3.

Primers	Annealing Temperature	$MgCl_2$ Concentration
42GF 5'- CCAACAGGCTGATGTTTATTCA -3'	58°C	2mM
42GR 5'- CCTCCAGTTGTCAAAATGTCA -3'		

Table C.5 PCR primer sequences, annealing temperature and $MgCl_2$ concentration for amplification of *LRRK2* exon 42, for subsequent agarose gel electrophoresis.
Abbreviations: F= forward primer; R= reverse primer; $MgCl_2$= magnesium chloride.

C.5 Screening for variations within *LRRK2* DNA and cDNA by PCR fragment analysis.

Specific PCR primers were designed to screen for the novel *LRRK2* mutation c.7187insGT (p.T2397STOP) in DNA, which was identified during my fPD study, by PCR fragment analysis (see section 5.3.2.6). The PCR products were screened for the mutation by PCR fragment analysis, as described in section 2.8.

PCR primer pairs specific for the cDNA sequence of *LRRK2* were also designed in order to investigate the effect of the L2063STOP and T2397STOP mutations on RNA expression, by PCR fragment analysis, and to sequence the cDNA in this region. These primers, along with annealing temperatures and $MgCl_2$ concentrations are listed below in tables C.6-C.7. Each PCR was optimised as covered in section 2.4, and the constituents of the reaction mixture were as listed in table 2.1. The standard cycling conditions were used for the PCRs on DNA (figure 2.3), but to ensure that PCR product abundance lay within the linear phase of PCR amplification, I used a PCR cycle length of 25 for both reactions with cDNA (see section 7.3.4). Either the forward or reverse PCR primer was labelled with a fluorescent compound called 6-Carboxyfluorescein (6FAM), so that after amplification the labelled PCR products could be separated with an automated sequencing system. The labelled primers were produced

by Sigma-Genosys. The same primer pairs were used for sequencing of the cDNA, but both the forward and reverse primers were unlabelled.

Primers	Annealing Temperature	$MgCl_2$ Concentration
49DGF 5'-CATAATGGTGGTGGTGTCATG-3' (6FAM)	58°C	1.5mM
49DGR 5'-GCCTCCTCCAGTTCCTATCC-3'		

Table C.6 PCR primer sequences, annealing temperature and $MgCl_2$ concentration for amplification of *LRRK2* exon 49 in DNA, for subsequent PCR fragment analysis. Abbreviations: F= forward primer; R= reverse primer; $MgCl_2$= magnesium chloride.

Primers	Annealing Temperature	$MgCl_2$ Concentration
42CGF 5'- AAAACATCAGAGGGCACACC-3' (6FAM) 42CGR 5'-ATGGGGCACAACCATATTCT -3'	58°C	1.5mM
49CGF 5'-CAAAATAGCCCTGTTGTGGAA -3' 49CGR 5'- TTCCGGTTGTAGCCCAATAC -3' (6FAM)	58°C	1.5mM

Table C.7 PCR primer sequences, annealing temperature and $MgCl_2$ concentration for amplification of *LRRK2* cDNA, for subsequent PCR fragment analysis. The primer pairs are specific for the cDNA sequence of *LRRK2* to investigate the effect of the L2063STOP and T2397STOP mutations on RNA expression, by PCR fragment analysis, and to sequence the cDNA in this region. Abbreviations: F= forward primer; R= reverse primer; $MgCl_2$= magnesium chloride.

Appendix D

The information which I have provided in this appendix relates to section 5.4.2 of the fPD study and section 6.4.2 of the sPD study.

D.1 fPD study

I have provided the clinical features and pedigrees of proband cases carrying the pathogenic (G2019S and R1441C) and the two putatively pathogenic *LRRK2* mutations (S1228T and M1869T).

D.1.1 Family 10

Two affected members of this family, III:1 and III:2 were studied, both carry the heterozygous G2019S mutation.

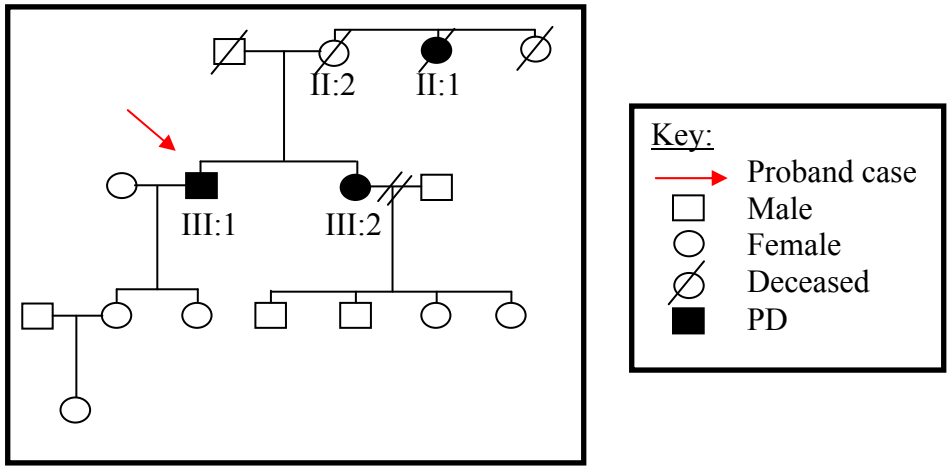


Figure D.1 Pedigree for family 10

The proband case III:1 is a right-handed male, was aged 71 at the time of evaluation and had onset of symptoms aged 54. His initial symptoms were tremor of the right hand, dragging of

his right foot and 'slowing down'. He also had dystonia of the right foot since early in the disease course. His symptoms had responded well to treatment with Levodopa (Sinemet Plus). He also had treatment-induced dyskinesias which at the time of evaluation had been present for three years.

Neurological examination revealed features typical of IPD with mask-like facies, asymmetrical bradykinesia and rigidity as well as postural instability and dyskinesias. His Hoehn and Yahr stage was 2, with a UPDRS III score of 17. There was no evidence of cognitive deficit with a MMSE score of 30. Anti-parkinsonian medication at time of evaluation was a combination of Levodopa and dopamine agonist (Ropinirole) (Levodopa equivalent dose of 550mg daily).

Subject III:2 is right-handed, was aged 66 at the time of evaluation, and had onset of symptoms aged 60. Her initial symptom was gait difficulty. She was initially commenced on Levodopa and subsequently has tried dopamine agonists (Cabergoline, Ropinirole and Pramipexole), Amantadine and Selegiline (MAOBI), but could not tolerate these medications.

Neurological examination revealed features typical of IPD with mask-like facies, asymmetrical resting tremor, bradykinesia and rigidity and postural instability. Her Hoehn and Yahr stage was 3, with a UPDRS III score of 33. There was no evidence of cognitive deficit with a MMSE score of 30.

According to family reports the other affected members of the family were: (i) a maternal aunt, subject II:1, who was diagnosed with PD at the age of 55. She was not reported to have had any tremor but had 'general slowing down and falls'. She was said to have had significant treatment benefit from Levodopa and there were no reported cognitive problems. She died at the age of 80, PD was listed in part II of the death certificate. (ii) A great uncle on the maternal

side of the family, subject I:1, who was said to have had tremor but was not definitely diagnosed with PD.

The mother of the proband (II:2) was unaffected by PD and died at the age of 90, the proband's maternal grandfather (I:2) was also said to be unaffected by PD.

Olfactory testing yielded UPSIT scores of 25 for subject III:1 and 30 for subject III:2.

D.1.2 Family 32

One affected member (II:1) and one unaffected member (II:2) of this family were studied.

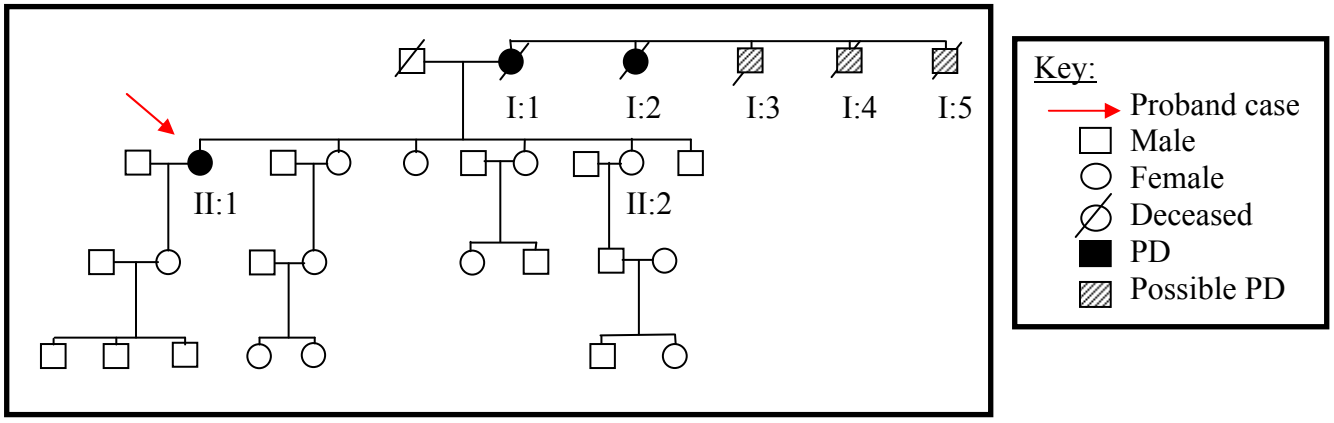


Figure D.2 Pedigree for family 32

The proband case II:1 carries the heterozygous G2019S mutation, whilst the unaffected member (II:2) does not. The proband is a right-handed female, was aged 70 at the time of evaluation and had onset of symptoms aged 59. Her initial symptoms were right-sided bradykinesia and micrographia, and she had dystonia of the left foot which began five years after onset of the disease. She was unable to tolerate Levodopa therapy. More recently she complained of tremor on the right arm and leg and dragging of the right foot.

Past medical history included temporal arteritis, gastric ulcer, irritable bowel syndrome and macular holes.

Neurological examination revealed mask-like facies, symmetrical rigidity, asymmetrical bradykinesia and postural instability. Her Hoehn and Yahr stage was 2, with a UPDRS III score of 29. There was no evidence of cognitive deficit with a MMSE score of 30. She was not taking any anti-parkinsonian medication at time of evaluation.

An MRI scan of her brain from 2005 revealed extensive patchy multi-focal high signal on T2 weighted scans, suggesting chronic small vessel disease.

Subject II:2 was unaffected by PD. She is left-handed and was aged 56 at the time of evaluation. Her past medical history included depression and irritable bowel syndrome. Neurological examination was normal. There was no evidence of cognitive deficit with a MMSE score of 30.

According to family reports the other affected members of the family were: (i) the mother of the proband, subject I:1, who was diagnosed with PD at the age of 59. She was said to have had tremor, worse on the left than the right, mask-like facies, 'general slowing down', shuffling gait, falls, and episodes of freezing. She had treatment benefit with Levodopa and Amantadine and had cognitive problems. She died at the age of 76. (ii) A maternal aunt, subject I:2, was diagnosed with PD in her mid fifties. She had tremor, rigidity and falls and was reported to have had treatment benefit from levodopa. She may also have undergone a neurosurgical procedure for her PD. (iii) Three other family members, all maternal uncles, subjects I:3, I:4 and I:5 were reported to have had possible PD, although the details available are limited. Subject I:3 had walking difficulties, subject I:4 had problems with speech and was given a diagnosis of PD. He did not apparently receive any treatment and died at the age of 88.

Olfactory testing yielded UPSIT scores of 24 for subject II:1 and 26 for subject II:2.

D.1.3 Family 33

One affected member (II:1) and four unaffected members (II:2, II:3, II:4 and III:1) of this family was studied.

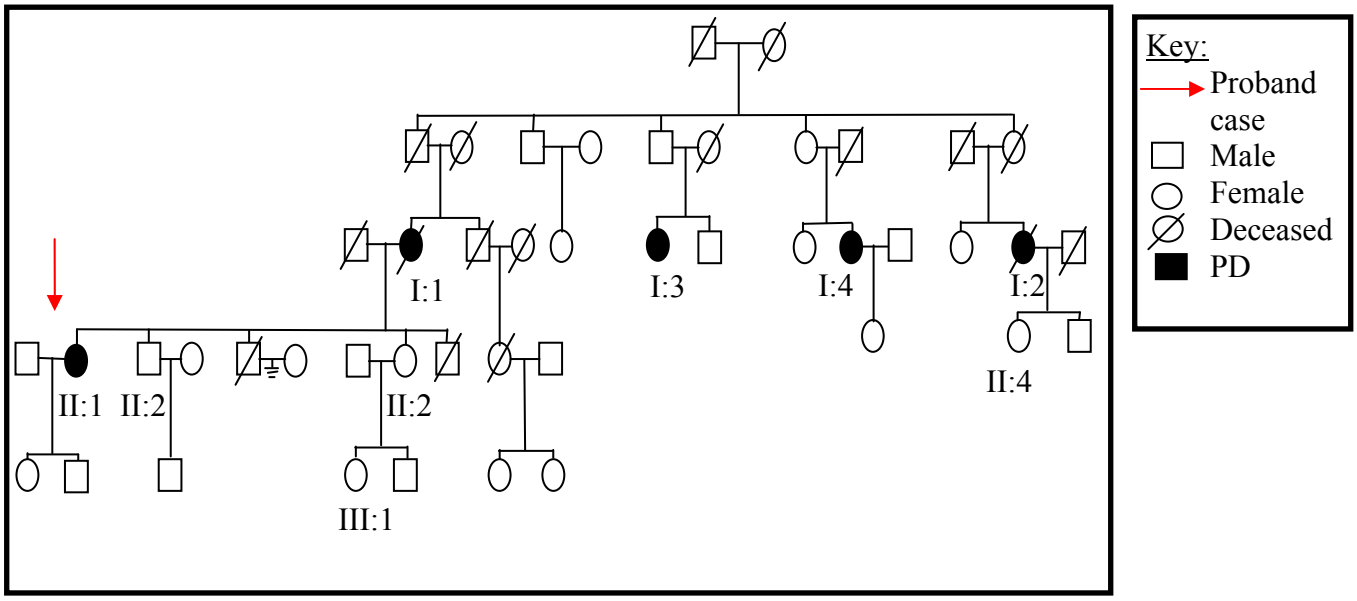


Figure D.3 Pedigree for family 33

The proband case, II:1, carries the heterozygous G2019S mutation, the unaffected members do not carry this mutation. The proband is a right-handed female, was aged 60 at the time of evaluation and had onset of symptoms aged 48. Her initial symptoms were left-sided rigidity and dystonia of the left foot. She had treatment benefit with Levodopa. She also had treatment induced dyskinesias and complained of wearing off phenomena and freezing. Her past medical history included ankylosing spondylitis.

Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical tremor, bradykinesia and rigidity as well as postural instability. Her Hoehn and

Yahr stage was 3, with a UPDRS III score of 23. She complained of some memory problems and hallucinations, which had been present for approximately a year before the date of evaluation, but her MMSE score was 30

Her anti-parkinsonian medication at time of evaluation was a combination of Levodopa, Entacapone (COMT inhibitor) and a dopamine agonist (Ropinirole) (Levodopa equivalent dose of 800mg daily).

The four unaffected family members studied were: (i) subject II:2, she was aged 66 at the time of evaluation and her neurological examination was normal. There was no evidence of cognitive deficit with a MMSE score of 30. (ii) Subject II:3, he was aged 54 at the time of evaluation. His past medical history included subarachnoid haemorrhage and nocturnal seizures. Neurological examination was normal. There was no evidence of cognitive deficit with a MMSE score of 30. (iii) Subject II:4, she was aged 66 at the time of evaluation. Her past medical history included hypertension and hypothyroidism. Neurological examination was normal and there was no evidence of cognitive deficit with a MMSE score of 30. (iv) Subject III:1, she was aged 42 at the time of evaluation. Her past medical history included coeliac disease. Neurological examination was normal and there was no evidence of cognitive deficit with a MMSE score of 30.

According to family reports the other affected members of the family were: (i) the mother of the proband, subject I:1. She was diagnosed with PD at the age of 75 and had asymmetrical tremor and rigidity. She had good treatment benefit with Levodopa. She died at the age of 95. (ii) A maternal aunt of the proband, subject I:2. She was diagnosed with PD at the age of 61 and had mask-like facies, asymmetrical bradykinesia and rigidity, but no tremor. She had good treatment benefit with Levodopa, and developed dyskinesias. She died at the age of 71. (iii) A

maternal aunt of the proband, subject I:3. She had received treatment for PD. She died at the age of 79. (iv) A maternal aunt of the proband, subject I:4, she has PD and was still alive at the time of the study.

Olfactory testing yielded UPSIT scores of 20 for subject II:1, 31 for subject II:2, 39 for subject II:3, 29 for subject II:4, and 30 for subject III:1.

D.1.4 Family 20

One affected member of this family was studied (II:1).

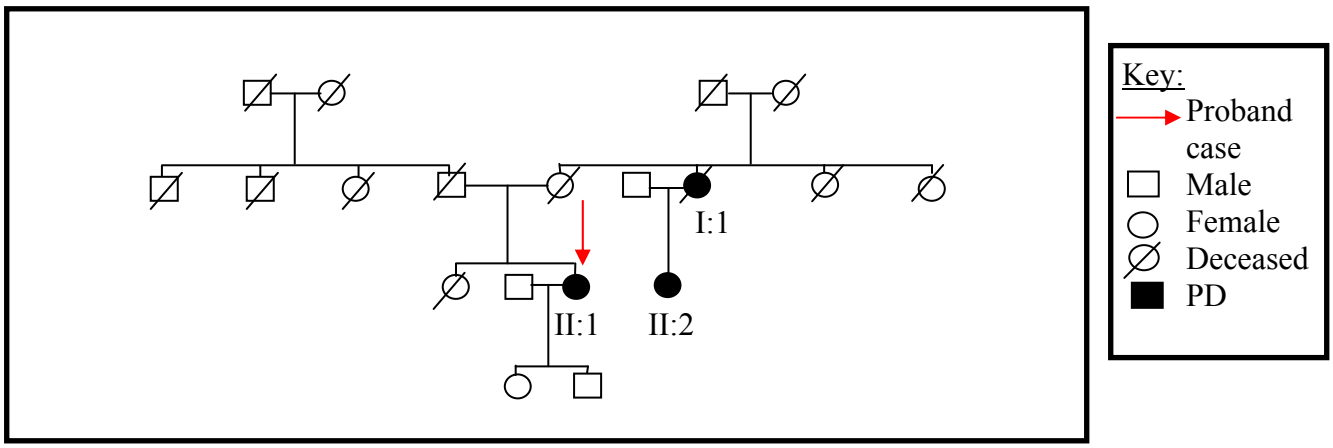


Figure D.4 Pedigree for family 20

The proband case, II:1, carries the heterozygous R1441C mutation. She is right-handed, was aged 72 at the time of evaluation and had onset of symptoms aged 71. Her initial symptoms were rigidity and gait difficulty, but also more recently unilateral tremor. She had not been commenced on treatment. Her past medical history included Polio at the age of three and a myocardial infarction.

Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical bradykinesia and rigidity as well as postural instability. Her Hoehn and Yahr

stage was 2, with a UPDRS III score of 15. She did not complain of any cognitive deficit and her MMSE score was 28.

According to family reports the other members of the family who had a definite diagnosis of PD were a maternal aunt, subject I:1, and a maternal cousin, subject II:2, who had symptoms of tremor, bradykinesia and dystonia.

Olfactory testing yielded a UPSIT score of 15 for subject II:1.

D.1.5 Family 1

One affected member (II:1) and one unaffected member (III:2) of this family were studied.

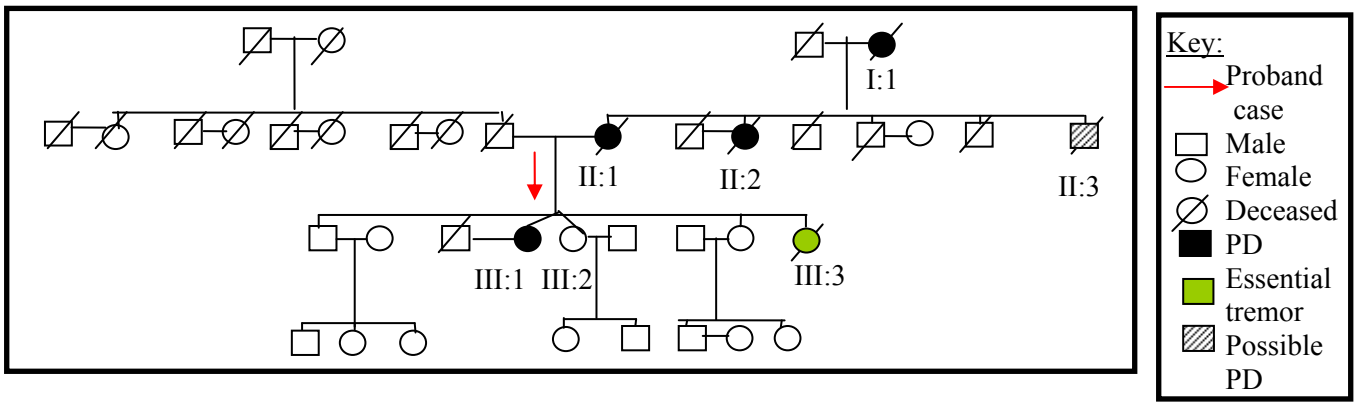


Figure D.5 Pedigree for family 1

The proband case, III:1, carries the heterozygous S1228T mutation, the unaffected family member (III:2) also carries the same mutation. The proband is a right-handed female, was aged 75 at the time of evaluation and had onset of symptoms aged 65. Her initial symptoms were neck rigidity and tremor of his left arm. She was first commenced on Bromocriptine therapy with good response. Her anti-parkinsonian medication at time of evaluation consisted of a combination of dopamine agonist (Cabergoline) and Levodopa (Levodopa equivalent dose of 525mg daily). Her past medical history included two transient ischaemic attacks.

Neurological examination revealed features typical of IPD, with mask-like facies, and asymmetrical resting tremor, rigidity and bradykinesia. as well as postural instability. Her Hoehn and Yahr stage was 3, with a UPDRS III score of 28. She did not complain of any cognitive deficit and her MMSE score was 30.

Subject III:2, identical twin sister of the proband, is right-handed and was aged 75 at the time of evaluation. Her past medical history was unremarkable and neurological examination was normal. She did not complain of any cognitive deficit and her MMSE score was 29.

According to family reports there were several other members affected by PD and some affected by other neurological conditions: (i) The mother of the proband, subject II:1, had a definite diagnosis of PD. She was diagnosed at the age of 65 and died at the age of 72. She was also reported to have had dementia. (ii) A maternal aunt, subject II:2, was said to have had PD, although she died at the age of 47. (iii) The maternal grandmother of the proband, subject I:1, had PD and also died at the age of 47. (iv) A maternal uncle, subject II:3, possibly had PD. (v) A sister of the proband, subject III:3, had a diagnosis of essential tremor.

D.1.6 Family 3

One affected member (II:1) and one unaffected member (II:2) of this family were studied.

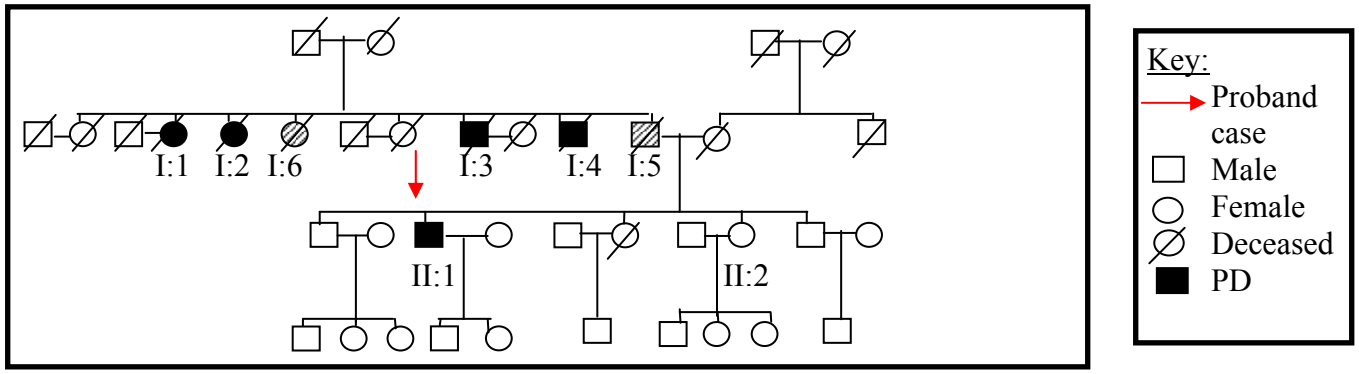


Figure D.6 Pedigree for family 3

The proband case II:1 carries the heterozygous M1869T mutation, subject II:2 does not. The proband is a right-handed male, was aged 67 at the time of evaluation and had onset of symptoms aged 64. His initial symptoms were right-sided bradykinesia, tremor and micrographia. He had not been commenced on any anti-parkinsonian medication. His past medical history included myocardial infarction, cerebrovascular attack, asthma and hypertension.

Neurological examination revealed findings typical of IPD, with mask-like facies, asymmetrical tremor, bradykinesia and rigidity. His Hoehn and Yahr stage was 1, with a UPDRS III score of 12. There was no evidence of cognitive deficit with a MMSE score of 30.

Subject II:2 is right-handed and was aged 63 at the time of evaluation. Her past medical history was unremarkable and neurological examination was normal. There was no evidence of cognitive deficit with a MMSE score of 30.

According to family reports other affected members were: (i) A paternal aunt, subject I:1, who had PD, she was diagnosed aged 38 and died aged 73. (ii) Another paternal aunt, subject I:2, had PD, she received Levodopa therapy and died at the age of 82 (iii) A paternal uncle, subject I:3, had PD and received Levodopa therapy, he died at the age of 83. (iv) Another paternal uncle, subject I:4, had PD. He received Levodopa therapy and died at the age of 87. (v) The father of the proband, subject I:5, had tremor but was never formally diagnosed with PD. He died at the age of 65 (vi) A paternal aunt, subject I:6, possibly had PD. She died at the age of 80.

D.2 sPD study

I have provided the clinical features of the four subjects who were identified as carriers of pathogenic (G2019S and R1441C) *LRRK2* mutations. In addition the demographics of the two controls, who were found to be heterozygous carriers of the putatively pathogenic M1869T mutation, and the novel p.P2093P variation, respectively, are also given.

All six individuals are Caucasian, PD237 is from the Birmingham PD study and the other three subjects and two controls (PP0293, PP0360, PP0071, PC109 and PC157) are from the PD GEN study.

D.2.1 Subject PD237

This subject carries the heterozygous G2019S mutation. He was aged 71 at the time of initial evaluation and had onset of symptoms aged 70. His initial symptoms were unilateral tremor, 'slowing down', reduced turning in bed and quiet speech. He was commenced on Levodopa therapy with good treatment effect. Examination revealed features typical of IPD, with mask-like facies, bilateral tremor, rigidity and bradykinesia which was worse on the right. There was no known family history of PD.

The subject was followed up for eleven years in clinic and was noted to have developed dystonia and treatment induced dyskinesias eight years after diagnosis. There was no evidence of cognitive dysfunction. His final recorded anti-parkinsonian medication consisted of a combination of Levodopa, Selegeline (MAOBI) and Entacapone (COMTI) (Levodopa equivalent dose of 800mg daily). He died at the age of 83 of a myocardial infarction.

D.2.2 Subject PP0293

This subject carries the heterozygous G2019S mutation. She was aged 78 at the time of entry to the PD MED/PD GEN study and had been diagnosed with PD at the age of 76. Her Hoehn and Yahr stage was 2.5 and she had a MMSE score of 30. She was commenced on treatment with a dopamine agonist (Ropinirole) with good treatment effect. There was no known family history of PD.

After two years of follow-up, her Hoehn and Yahr stage remained at 2.5 and she was not noted to have developed any treatment induced dyskinesias, motor fluctuations or cognitive dysfunction. Her latest recorded anti-parkinsonian medication consisted of Ropinirole at a dose of 25mg per day.

D.2.3 Subject PP0360

This subject carries the heterozygous G2019S mutation. He was aged 61 at the time of entry to the PD MED/PD GEN study and had been diagnosed with PD at the age of 60. His Hoehn and Yahr stage was 1 and he had a MMSE score of 28. He was commenced on treatment with Selegeline (MAOBI). There was no known family history of PD.

After four years of follow-up his Hoehn and Yahr stage progressed to 2.5, he was not noted to have developed any treatment induced dyskinesias or motor fluctuations. His anti-parkinsonian medication was changed from Selegeline, he was first tried on two dopamine agonists sequentially (Pergolide and Ropinirole) both of which were stopped due to side effects. His latest recorded anti-parkinsonian medication consisted of Levodopa at a dose of 450mg per day.

D.2.4 Subject PP0071

This subject carries the heterozygous R1441C mutation. She was aged 71 at the time of entry to the PD MED/PD GEN study and had been diagnosed with PD at the age of 61. Her Hoehn and Yahr stage was 3 and she had a MMSE score of 29. She had initially been treated with Levodopa from the time of diagnosis. A dopamine agonist had been added to treatment alongside the Levodopa, at the time of entry to the study, but was withdrawn after eight months because of confusion. There was no known family history of PD.

The subject was followed up for six years as part of the study and died aged 77. At the age of 73 her Hoehn and Yahr stage had progressed to 5 and she had been institutionalized. At her final assessment aged 76, her Hoehn and Yahr stage was recorded as 5, but she was not noted to have developed any cognitive dysfunction, with a MMSE score of 30. At this assessment her anti-parkinsonian medication was Levodopa at a daily dose of 900mg.

D.2.5 Controls PC109 and PC157

Individual PC109 carries the heterozygous M1869T mutation and was aged 46 at the time of entry to the PD GEN study. Individual PC157 carries the novel heterozygous P2093P variation and was aged 49 at the time of entry to the PD GEN. Both individuals are female, are the spouses of subjects who had entered the study, were unaffected by PD and had no known family history of PD.

Appendix E

The information which I have provided in this appendix relates to the fPD study in chapter 5. Where control data on *LRRK2* variations identified in my study was not available in order to perform statistical analyses, I used the published control data from a North American cohort of 275 unrelated neurologically normal Caucasian individuals. Of this cohort 127 were ascertained as spouses of an individual affected with PD, the remainder were volunteers. None of the controls had any first degree relative with a known primary neurological disorder [666]. The demographics of this control cohort are listed below in table E.1.

Number of controls	Male (%)	Female (%)	Mean age (range)
275	133 (48)	142 (52)	68 (55-88)

Table E.1 Demographics of control cohort used for statistical analysis in my study of *LRRK2* variations in fPD. Control cohort data obtained from study by Paisan-Ruiz al. 2008 [666].

The minor allele frequencies of previously described non-synonymous and synonymous coding sequence variations, and non-coding (intronic) sequence variations, in patients from my study is compared to control data in tables E.2-E.4 below.

Nucleotide Change	*LRRK2* exon	Genbamk reference number	Predicated protein change	Minor allele Freq. in patients	Minor allele Freq. in Controls	P value
c.1653C>G	14	rs7308720	p.N551K	0.03	0.07	0.090
c.2167A>G	18	rs10878307	p.I723V	0.04	0.07	0.478
c.4193G>A	30	rs7133914	p.R1398H	0.04	0.07	0.166
c.4624C>T	32	rs33958906	p.P1542S	0.03	0.02	0.690
c.4934T>A	34	rs11564148	p.S1647T	0.34	0.29	0.111
c.7190C>T	49	rs3761863	p.T2397M	0.30	0.39	0.104

Table E.2 Minor allele frequencies of six previously described *LRRK2* coding sequence variations, which lead to non synonymous amino acid substiutions, identified in this study with control data. Control data from Paisan-Ruiz et al. 2008 [666].

Nucleotide Change	*LRRK2* exon	Genbank reference number	Predicated protein change	Minor allele Freq. in patients	Minor allele Freq. in Controls	P value
c.578C>T	5	rs10878245	p.L153L	0.34	0.41	0.185
c.2857T>C	22	rs7966550	p.L953L	0.12	0.15	0.474
c.4269G>A	30	rs11175964	p.K1423K	0.04	0.07	0.233
c.4872A>C	34	rs1427263	p.G1624G	0.29	0.37	0.034
c.4911G>A	34	rs11176013	p.K1637K	0.41	0.47	0.213
c.5457C>T	37	rs10878371	p.G1819G	0.41	0.47	0.144
c.6324G>A	43	rs10878405	p.E2108E	0.33	0.30	0.151
c.7155A>G	48	rs33962975	p.G2385G	0.14	0.13	0.583

Table E.3 Minor allele frequencies of eight previously described synonymous *LRRK2* coding sequence variations identified in this study with control data. Control data from Paisan-Ruiz et al. 2008 [666].

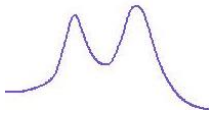
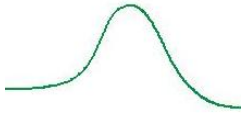
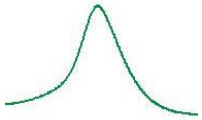
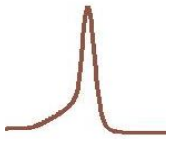
Nucleotide change	*LRRK2* intron	Genbank reference number	Minor allele Freq. in patients	Minor allele Freq. in Controls	P value
IVS4+38A>T	4	rs2131088	0.09	0.08	0.826
IVS10-10C>A	9	rs7955902	0.41	0.38	0.100
IVS11+130G>A	11	rs7969677	0.12	0.20	0.132
IVS14+68G>C	14	rs10784462	0.46	0.56	0.238
IVS30+52insGT	30	rs10650388	0.55	0.62	0.068
IVS34-31T>C	33	rs1896252	0.57	0.52	0.843
IVS34+32A>G	34	rs11564205	0.18	0.14	0.286
IVS36+36T>C	36	rs7137665	0.34	0.38	0.051
IVS43+53G>A	43	rs11176143	0.12	0.11	0.697

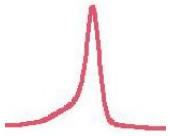
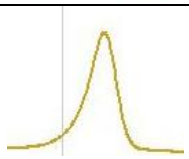
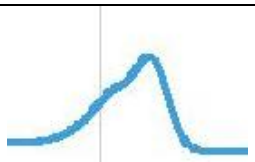
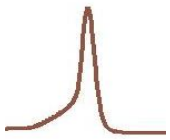
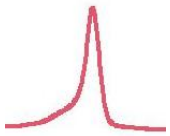
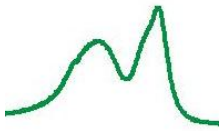
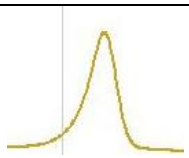
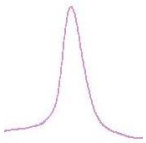
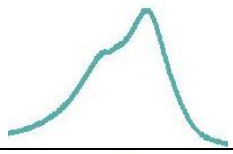
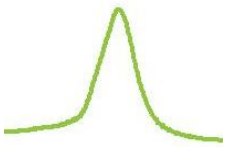
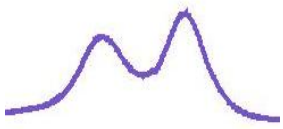
Table E.4 Minor allele frequency of nine previously described *LRRK2* non-coding (intronic) single nucleotide polymorphisms (SNPs) identified in this study in patients with control data. Control data from Paisan-Ruiz et al. 2008 [666].

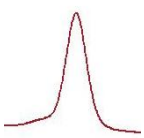
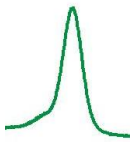
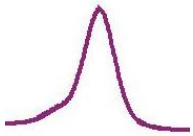
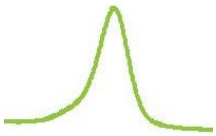
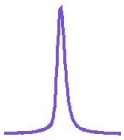
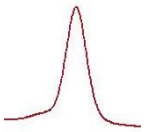
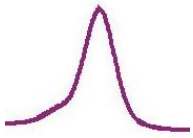
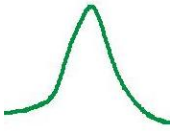
Appendix F

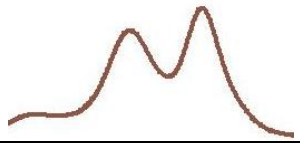
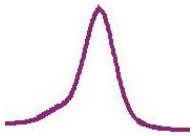
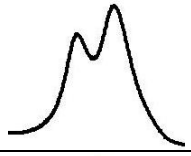
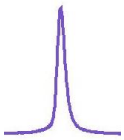
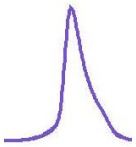
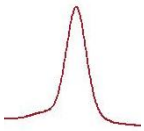
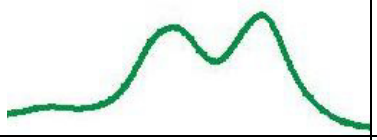
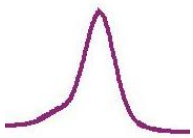
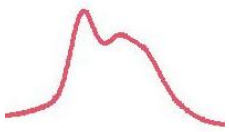
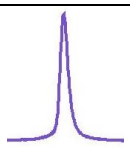
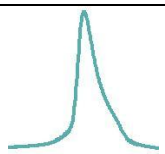
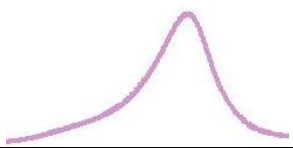
The information which I have provided in this appendix relates to the work carried out in chapter 6, the screening for *LRRK2* variants in sPD in eight exons: 24, 25, 31, 32, 38, 40, 41 and 42 by denaturing high performance liquid chromatography (DHPLC). Table F.1 below demonstrates DHPLC chromatograms for different variants in all eight exons, at different column temperatures, for subjects known to be wild-type or heterozygous carriers of these variants. Some variants, for example 6187delCTCTA (p.L2063 STOP) in exon 42, were not detected in the sPD cases, but are provided here as an illustration of the technique.

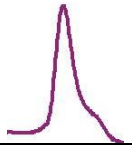
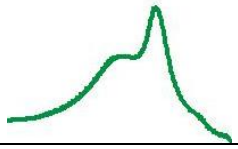
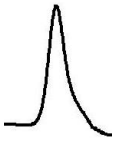
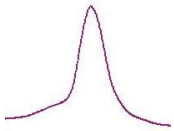
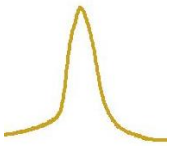
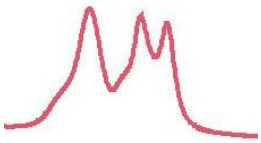
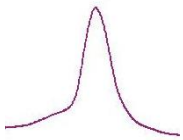
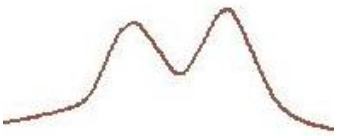
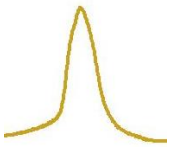
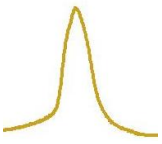
The DHPLC chromatograms show one peak for the subjects who were wild-type, representing homoduplexes only, and two peaks for the subjects carrying the variants, representing a mixture of homoduplexes and heteroduplexes. This technique allows discrimination between wild-type and mutants for these variants. For each variant at least two column temperatures were required to cover the entire region, and in most cases there is an optimal column temperature for each variant to allow discrimination between wild-type and mutant.

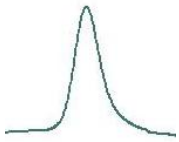
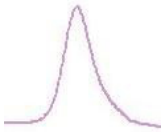
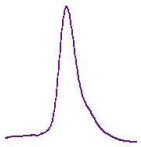
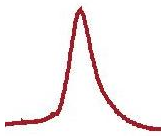
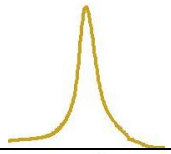
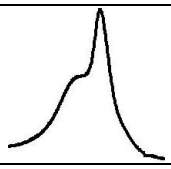
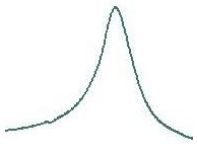
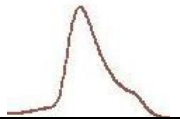
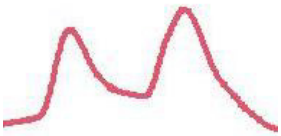
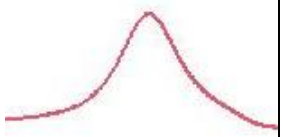
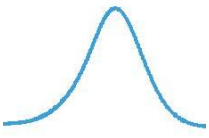
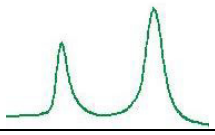
LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild type	Variant	
24	55.0°C			**IVS24+31T>C**
	57.3°C			**IVS24+31T>C**
	55.0°C			**IVS24+46G>T**
	57.3°C			**IVS24+46G>T**
25	54.1°C		N/A	
	56.8°C		N/A	
	57.6°C		N/A	
31	59.5°C			c.4321C>T R1441C

LRRK2 **Exon**	**DHPLC column temperatures**	**DHPLC Chromatograms**		**Nucleotide change & predicated protein change**
		Wild type	**Variant**	
31	60.0°C			c.4321C>T R1441C
	61.5°C			c.4321C>T R1441C
	59.5°C			**c.4364delAT** **D1455G**
	60.0°C			**c.4364delAT** **D1455G**
	61.5°C			**c.4364delAT** **D1455G**
32	54.6°C			c.4624C>T P1542S
	57.0°C			c.4624C>T P1542S
	54.6°C			c.4541G>A R1514Q
	57.0°C			c.4541G>A R1514Q

LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
38	51.0°C			c.5606T>C M1869T
	52.2°C			c.5606T>C M1869T
	55.0°C			c.5606T>C M1869T
	57.5°C			c.5606T>C M1869T
	51.0°C			IVS38-9A>G
	52.2°C			IVS38-9A>G
	55.0°C			IVS38-9A>G
	57.5°C			IVS38-9A>G

LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
38	51.0°C			IVS38+35G>A
	52.2°C			IVS38+35G>A
	55.0°C			IVS38+35G>A
	57.5°C			IVS38+35G>A
	51.0°C			**IVS38+54T>C**
	52.2°C			**IVS38+54T>C**
	55.0°C			**IVS38+54T>C**
	57.5°C			**IVS38+54T>C**
	51.0°C			**IVS38+80A>G**

LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
38	52.2°C			**IVS38+80A>G**
	55.0°C			**IVS38+80A>G**
	57.5°C			**IVS38+80A>G**
40	58.8°C			IVS40+34T>C
	61.3°C			IVS40+34T>C
	58.8°C			IVS40+48C>T
	61.3°C			IVS40+48C>T
	58.8°C			IVS40+34T>C & IVS40+48C>T
	61.3°C			IVS40+34T>C & IVS40+48C>T

LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
41	53.8°C			C.6055G>A G2019S
	57.8°C			C.6055G>A G2019S
	59.2°C			C.6055G>A G2019S
	60.0°C			C.6055G>A G2019S
42	54.7°C			C.6241A>G N2081D
	55.9°C			C.6241A>G N2081D
	56.9°C			C.6241A>G N2081D
	58.0°C			C.6241A>G N2081D
	54.7°C			**c.6187delCTCTA L2063STOP**

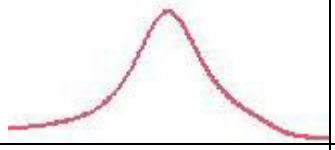
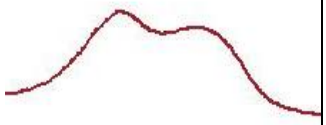
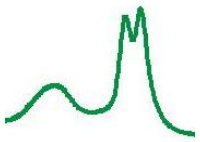
LRRK2 Exon	DHPLC column temperatures	DHPLC Chromatograms		Nucleotide change & predicated protein change
		Wild Type	Variant	
42	**55.9°C**			**c.6187delCTCTA L2063STOP**
	56.9°C			**c.6187delCTCTA L2063STOP**
	58.0°C			**c.6187delCTCTA L2063STOP**
	54.7°C			**c.6279delT P2093P**
	55.9°C			**c.6279delT P2093P**
	56.9°C			**c.6279delT P2093P**
	58.0°C			**c.6279delT P2093P**

Table F.1 Screening for *LRRK2* variants in eight exons: 24, 25, 31, 32, 38, 40, 41 and 42 by denaturing high performance liquid chromatography (DHPLC). PCR reactions were performed as described in section 6.3.2.4 and then DHPLC used to screen for variants. This table demonstrates DHPLC chromatograms, at different column temperatures, for the eight exons. At the optimal temperature for each variant the chromatograms show one peak for the subjects who were wild-type, representing homoduplexes only, and two peaks for the subjects carrying the variants, representing a mixture of homoduplexes and heteroduplexes. This technique allows discrimination between wild-type and mutants for these variants. Pathogenic *LRRK2* mutations are shown in red, with variants described for the first time in this study (fPD or sPD) in bold, the other variants listed here were identified in this study but have been described previously.

Appendix G

The information which I have provided in this appendix relates to the presentation of data from this thesis.

G.1 Association of British Neurologists

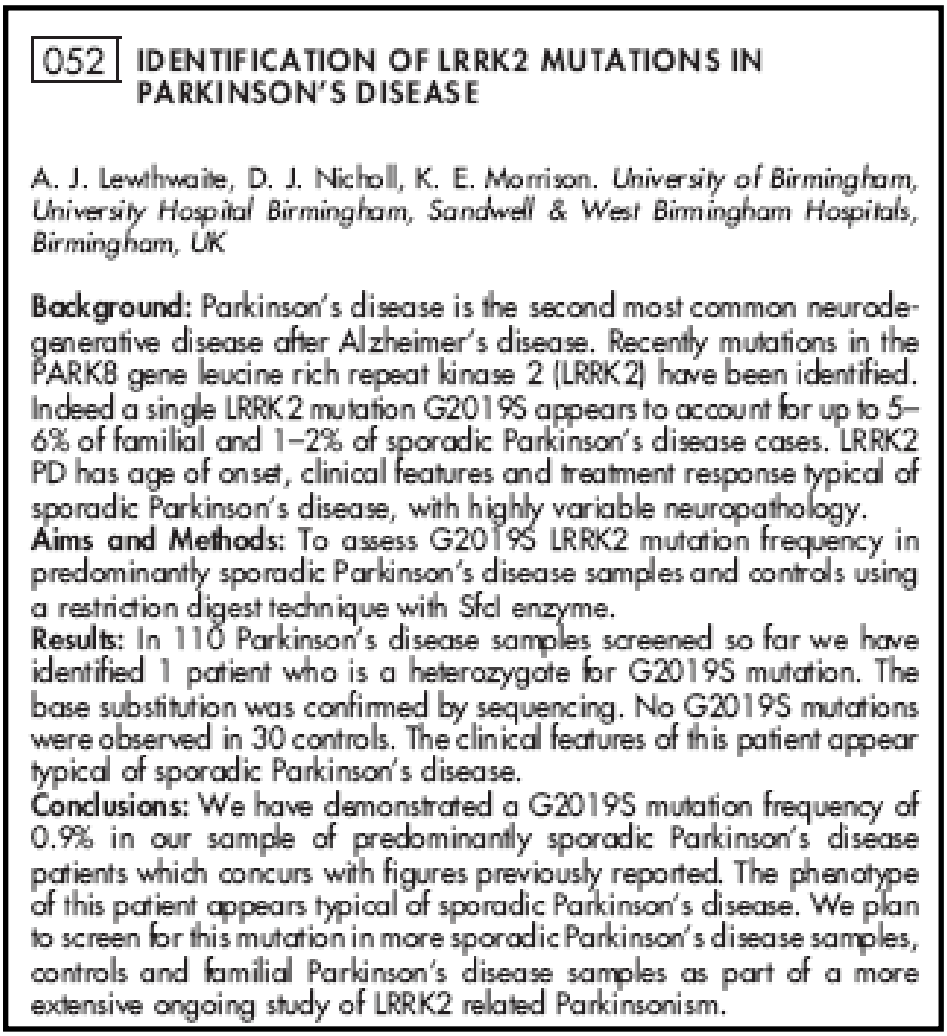
I presented data in poster format at the Association of British Neurologists (ABN) scientific meeting in April 2006, in Brighton. This poster was accepted for presentation after submission of an abstract, which were subsequently published: Identification of *LRRK2* mutations in Parkinson's disease. AJ Lewthwaite, DJ Nicholl & KE Morrison. *JNNP* 2006; **77:** 1397 (see figure G.1).

G.2 Movement Disorders Society

I also presented data at the International Congress of The Movement Disorder Society (MDS), Istanbul in June 2007. These posters were accepted for presentation after submission of abstracts, which were subsequently published:

(i) Screening for *LRRK2* mutations in familial Parkinson's disease. AJ Lewthwaite, TD Lambert, NW Wood, DJ Nicholl & KE Morrison. *Movement Disorders* 2007; **22 Supl 16:** S102-S103 (see figure G.2)

(ii) Clinical and imaging characteristics of a dominant kindred with benign parkinsonism and dopa-responsive dystonia. AJ Lewthwaite, TD Lambert, V Bonifati, DJ Nicholl & KE Morrison. *Movement Disorders* 2007; **22 Supl 16:** S266-S267 (see figure G.3).



052 IDENTIFICATION OF LRRK2 MUTATIONS IN PARKINSON'S DISEASE

A. J. Lewthwaite, D. J. Nicholl, K. E. Morrison. *University of Birmingham, University Hospital Birmingham, Sandwell & West Birmingham Hospitals, Birmingham, UK*

Background: Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's disease. Recently mutations in the PARK8 gene leucine rich repeat kinase 2 (LRRK2) have been identified. Indeed a single LRRK2 mutation G2019S appears to account for up to 5–6% of familial and 1–2% of sporadic Parkinson's disease cases. LRRK2 PD has age of onset, clinical features and treatment response typical of sporadic Parkinson's disease, with highly variable neuropathology.
Aims and Methods: To assess G2019S LRRK2 mutation frequency in predominantly sporadic Parkinson's disease samples and controls using a restriction digest technique with SfcI enzyme.
Results: In 110 Parkinson's disease samples screened so far we have identified 1 patient who is a heterozygote for G2019S mutation. The base substitution was confirmed by sequencing. No G2019S mutations were observed in 30 controls. The clinical features of this patient appear typical of sporadic Parkinson's disease.
Conclusions: We have demonstrated a G2019S mutation frequency of 0.9% in our sample of predominantly sporadic Parkinson's disease patients which concurs with figures previously reported. The phenotype of this patient appears typical of sporadic Parkinson's disease. We plan to screen for this mutation in more sporadic Parkinson's disease samples, controls and familial Parkinson's disease samples as part of a more extensive ongoing study of LRRK2 related Parkinsonism.

Figure G.1 Abstract submitted to the Association of British Neurologists (ABN) for scientific meeting in April 2006. This data was presented as a poster and the abstract subsequently published: Identification of *LRRK2* mutations in Parkinson's disease. AJ Lewthwaite, DJ Nicholl & KE Morrison. *JNNP* 2006; **77:** 1397.

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Screening for *LRRK2* mutations in UK familial Parkinson's disease patients

A.J. Lewthwaite, T.D. Lambert, N.W. Wood, D.J. Nicholl, K.E. Morrison (Birmingham, West Midlands, United Kingdom)

Objective: To determine frequency of known and potentially novel *LRRK2* mutations in familial Parkinson's disease (PD) patients in the United Kingdom (UK).

Background: Genetic influence on PD has been recognized as far back as 19th century. Indeed up to 10% of PD patients may have a positive family history. Study of familial PD has led to identification of a number of PD genes, most recently *LRRK2*. This is a large gene with 51 exons and putative protein kinase function. Importantly a single *LRRK2* mutation G2019S appears to account for up to 5-6% of familial and 1-2% of sporadic PD cases, although the frequency of this mutation appears to be much higher in North African familial PD cases.

Methods: We recruited patients with PD from around the UK with a history of at least 1 other affected family member and apparently autosomal dominant inheritance. Patients either self-referred or were referred by colleagues. Each affected participant had detailed clinical assessment of parkinsonian features including use of UPDRS and Hoehn & Yahr scales and Video recordings. We are currently completing screening these familial PD patients by genomic sequencing for known and novel *LRRK2* mutations in all 51 exons.

Results: We recruited 51 affected participants from 44 families which have 2 or more members affected by PD. There are 28 males and 23 females, average age of affected participants is 69, range 39 to 89. Average age of disease onset is 58 with an average disease duration of 11 years. Sequencing results from 44 probands show heterozygous G2019S mutations in 3 probands (6.8%) and heterozygous R1441C mutation in 1 proband (2.3%). These are similar to mutation frequencies identified in previous studies. The phenotype associated with these *LRRK2* mutations generally appears to be of typical PD. Where mutations have been identified in probands and samples are available we have also screened other affected and unaffected family members. Further sequencing results from remaining exons are awaited.

Conclusions: In this large cohort of UK PD families we are screening for mutations in all 51 *LRRK2* exons. From data available to date we have identified pathogenic *LRRK2* mutations in 4 probands (9.1%). This supports results from previous studies and further emphasises the importance of this gene in our understanding of the pathogenic mechanisms involved in PD.

Figure G.2 Abstract submitted to the Movement Disorder Society (MDS) for International Congress in June 2007. This data was presented as a poster and the abstract was subsequently published: Screening for *LRRK2* mutations in familial Parkinson's disease. AJ Lewthwaite, TD Lambert, NW Wood, DJ Nicholl & KE Morrison. *Movement Disorders* 2007; **22 Supl 16:** S102-S103.

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Clinical and imaging characteristics of a dominant kindred with benign Parkinsonism and dopa-responsive dystonia

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Objective: To describe clinical and imaging characteristics of a dominant kindred with benign Parkinsonism and Dopa-Responsive Dystonia.

Background: During our study of familial Parkinson's disease (PD) we identified a family in which there are currently 5 affected members in two generations. Four of the affected individuals are male and one female. The affected individuals have been examined (AJL) and show a phenotype ranging from slowly progressive dopa responsive parkinsonism to dopa-responsive dystonia (DRD). From family reports there were also two members of the previous generation who may have had parkinsonism.

Methods: Each affected participant has had detailed clinical assessment of parkinsonian features including use of UPDRS and Hoehn & Yahr scales and Video recordings (AJL). Each affected individual has also had Dopamine Transporter (DAT) scans performed. Furthermore 6 members of the family (5 affected and one unaffected) have been screened for mutations in PD genes: *Parkin*, *DJ-1*, *PINK1* and *LRRK2* and for mutations in GTP cyclohyrolase 1 gene (*GCH1*), the most common cause of DRD.

Results: After extensive clinical assessment (AJL) 3 individuals from the kindred have been diagnosed as having benign dopa-responsive parkinsonism and 2 with DRD. The median age of disease onset is 50 with a median duration of 20 years. DAT scan images are available on 4 affected individuals and 1 unaffected sibling. Clinical details and DAT scan results are summarised in the table below. All 51 exons of *LRRK2* have been sequenced and no mutations found, results from screening of other genes are awaited.

Conclusions: Accurate clinical diagnosis has been challenging in this family, indeed patient 5:5 was treated for almost 20 years for PD, the diagnosis only recently being revised to DRD. DAT imaging has supported the clinical diagnoses in all but one case, patient 5:4. This patient has had slowly progressive dopa-responsive parkinsonism for 18 years but has a normal DAT scan. This result suggests that this individual may fall into the category of patients who have parkinsonism with a normal DAT scan or that there may be overlap between Parkinsonism and DRD in this kindred. We await the outstanding molecular genetic screening results to help further clarify the diagnoses in this family.

Table 1: Patient age, gender, diagnosis, age of disease onset, disease duration and DAT scan result

Patient	Age	Gender	Clinical diagnosis	Age of disease onset	Disease duration (years)	DAT scan
5:1	82	Male	Parkinsonism	58	24	Abnormal
5:2	80	Male	Normal	N/A	N/A	Normal
5:3	78	Male	Parkinsonism	75	3	Abnormal
5:4	68	Male	Parkinsonism	50	18	Normal
5:5	66	Male	DRD	44	22	Normal
5:6	26	Female	DRD	6	20	Not done

Figure G.3 Abstract submitted to Movement Disorder Society (MDS) International Congress in June 2007. This data was presented as a poster and the abstract subsequently published: Clinical and imaging characteristics of a dominant kindred with benign parkinsonism and dopa-responsive dystonia. AJ Lewthwaite, TD Lambert, V Bonifati, DJ Nicholl & KE Morrison. *Movement Disorders* 2007; **22 Supl 16:** S266-S267.

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