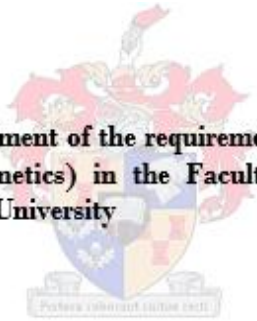
Screening for disease-causing genes in Black South African patients with Parkinson's Disease

By

Claudia Ntsapi



Thesis presented in fulfilment of the requirements for the degree of Master of Science (Human Genetics) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Associate Professor Soraya Bardien
Faculty of Medicine and Health Sciences
Department of Biomedical Sciences

Co-supervisor: Dr. Sihaam Boolay
Faculty of Medicine and Health Sciences
Department of Biomedical Sciences

March 2015

DECLARATION

I, the undersigned, hereby declare that the entirety of the work contained in this thesis is my own original work (save to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: …C. Ntsapi……Date: ……March 2015……

ABSTRACT

Parkinson's disease (PD) is an increasingly common neurodegenerative disorder involving the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, which leads to disruption of the motor circuit. Neuronal cell loss in excess of 50% of normal levels is required for the development of clinical symptoms, including tremor, muscular rigidity, bradykinesia, and postural instability. There is currently no available drug to prevent, reverse, or halt the progression of the disease, but limited symptomatic treatment is available. Increasing age is considered to be one of the strongest risk factors for PD. As current epidemiological studies project that worldwide populations will increase significantly in age, this has important implications for PD.

Although the molecular mechanisms underlying neuronal degeneration leading to PD remains unknown, it has becoming increasingly clear that genetic factors (alone or in combination with environmental factors), contribute to the complex pathogenesis of the disease. Eight validated genes have been directly implicated in the aetiology of PD, namely: *parkin*, *PINK1*, *DJ-1*, *ATP13A2*, *SNCA*, *LRRK2, VPS35,* and *EIF4G1*. These genes have been predominantly identified and studied in European, North American and Asian populations; whereas results from Sub-Saharan African (SSA) populations, especially those of Black African ancestry remain underrepresented in genetic studies. Given the fact that results obtained from other populations may not be transferable to SSA populations as well as the projected increase in this disorder worldwide, it is crucial that comprehensive genetic studies are performed in traditionally understudied populations of SSA.

To this end, the aim of the present study was to investigate the molecular aetiology of PD in a group of Black South African (SA) PD patients. A total of 47 patients were recruited for the study, and 26% had a positive family history of the disorder with the average age at onset being 55.3 ± 11.2 years. Mutation screening of the known PD genes was performed. Additionally, mutation screening of *GBA* was also performed, as heterozygous mutations in this gene serve as the strongest risk factor for PD. A variety of mutation-screening techniques was employed, namely: Sanger sequencing and high-resolution melt (for detection of missense mutations and small insertions or deletions), and multiplex-ligation dependent probe amplification assay (for detection of copy number changes). Furthermore, next generation sequencing was used for systematic screening of the known PD genes plus an additional 160 candidate genes. In addition, genotyping of CAG repeat expansions in the genes encoding ataxin-2 (*SCA2*) and ataxin-7 (*SCA7*) was performed using fluorescently-labelled polymerase chain reaction primers followed by electrophoresis on the ABI 3130xl Genetic Analyzer.

Mutation screening revealed that the known PD genes do not play a significant causal role in disease pathogenesis in the current group of Black SA patients, as only 2 out of 47 (4.3%) patients screened harboured mutations in *parkin*. One patient had a heterozygous exon 2 duplication and a heterozygous exon 9 deletion, and another patient had a heterozygous exon 4 deletion and a heterozygous G430D mutation. Moreover, a Q311K, change in *parkin* and four novel variants (I610T, H1758P, N2133S, and T2423S) were found in *LRRK2*, however the pathogenicity of these variants needs to be investigated further. None of the patients had pathogenic repeat expansions at the *SCA2* and *SCA7* loci, thereby excluding these two spinocerebellar ataxia subtypes as genetic modifiers of PD, or as a cause of the disease phenotype in this group of patients. No pathogenic mutations were found in any of the remaining known PD genes, it is therefore likely that these patients harbour mutations in novel PD-causing genes.

This is the first molecular genetic study exclusively of Black SA PD patients, and the first comprehensive analysis of all the known PD genes in a SSA population. Common pathogenic mutations in these genes, previously shown to be disease causing in several European populations, were not detected in the present study and can therefore be excluded as the cause of PD in this group of patients. However, given the relatively small sample size, further mutation screening of a larger cohort could prove beneficial in the elucidation of novel disease-causing genes which may potentially refute/substantiate a current hypothesis which suggests that the genetic aetiology of PD is distinctly different across various ethnic populations groups worldwide. Continued effects to screen this group of patients may shed insight into population specific genetic determinants, which may aid in the identification of novel drug targets.

OPSOMMING

Parkinson se siekte (PD) is 'n toenemend algemene neurodegeneratiewe siekte wat die progressiewe verlies van dopaminergiese neurone in die substantia nigra pars compacta behels, wat lei tot die ontwrigting van die motororiese senuweestelsel. 'n Neuronale verlies van meer as 50% van die normale vlakke is nodig vir die ontwikkeling van die kliniese simptome, insluitende bewing, spier rigiditeit, bradykinesia, en posturale onstabiliteit. Alhoewel beskikbare medikasies vir PD wel simptome onderdruk, is dit nie in staat om die siekte te voorkom of vordering daarvan te verhoed nie. Een van die mees bedeidende risikofaktore vir PD is toenemende ouderdom. Huidige epidemiologiese studies voorspel 'n toename in populasie ouderdom wat belangrike implikasies vir die voorkoms van PD kan inhou.

Die molekulere meganismes onderliggend aan die neurale agteruitgang in PD is steeds onbekend, maar dit blyk dat 'n aantal genetiese faktore (in kombinasie met omgewingsfaktore) bydra tot die komplekse patogeniese agteruitgang van die siekte. Agt bevestigde gene is gevind om direk betrokke te wees by die etiologie van PD, naamlik: *parkin*, *PINK1*, *DJ-1*, *ATP13A2*, *SNCA*, *LRRK2, VPS35,* en *EIF4G1*. Hierdie gene is oorwegend geïdentifiseer en bestudeer in die Europese, Noord-Amerikaanse en Asiatiese bevolkings; terwyl die bestudering van Sub -Sahara- Afrika (SSA) bevolkings, veral dié van Swart Afrika afkoms, onderverteenwoordig bly in genetiese studies. Siende dat resultate verkry vanaf ander bevolkings nie verwant is aan die SSA bevolking nie, en ook nie 'n voorspelling van wereldwye toename van die siekte kan verteenwoordig nie, is dit van belang dat omvattende genetiese studies uitgevoer word op hierdie onderbestudeerde bevolkings van SSA.

Desnieteenstaande, die doel van die studie was om die molekulere etiologie van PD in 'n groep Swart Suid-Afrikaanse (SA) PD pasiente te ondersoek. 'n Totaal van 47 pasiente is gewerf vir die studie waarvan 26% 'n famielie geskiedenis het vir die afwyking. Die gemiddelde ouderdom van aanvang vir die pasiente was 55.3 ± 11.2 jaar. Mutasie volgorderbepaling vir alle bekende PD gene is uitgevoer. Addisionele mutasie volgorderbepaling vir die *GBA* is ook uitgevoer siende dat heterosigotiese mutasies in die geen dien as 'n sterk risikofaktor vir die ontwikkeling van PD. 'n Verskeidenheid mutasie volgordebepalingstegnieke is geinkorporeer vir die studie, naamlik: Sanger volgordebepaling en hoë resolusie smelt tegniek (vir die identifisering van missense mutasies asook kleinskaalse invoegings of weglatings), en multiplex-afbinding afhanklike ondersoek versterkingstoets (vir die opsporing van veranderinge in kopiegetal). Verder is volgende generasie volgordebepaling gebruik vir die sistematiese ondersoek van die bekende PD gene, asook vir 160 kandidaat gene wat vooraf bepaal is. Fluoreserend-geetiketeerde polymerase ketting reaksie primers is gebruik vir genotipering van

CAG herhalings uitbreidings in die ataxin-2 (*SCA2*) en die ataxin-7 (*SCA7*) gene, gevolg deur elektroforese met behulp van die "ABI 3130x1 Genetic Analyzer".

Mutasie volgordebepaling het voor die lig gebring dat die bekende PD gene klaarblyklik nie 'n beduidende oorsaaklike rol speel in die patogenese van die siekte in die huidige groep Swart SA pasiente nie, want slegs 2 uit die 47 (43%) pasiente koester mutasies in *parkin*. Een van die pasiente besit 'n heterosigotiese duplikasie van ekson 2 en 'n heterosigotiese weglating van ekson 9; in die ander pasient is 'n heterosigotiese ekson 4 weglating en 'n heterosigotiese G430D mutasie geïdentifiseer. Verder is 'n Q311K verandering in *parkin* gevind asook vier nuwe variante (I610T, H1758P, N2133S en T2423S) in die *LRRK2* geen. Die patogenisiteit van hierdie variante moet egter nog bepaal word. Geen patogeniese herhaalings uitbreidings is gevind in die *SCA2* en *SCA7* lokusse nie, en die moontlikheid van die twee spinocerebellar ataksie subtipes as genetiese bepalers vir PD is uitgekanselleer. Geen patogeniese mutasies is gevind in enige van die oorblywende bekende PD gene nie, dit is dus waarskeinlik dat die pasiente wel mutasies in nuwe PD-geassosieerde gene besit.

Hierdie is die eerste molekulere genetiese studie uitgevoer op uitsluitlik Swart SA PD pasiente, en ook die eerste omvattende ondersoek van al die bekende PD gene in 'n SSA bevolking. Die algemene patogeniese mutasies in gene, wat voorheen bewys is as siekte veroorsakend in 'n aantal Europese bevolkings, is nie bespeur in die huidige studie nie en kan dus nie verantwoordelik gehou word vir die voorkoms van PD in hierdie pasiente nie. Maar, siende dat die steekproefgrootte in hierdie studie relatief klein was, kan addisionele volgordebepaling van 'n groter pasient groep voordelig wees in die bepaal van nuwe siekte-veroorsakende gene wat die potensiaal het om huidige hipoteses, dat die genetiese etiologie van PD duidelik verskil oor verskeie etniese groepe wereldwyd, te weerlê of bevestig. Die voortsetting van genetiese ondersoeke onder hierdie groep pasiente kan bydra tot insig met betrekking tot bevolking-spesifieke genetiese bepalers, en uiteindelik tot die identifisering van nuwe teikens vir medikasies teen die siekte.

List of Abbreviations and Symbols

α	Alpha
aSYN	α-synuclein protein
β	Beta
® / ™	Registered trademark
%	Percentage
C^0	Degrees Celsius
CI	confidence interval
A	Alanine (Ala)
AAO	Age at onset
AD	Alzheimer's disease
AD	Autosomal dominant
ADPD	Autosomal dominant form of Parkinson's disease
ALP	Autophagy-lysosome pathway
ANK	Ankyrin repeat domain
AR	Autosomal recessive
ARPD	Autosomal recessive form of Parkinson's disease
ARM	Armadillo domain
ATP	adenosine-5'-triphosphate
ATP13A2	ATPase type 13 A2
Alu	Short interspersed nuclear element
bp	Base pair(s)
Cys	cysteine
CE	capillary electrophoresis
CAF	Central Analytical Facility
CBD	corticobasal degeneration
CNS	Central nervous system
CNV(s)	copy number variant(s) / copy number variation
COMT	catechol-O-methyltransferase
COR	Carboxy terminal of ROC
D	Aspartic acid (Asp)
DA	Dopamine
DAT	Dopamine transporter
ddNTP	Di-deoxyribonucleotide triphosphate
DBS	Deep Brain Stimulation
DLB	Dementia with Lewy bodies (or diffuse Lewy body disease

DNA	Deoxyribonucleic acid
ddNTP	Di-deoxyribonucleotide triphosphate
dNTP(s)	Deoxynucleotide triphosphate(s)
dsDNA	Double stranded DNA
dTTP	deoxythymidine triphosphate
DMSO	Dimethyl sulphoxide
E	Glutamic acid (Glu)
EDTA	Ethylene diaminetetraacetic acid
EIF4G1	Eukaryotic translation initiation factor 4-gamma
EOPD	Early-onset Parkinson's disease (AAO <50)
ER	endoplasmic reticulum
EtBr	Ethidium bromide
et al.	Et alia (Latin abbreviation for "and others")
e.g.	Example given
ExoI	Exonuclease I
Fluoro-dopa	Fluorine-18-labelled-dopa
G	guanine
G	glycine (Gly)
H	histidine (His)
GBA	glucocerebrosidase
HRM	High-resolution melt
in vitro	Latin abbreviation for "in a test tube"
IBR	In-between RING
i.e.	Id est (Latin abbreviation for "that is"/ " in essence")
IVS	Intron/ intravenous sequence
K	lysine (Lys)
kb	kilobase
KRS	Kufor-Rakeb syndrome
L	leucine (Leu)
LB(s)	Lewy body / Lewy bodies
LBD	Lewy body dementia
L-dopa	levo-3,4-dihydroxyphenylalanine
LOPD	late-onset Parkinson's Disease (AAO > 50)
LRRK2	Leucine rich repeat kinase 2 (dardarin)
MAF	Minor allele frequency
MAO	Monoamine oxidase
MAPKKK	Mitogen–activated protein kinase kinase kinase

M	Methionine (Met)
MAPT	Mictrotubule-associated protein tau
$MgCl_2$	magnesium chloride
MLPA	Multiplex ligation-dependent probe amplification
MIM	Mendelian Inheritance in Man
mM	Millimolar
Mn	manganese
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+	1-methyl-4-phenylpyridine
MRI	Magnetic resonance imaging
MSA	multiple system atrophy
MTS	Mitochondrial targeting domain
mRNA	Messenger ribonucleic acid
N	asparagine (Asn)
n	Sample size
NAC	Non-amyloid-B component
NCBI	National Centre for Biotechnology Information
NGS	Next generation sequencing
n.a.	not available / not applicable
ng	Nanogram
ng/µl	Nanograms per microlitre
NIA	National Institute on Aging
NO	nitric oxide
OR	Odds ratio
P	proline (Pro)
p-value	Probability value
PARK2	Parkin
PCR	polymerase chain reaction
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PINK1	PTEN-induced putative kinase 1
PSP	progressive supranuclear palsy
PSGBA	glucocerebrosidase homologous pseudogene
Q	glutamine (Gln)
R	arginine (Arg)
RING	Really interesting new gene
ROC	Ras of complex proteins

ROH(s)	region(s) of homozygosity
ROS	reactive oxygen species
S	serine (Ser)
SAP	Shrimp alkaline phosphatase
SB	Sodium borate buffer
SCA	Spinocerebellar ataxia
SCA2	Spinocerebellar ataxia subtype 2
SCA7	Spinocerebellar ataxia subtype 7
SD	Standard deviation
SNpc	substantia nigra pars compacta
SNCA	a-synuclein gene
SNP	single nucleotide polymorphism
SD	standard deviation
SSA	sub-Saharan Africa
SA	South Africa
ssDNA	Single stranded DNA
STN	subthalamic nucleus
Taq	DNA polymerase (EC 2.7.7.7) isolated from *Thermus aquaticus*
Tm	Melting temperature
TP-PCR	Tandem repeat-primed PCR
UPDRS	Unified Parkinson's Disease Rating Scale
Ub	Ubiquitin
UBL	Ubiquitin-like domain
UPD	Unique Parkin domain
UPS	ubiquitin proteasome system
U	One unit, defined as the amount of enzyme that incorporates 10nmoles of dNTPs into acid-insoluble form in 30 minutes at 72°C.
UV	Ultraviolet
v.	version
VPS35	Vacuolar protein sorting-associated protein 35
WT	wild type
WES	Whole exome sequencing
Zn	zinc
µg/ml	Micrograms per millilitre
µM	Micromolar
µL	Microliter

List of Figures

Chapter 3:

List of Tables

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Associate Professor Soraya Bardien, for giving me the opportunity to undertake this study and for all the guidance and support throughout the course of my MSc studies. I want to express my deepest gratitude for taking the time to mentor me; you have inspired me to pursue my goals with hard work and dedication. Thank you, once again, for your time, support and patience. I will forever cherish the lessons you taught me, on both personal and professional level.

I would also like to thank my co-supervisor Dr. Sihaam Boolay, for being so generous with her time, and always willing to lend a helping hand. Your assistance in the completion of this thesis was immeasurable and I could not have accomplished as much as I have without your support and constant words of encouragement. I am so grateful to have had the privilege of working alongside you throughout my MSc studies, and I appreciate all you have done for me more than words can say.

I would also like to thank Professor Alexis Brice and Dr Suzanne Lesage for the opportunity to work at the Brain & Spine Institute, in Paris, France. The time we spent in your research laboratory was testament of what is possible given hard work, and dedication.

Next, I would like to thank all my colleagues in the MAGIC lab; particularly those in the Parkinson's disease research group, who each had a hand in the completion of this thesis. To Ms. Celia van der Merwe, thank you for the countless hours spent assisting me in the lab. To Ms Brigitte Glanzmann, you truly are one of the most amazing people that I have had the pleasure of meeting, and I am so grateful for your friendship, and the time and patience you dedicated to assisting me in completion this thesis. It has been an absolute joy learning from you, your positivity and humour have made these last two years unforgettable. I will forever cherish the memories we made in Paris.

My work would not have been possible without the generous financial support of the National Research Foundation (NRF), the Harry Crossley Foundation, the Medical Research Council (MRC), as well as conference funding provided by the South African Society for Human Genetics (SASHG), all of which is gratefully acknowledged in furthering the cause of Parkinson's disease research.

To Clement, thank you for supporting my every endeavour without fail. For better or worse, you have been there. To my family, thank you for being my personal "cheer squad" over the years, for loving me, and always willing to sacrifice to give me the freedom to follow my dreams. I couldn't have made it to the finish line without you.

Isaiah 55: 8 – 9.

Table of Contents

Outline of the Thesis

Chapter one provides a detailed look at Parkinson's disease from both a global and local perspective. In addition, available literature is presented to assess the role of known genetic causes of the disorder, and potential disease mechanisms implicated in PD pathogenesis. Lastly, a detailed outline of the mutation screening techniques is provided, as well as the overall aims and objectives of the present study.

Chapter two is the detailed experimental chapter.

Chapter three provides a summary of all the results from the present study.

Chapter four provides a detailed discussion of all the results including a brief look at the study limitations, including the general conclusions that can be drawn from the entire study, and a short outline of future work which may be carried out to further the genetic knowledge on Black South African PD patients.

CHAPTER 1: LITERATURE REVIEW

1.1 General introduction

Parkinson's disease (PD) was already known for decades in Ayurveda India under the pseudo-name "Kampavata" before being formally documented in a monograph by Dr. James Parkinson in 1817, in which he described six cases of "involuntary tremulous motion with lessened muscular power" (Parkinson, 2002; Kempster et al., 2007). Decades passed before his work was finally recognized by French neurologist Jean Martin Charcot who named the disorder "la maladie de Parkinson" (i.e. Parkinson's disease) (Camacho and Francisco, 2012). Nearly fifty years passed thereafter, before a neurologist by the name of Fritz Heinrich Lewy first described inclusion bodies as one of the hallmark features of PD pathology (Holdorff, 2002). Although these inclusion bodies, often referred to as Lewy bodies (LBs), are regarded as the pathological hallmark for PD, they are not common to all cases nor are they disease-specific, and have been described in a number of other conditions including Lewy body dementia (LBD), multiple system atrophy (MSA), corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP) (Halliday et al., 2011). These other neurodegenerative disorders are often classified as "parkinsonism", or "Parkinson-plus syndromes", which are clinically characterized by the presence of selective PD features, in addition to other symptoms which are not characteristic of the disorder (Nacimiento, 2006). Unless stated otherwise, PD in this text refers either to the sporadic/idiopathic form of the disease, which has no identifiable environmental or genetic cause, or familial PD, which typically has an underlying genetic cause.

1.1.1 Clinical features and disease pathology of PD

The four cardinal symptoms of PD relate to motor dysfunction. Clinically, these symptoms manifest as resting tremor (initially affects muscle movement on one side of the body at disease onset), bradykinesia (i.e. slowness in the initiation of voluntary movement, with progressive reduction in speed and amplitude of repetitive actions), rigidity (due to increased contraction of both agonist and antagonist muscles), and postural reflex impairment which is more common in stages of the disease (Fahn, 2003). Nonmotor symptoms may include depression, sleep disorders, hallucinations and delirium (Amar et al., 2014). Psychiatric symptoms, including anxiety and mood disorders, have been reported in nearly 40% of PD cases, while autonomic dysfunction (e.g. constipation, loss of smell and sleep disorders) are universal to all PD cases (Dissanayaka et al., 2014). Although PD symptoms become progressively more debilitating over time, they are not directly fatal, with

the most frequent cause of death in the majority of reported cases being pneumonia, sepsis, fractures, and falls, respectively (Hely et al., 2008; Pennington et al., 2010).

The neuropathological hallmark of PD is characterized by the depigmentation of specific neuronal populations in the brain, most notably the dopaminergic neurons of the substantia nigra pars compacta (SNpc) of the midbrain (Figure 1.1). Dopaminergic neurons are the main source of dopamine (DA) neurotransmitter in the central nervous system (CNS), with a prominent role in voluntary movement and behavioural processes (Chinta and Andersen, 2005). Dopaminergic cell loss in excess of 50% is required to trigger the onset of PD symptoms (Farrer, 2006). Evidence of dopaminergic loss is often accompanied by the presence of LBs, which are abnormal proteinaceous aggregates (Figure 1.2) enriched in a-synuclein and ubiquitinated proteins (Poulopoulos et al., 2012).

1.1.1.1 *The role of dopamine in PD*

DA is an essential neurotransmitter which plays a key role in controlling voluntary movement, balance, cognition, and neuroendocrine secretion for the normal functioning of the CNS (Salamone and Correa, 2012). Additionally, DA also plays a role in the brain's complex system of motivation and reward (Jaber et al., 1996). Consequently, depletion of DA within the SNpc (Figure 1.3), results in the dysregulation of essential motor circuits, responsible for controlling and maintaining voluntary movement (Gancher, 2002).

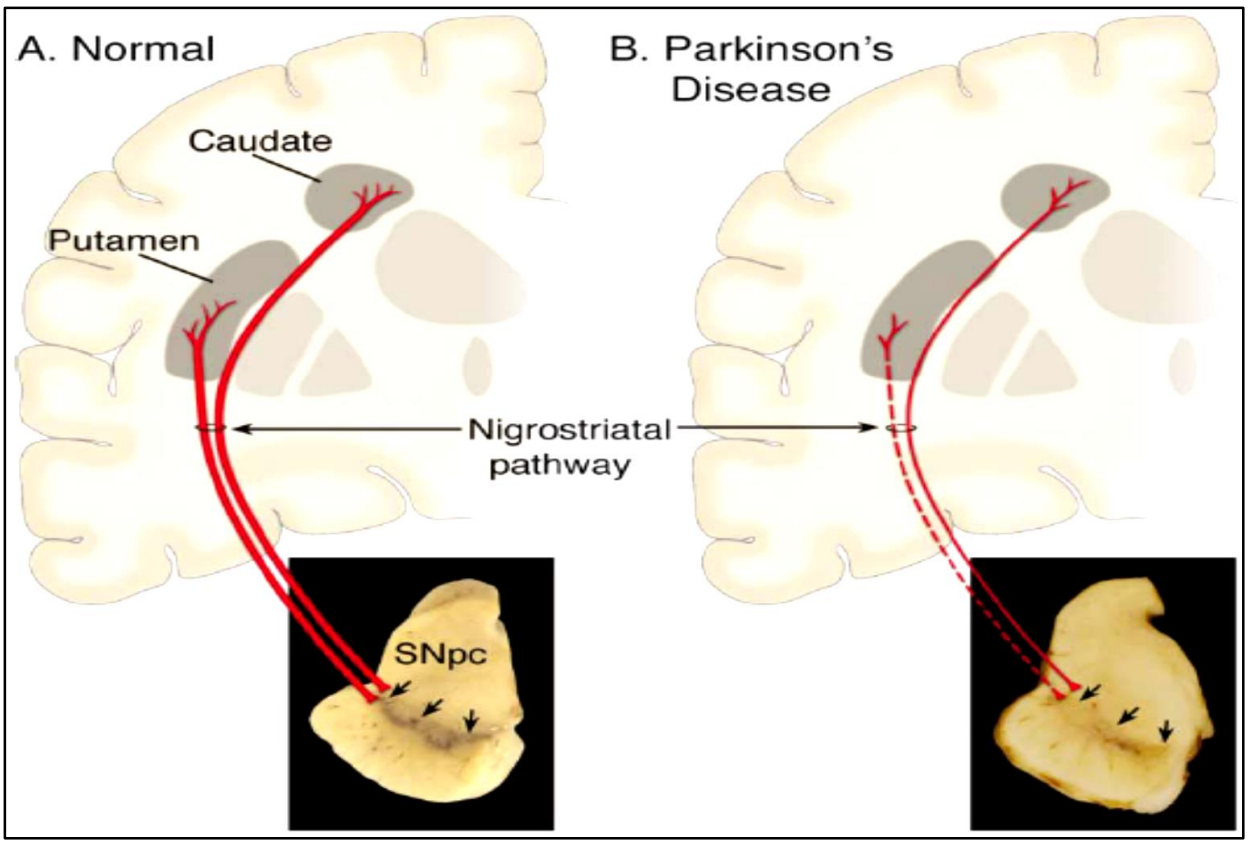


Figure 1.1 Neuropathology of PD. (**A** and **B**) Schematic illustration of the impaired nigrostriatal pathway (shown in red) in a PD patient compared to a healthy control. The PD SNpc shows prominent loss of dopaminergic neurons projecting to the putamen (dashed red line), and a modest loss of those projecting to the caudate (thin red solid line). The schematic illustration also demonstrates depigmentation of the SNpc (i.e. loss of dark-brown pigment - neuromelanin; as indicated by arrows), due to the excessive loss of dopaminergic neurons [taken from (Dauer and Przedborski, 2003)]. The Nigrostriatal pathway is the main circuit in the CNS, involved in the transmission of electrochemical signals which facilitate both voluntary and involuntary movement.

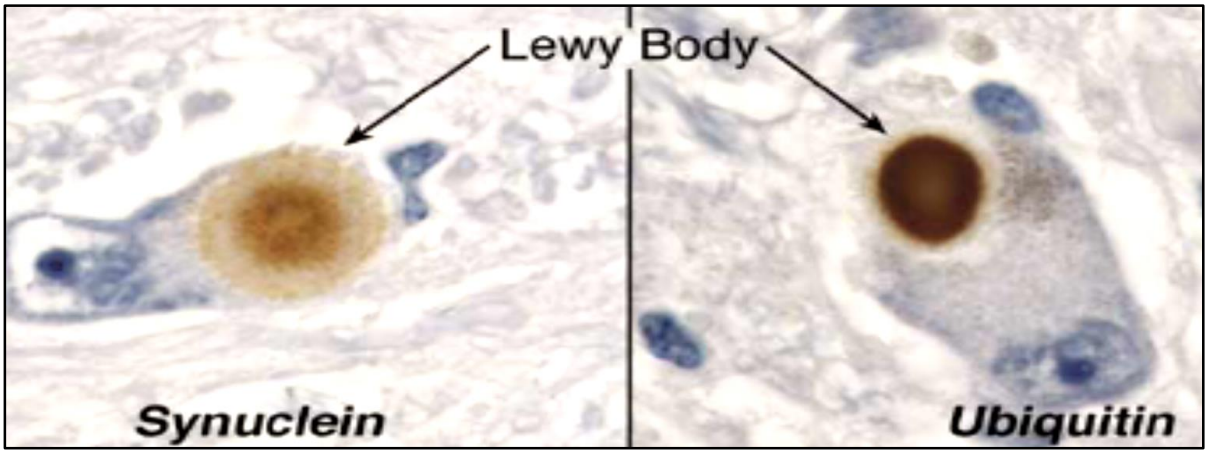


Figure 1.2 Post-mortem immunohistochemical labelling of LBs in human brain tissue from an individual with PD. Protein specific antibodies are used for the detection of α-synuclein and ubiquitin, which form part of LBs [taken from (Dauer and Przedborski, 2003)].

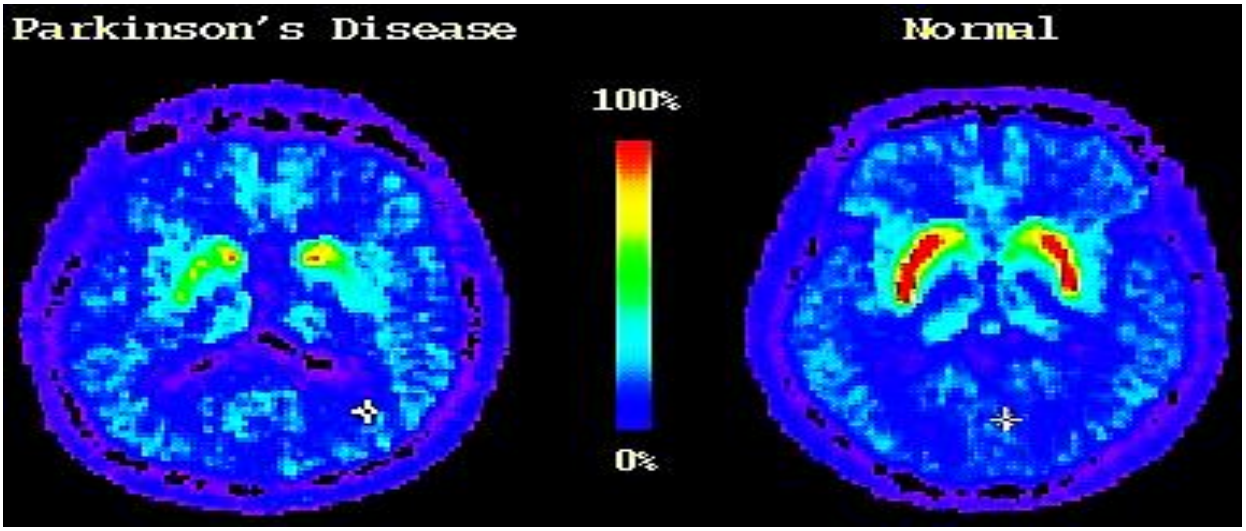


Figure 1.3 DaTSCAN (dopamine transporter) image showing the uptake of ^{18}F-DOPA (Fluoro-dopa-18) in the brain of a healthy volunteer compared to that of an individual with PD. ^{18}F-DOPA is used as a radiotracer which measures DA distribution in the basal ganglia (i.e. the brains 'control centre', includes the SNpc). The bright orange areas in the scan show a robust uptake of fluorodopa, which indicates normal DA distribution and function. Green to blue areas are indicative of significantly less fluorodopa uptake, which indicates a considerable loss of DA receptors, as observed in PD patients [(taken from (Brooks, 2004)].

1.1.2 Treatment of PD

There is currently no treatment to prevent, reverse, or halt the process of neurodegeneration in PD. Two primarily symptomatic approaches are therefore used in the treatment of the disorder. Firstly, a disease modifying approach which slows down disease progression, and secondly, a symptomatic treatment approach with the specific aim of alleviating the more debilitating symptoms of the disease (Freire and Santos, 2010). Available symptomatic treatments currently include medical and surgical options. Anticholinergics are typically used in patients in whom tremor is the prominent symptom, but individual drug response is highly variable. Other medications include monoamine oxidase inhibitors (MAOIs), catechol-O-methyltransferase (COMT) inhibitors, DA agonists, and the more commonly used levodopa (L-DOPA) replacement therapy (Warren et al., 2014). Since its first discovery nearly forty-five years ago, L-DOPA remains the most effective symptomatic PD drug, and forms the backbone of a variety of other treatment options throughout the course of the disease (Barbeau, 1969). L-DOPA alleviates the severity of tremor, rigidity, and mildly improves muscle control and overall balance (Freire and Santos, 2010). However, as the disease progresses, patients often have to contend with a range of adverse side effects as L-DOPA becomes increasingly less effective, with patients often experiencing prolonged 'wearing-off' periods, characterized by increased motor fluctuations, dyskinesia (i.e. uncontrolled spasmodic or repetitive motions), and dystonia (i.e. involuntary muscle contractions) (Marsden, 1994; Thanvi and Lo, 2004). Consequently, surgical treatment is considered to be the best treatment option for patients with advanced PD, or for whom drug therapy is no longer sufficient. Deep brain stimulation (DBS) is the most commonly used surgical treatment, involving the implantation of deep-brain electrodes which send electrical impulses to specific parts of the brain (Wertheimer et al., 2014). Controlled electrical stimulation of selective regions of the brain (e.g. the motor thalamus, or the subthalamic nucleus), is facilitated through the reduction of neural activity in the tissues surrounding the surgically implanted electrodes (Freund, 2005). The motor thalamus is primarily targeted for the alleviation of tremor; while DBS of the subthalamic nucleus is performed for the treatment of bradykinesia, rigidity, and drug-induced dyskinesia (Rodriguez-Oroz et al., 2005). Some of the more prominent disadvantages of DBS include the exorbitant cost of the procedure and the need for repeated adjustment of the stimulation parameters (Obeso et al., 2001). DBS is only recommended for patients with severe motor fluctuations which can no longer be controlled by medication, or those who are intolerant to medication, as long as they do not have severe cognitive impairment (Bronstein et al., 2011).

1.1.3 The incidence and prevalence of PD

1.1.3.1 *Incidence*

Standardized incidence rates for PD, determined from prospective population-based studies, are estimated to be 8 to 18/100,000 person-years, with a 1.5% lifetime risk of developing the disease (de Rijk et al., 1995). Incidence rates for PD across all age groups are reported to range from 1.5-22/100,000 person-years, while studies which are solely restricted to older populations (≥ 60 years of age) report an overall incidence rate of 529/100,000 person-years, with an estimated 59,000 new cases reported each year in the United States alone (Hirtz et al., 2007). Variations in PD incidence across different ethnic groups may provide clues about the aetiology of the disorder, such as differences in environmental exposures, susceptibility alleles, or population specific PD causative genes. However, such comparative studies continue to be hampered by differences in case ascertainment, sample size, the diagnostic criteria used, methodological restrictions, and biased reporting (Brewis et al., 1966; Marttila and Rinne, 1976).

1.1.3.2 *Prevalence*

The prevalence rate of PD in European countries is estimated to be approximately 108 to 257/100,000 persons per year, but this figure varies from country to country. When only older age groups (≥ 60 years) were included in the study, the prevalence rate varied from 1280 to 1500/100,000 persons per year (von Campenhausen et al., 2005). The prevalence of PD in Asian countries is slightly lower, with figures varying from 51.3 to 176.9/100,000 persons per year across all age groups (Muangpaisan et al., 2009). However, the prevalence of PD is thought to be even lower in African countries, with the crude prevalence varying from 7 to 31.4/100,000 persons per year (Okubadejo et al., 2006; Okubadejo, 2008). Moreover, these figures are generally lowest in the east and west African countries, where life expectancy is thought to be the lowest (Richards and Chaudhuri, 1996). In contrast, PD prevalence rates are comparatively higher in north African countries, but still significantly lower than that reported for European countries (McInerney-Leo et al., 2004). However, these estimates are often subjective, and vary considerably due to differences in population structure, the diagnostic criteria used, and a high percentage of underreporting, particularly in African countries which are generally faced with pressing political issues and limited access to health care facilities (Bower and Zenebe, 2005; Pearce and Wilson, 2007). Thus, it is not known whether PD is more common in certain African countries than others, what is certain is that the number of persons 60 years and older is increasing worldwide due to the

current trend of population aging, which is thought to result in an increased prevalence of age-related diseases such as PD (Lee and Mason, 2011). This trend has previously been described in an analysis by Dorsey et al. (2007), who using published PD prevalence studies from Europe's five most populous countries (Germany, France, the United Kingdom, Italy, and Spain) and the ten most populous nations worldwide (China, India, United States, Indonesia, Brazil, Pakistan, Bangladesh, Russia, Nigeria, and Japan), estimated that the number of individuals with PD over age 50 was between 4.1 and 4.6 million in 2005. By 2030, this number is expected to increase by a factor of two, to between 8.7 and 9.3 million people with PD (Dorsey et al., 2007). Results from a similar study by Bach et al. (2011) projected a 1.6 fold increase between 2010 and 2035 in the number of PD affected individuals over the age of 50 years (Figure 1.4), which is comparable with the earlier projections by Dorsey et al. (2007).

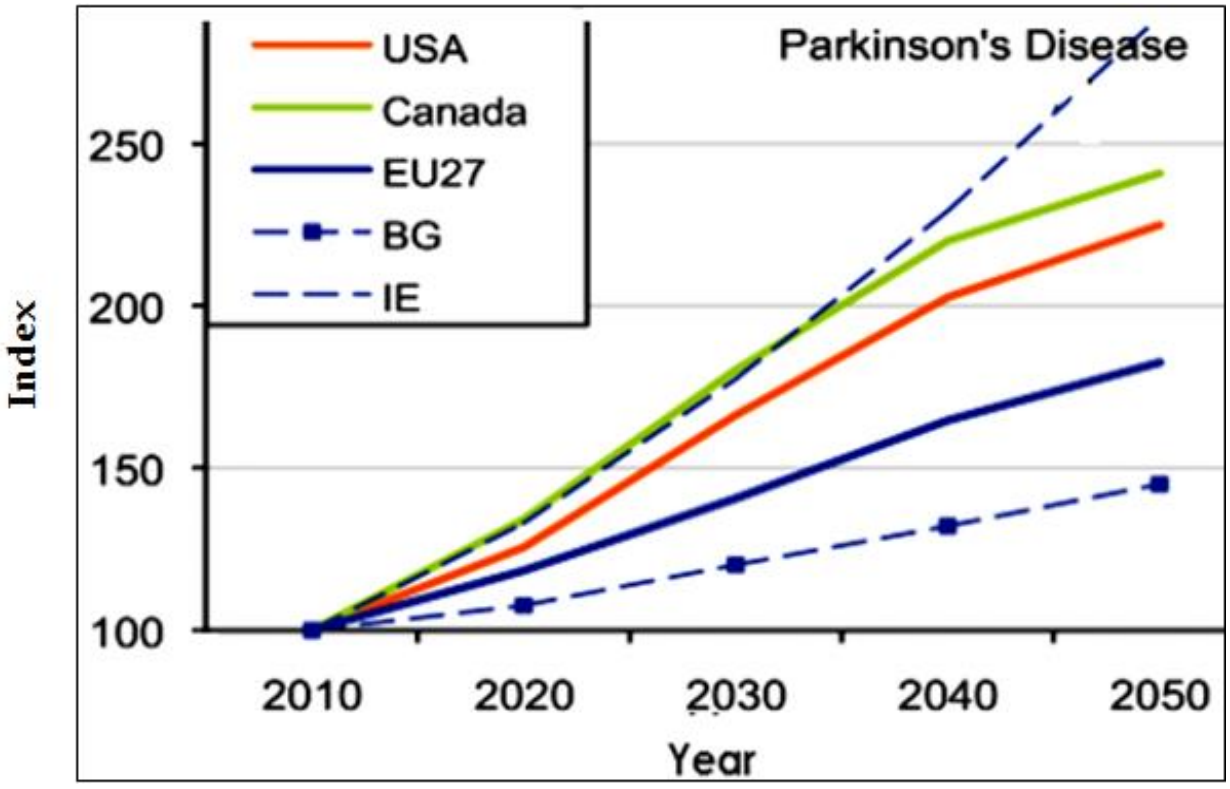


Figure 1.4 Projected increase in the number of individuals over age 50 with PD by country, 2010 through to 2050. 27 European countries (EU37), the United States of America (USA), Bulgaria (BG), Ireland (IE) and Canada are graphically represented [taken from (Bach et al., 2011)].

1.2 The aetiology of PD

The majority of PD cases are thought to arise due to a combination of environmental factors that confer an increased risk for the disease, and genetic factors that may cause or modify disease susceptibility. Over the last decade, the aetiology of PD has shifted considerably towards the genetic hypothesis following the identification of a growing number of PD-causative genes. An estimated 5-10% of PD cases are thought to stem from purely inherited genetic factors, but even these cases where there appears to be a deterministic genetic cause, are often complicated by incomplete penetrance (Vaughan et al., 1998). Incomplete penetrance is evident either when carriers of a disease-causing mutation die before the manifestation of the disease, or if the effect of the mutation is not strong enough to manifest the disease phenotype in all carriers. This observation is thought to be strong evidence of the existence of a complex gene-to-gene-interaction, or a gene-environment interaction which may cause an individual to have an increased susceptibility to certain PD triggers (Sveinbjörnsdottir et al., 2000). In a classic twin study design, concordance rates in monozygotic (i.e. genetically identical) and dizygotic (i.e. share half of their genome) twins are compared; if concordance rates are higher in monozygotic twins, genetic factors are thought to be the primary cause of a disease. Findings from early twin studies showed little concordance in monozygotic twins when PD developed after the age 50 years (Ward et al., 1983; Marttila et al., 1988), but complete concordance was reported for disease onset before the age of 50 years (Tanner et al., 1999). These findings suggest that genetic factors may be responsible for a significant proportion of early-onset cases (≤ 50 years), and less so for late-onset PD cases (≥ 60 years).

1.2.1 Demographic, environmental and lifestyle risk factors for PD

1.2.1.1 *Gender and increasing age*

Increasing age serves as one of the strongest risk factors for the majority of neurodegenerative diseases, including PD (Benito-León et al., 2003). Work by Bower et al. (1999) studied the age and gender specific incidence of PD in Olmstead County, where they found that the incidence of PD in men 60 years and older, to be 50% higher than that reported for women. Van Den Eeden and colleagues (2003) reported a similar finding in the Kaiser Permanente population in the mid-1990s. They reported an incidence rate of 120 in 100,000 men between 70 and 80 years of age, while an incidence rate of 80 in 100,000 was reported for women in the same age bracket.

The loss of selective female hormones, as a consequence of increasing age, is also thought to be a risk factor for PD. One such example is the loss of estrogen production in elderly women (60 years and older). Estrogen is thought to confer a neuroprotective effect, and there is strong evidence to suggest that early menopause, a hysterectomy or removal of the ovaries may increase the risk for PD (Popat et al., 2005). Studies in a small clinical trial consisting of postmenopausal women with PD, showed significant improvement of motor function in patients who received estrogen replacement therapy, compared to those who had not (Tsang et. al, 2000). Estrogen is therefore thought to confer a neuroprotective role against PD, but the mechanism of this proposed effect is still unclear.

Interestingly, α-synuclein protein aggregation (the major component of LBs), has been shown to increase significantly with age in the SN, thereby facilitating the loss of dopaminergic neurons (Collier et al., 2011). Furthermore, accumulation of reactive oxygen species (ROS) is thought to be a common feature associated with the ageing brain, and is partially attributed to mitochondrial dysfunction due to decreased mitophagy (i.e. degradation of damaged/dysfunctional mitochondria) with ageing (Mammucari and Rizzuto, 2010; Green et al., 2011).

1.2.1.2 *Occupational toxin exposure*

Evidence in support of the environmental hypothesis, stems from the ground-breaking observations by Langston et al (1983) of a group of drug addicts in California who developed an acute parkinsonian syndrome which led to the identification of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP). MPTP is a contaminant of a synthetic opiate that may cause parkinsonism through its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) (Langston et al., 1983). Further investigation of this toxin later revealed that MPTP is metabolized to MPP+, which is subsequently transported by DA transporters into dopaminergic neurons where it ultimately inhibits mitochondrial function with long term exposure, and mimics the major clinical features of PD (Langston and Irwin, 1986). Apart from the young age of disease onset, and the associated pathology showing evidence of nigral cell loss without LB pathology, this form of parkinsonism was phenotypically similar to that described for sporadic PD cases, with good response to L-DOPA therapy (Langston and Irwin, 1986). The MPTP scenario was proof that long term exposure to an environmental agent with similar properties, or mechanisms as MPP+, may significantly alter dopaminergic neurotransmission in the SNpc, and could potentially result in movement abnormalities with strong resemblance to PD. Setting aside this earlier report of the few individuals

with MPTP-induced parkinsonism, MPTP is extremely rare in the environment, and is therefore regarded as a negligible cause of PD (McCormack et al., 2002).

Selective occupations are considered to be strong risk factors for PD. In general, farmers and those with agricultural or rural occupations are thought to have a higher risk of developing PD, possibly due to long term exposure to selective pesticides, or other environmental factors (Greenamyre and Hastings, 2004; Hancock et al., 2008). Studies by Tanner and colleagues suggests a 3-fold increased risk of PD with long-term occupational exposure to paraquat and rotenone (Tanner et al., 2011). Paraquat is a widely used broad-spectrum herbicide, which is thought to have neurotoxic properties due to its structural similarity to the active metabolite of MPTP (i.e. MPP^+) (Wesseling et al., 2001). Paraquat is also known to generate ROS by redox cycling; a mechanism which has been shown to induce dopaminergic damage *in vivo* (Fishellett et al., 1974). Additionally, systemic injection of paraquat has been shown to elicit a dose-dependent decrease in voluntary movement, and DA cell count in the SNpc of mice models (Brooks et al., 1999; McCormack et al., 2002).

Similar to paraquat, long-term exposure to rotenone is also thought to have an equally destructive effect on dopaminergic neurons as paraquat. Rotenone is a naturally occurring compound that is found in the roots and leaves of several plant species, and is extensively used as an insecticide (Cannon and Greenamyre, 2011). Rotenone has previously been shown to be a selective inhibitor of mitochondrial complex I, which is interestingly one of the mechanisms implicated in PD pathogenesis (Ravanel et al., 1984; Parker et al., 1989).

Mining and welding are two other frequently discussed occupations, which generally involve long-term exposure to high levels of various metals (e.g. zinc, copper, lead, and manganese). The impact of prolonged metal exposure is evident from *in vivo* brain imaging, which reveal a consistent increase in iron concentration in selective regions of the brain, where it is thought to contribute to process of neurodegeneration (Gerlach et al., 2006; Oakley et al., 2007). High levels of manganese (Mn) exposure has also been linked to striatal DA depletion, extrapyramidal dysfunction, and parkinsonism (Khalid et al., 2011). Additionally, Mn is thought to be a putative modulator of DA release, with a neurotoxic effect on mitochondrial function leading to the impairment of cellular energy metabolism (Schapira, 2007).

How, and if environmental factors interact with genetic factors to trigger the onset of PD, remains unclear. Although numerous studies have reported a positive association between selective

pesticide exposure and PD, a positive association has not been consistently made for a single agent/toxin to date. The general conclusion is that outside of a few rare cases, it is highly unlikely that exposure to a single environmental agent on its own, may account for the significant number of sporadic PD cases

1.2.1.3 *Dose-dependent cigarette smoking, and coffee consumption*

Despite the numerous adverse health effects associated with cigarette smoking, a growing number of studies suggests a reduced risk of PD in long term smokers (Hernán et al., 2002; Demers et al., 2014). In a study by Quik et al. (2006), laboratory animals were treated with a compound known to destroy dopaminergic neurons of the SNpc, with half of these animals having received nicotine in their water supply. The researchers found that the levels of DA transmission were greater in the nicotine-treated animals than in the controls. Another study assessed the role of nicotine in transgenic rats with movement abnormalities similar to that observed in PD patients (Bordia et al., 2010). Systemic dose-dependent nicotine injection was shown to significantly improve the voluntary movements in these rats, but the mechanisms underlying this association remain unclear. However, a number of theories have been proposed to explain the putative neuroprotective effect of smoking, the most likely of which involves the ability of nicotine to inhibit *monoamine oxidase B* (MAO-B), and stimulate acetylcholine receptors, which is thought to have a role in DA release (Fowler et al., 1996; Quik and Kulak, 2002). However, given that more than 4000 components make up cigarettes, no single agent/substance can be conclusively isolated as the neuroprotective agent responsible for this effect (Demers et al., 2014). It should also be noted that the number of years spent smoking, rather than the number of cigarettes smoked per day, is associated with a reduced risk of PD (Kachergus et al., 2005).

Similarly, a dosage-dependent reduced risk of PD has been suggested for coffee consumption (Hernán et al., 2002). Case-control studies have consistently reported this correlation in several follow-up studies (Paganini-Hill, 2001; Qi and Li, 2013). The proposed mechanism by which caffeine confers this effect has been demonstrated in numerous animal studies, in which dose-dependent caffeine injection was shown to mildly improve motor deficits in transgenic mice, possibly via the inhibition of the adenosine A_2 receptor which is thought to moderately alleviate the severity of selective PD motor deficits (Morelli and Wardas, 2001; Xu et al., 2005).

Despite the increasing number of environmental factors that have been investigated for their potential role in PD, only a positive family history, advancing age, dose-dependent caffeine consumption, and long-term smoking have provided consistent results of a strong association with risk for PD.

1.3 The genetic aetiology of PD

Despite extensive progress over the last decade, the aetiology of PD remains poorly understood. Without a conclusive answer as to what causes the disease, it may prove difficult to uncover novel drug targets or develop more effective drug treatments for the disorder. Although the majority of reported PD case are of sporadic origin (~90%), the last decade has resulted in a growing body of literature in support of the genetic hypothesis for PD. It is mainly through the study of rare large families with clear Mendelian inheritance, that several causative genes have been identified, proving that the contribution of genetics to PD may be greater than previously thought. A summary of the most established genes is outlined in Table 1.1.

Table 1.1 List of genes directly implicated in familial PD [taken from (Trinh and Farrer, 2013)].

Gene	Locus	AAO (years)	Mutations identified	Protein	Protein Function
Dominantly inherited genes associated with late-onset PD					
SNCA	PARK1/4	30–80	A30P; E46K; A53T; gene duplications and triplications	a-synuclein	Plays a role in synaptic vesicle recycling and promotes exocytosis
LRRK2	PARK8	32–79	7 point mutations	leucine rich repeat kinase 2	Plays a role in chaperone-mediated autophagy, cellular and protein interactions and cell signalling
VPS35	PARK17	40–68	Missense: D620N	vacuolar protein sorting 35	Mediates cargo recognition of early endosomes and membrane recruitment
EIF4G1	PARK18	50-80	Missense: R1205H	eukaryotic translation initiation factor 4 gamma 1	mRNA translation processes
Recessively inherited juvenile genes associated with early-onset PD					
parkin	PARK2	12-58	Various point mutations; exonic rearrangements	Parkin	Ubiquitination and proteasomal function
PINK1	PARK6	18-56	Various point mutations; rare, large deletions	PTEN putative induced kinase	involved in mitochondrial maintenance
DJ – 1	PARK7	24–39	Point mutations; large deletions	Oncogene DJ-1	involved in mitochondrial maintenance and protection during oxidative stress
ATP13A2	PARK9	10–33	Point mutations	P5 subfamily of ATPase's	Plays a role in lysosome-mediated autophagy

AAO = Age at onset

1.3.1 Inheritance patterns

1.3.1.1 *Autosomal dominant PD*

Autosomal dominant (AD) disorders generally present with a clinical and pathological phenotype similar to that observed in sporadic PD cases (Carr et al., 2003). Dominant inheritance is generally characterized by the presence of one mutation, on one allele (i.e. one gene copy inherited from either parents - heterozygous) is sufficient to cause disease. Additionally, at least one carrier of the disease-causing allele must be present in every successive generation, and the disease-causing allele must be transmitted to at least 50% of all the offspring of an affected parent (Klein and Schlossmacher, 2006).

1.3.1.2 *Autosomal recessive PD*

Autosomal recessive (AR) disorders are generally defined by the following: (1) two identical mutations [i.e. one on each allele, inherited from both parents, (homozygous)], or two different mutations [i.e. one on each allele, inherited from both parents, (compound-heterozygous)] are required to cause disease and (2); the presence of several affected members in one generation, with clinically asymptomatic parents and (3), the offspring of affected parents have a 25% risk of inheriting the disease-causing alleles. Lastly, patients with ARPD generally present with an earlier age of disease onset, and often demonstrate slower disease progression (Klein et al., 2007). It should be noted that heterozygous mutations may also result in disease. The most common pathogenic mechanism for heterozygous loss-of-function mutations is thought to be haploinsufficiency (i.e. wherein a single functional copy of a gene does not produce enough protein to bring about a wild-type condition) (Huang et al. 2010). Variable penetrance of heterozygous mutations is thought to be due to environmental factors, epigenetic effects, genetic modifiers or a combination of the three (Klein et al., 2007).

An estimated 5–10% of individuals with PD are classified as familial patients because they have been shown to carry heritable, disease-associated mutations in a series of genes referred to as the *PARK* genes (Lesage and Brice, 2009). To date, eight *PARK* genes have been identified which encode: *SNCA* (a-synuclein - *PARK*1/4), *LRRK2* (leucine-rich repeat kinase 2 - *PARK*8), *VPS35* (vacuolar protein sorting 35 - PARK17), and *EIF4G1* (eukaryotic translation initiation factor 4-gamma 1 - PARK18), all of which show a dominant inheritance pattern. The remaining four genes are associated with a recessive inheritance pattern and encode: *parkin* (*PARK*2), *PINK1* (PTEN-

Induced Kinase 1 - *PARK*6), oncogene *DJ-1* (*PARK*7), and *ATP13A2* (ATPase type 13A2 - *PARK*9) (Spatola and Wider, 2014). These genes will be discussed in more detail in the following sections.

1.3.2 PD-causing genes

1.3.2.1 *SNCA / PARK1/4*

A key discovery in the elucidation of familial PD genes was the identification of an A53T mutation in *SNCA,* first identified in a large Italian pedigree with ADPD (Polymeropoulos et al., 1996). This was the first real evidence that a single gene mutation could cause PD, and this same mutation was subsequently identified in five unrelated Greek families (Golbe et al., 1996). Since then, four additional mutations, namely: A30P, E46K, H50Q, and G51D have been discovered (Krüger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Lesage et al., 2013). Mutations in SNCA are extremely rare, with even fewer reports of gene duplications, or triplications in families with *SNCA* mutations (Singleton et al., 2003; Fuchs et al., 2007).

Only the clinical phenotypes of the A30P, E46K, and A53T mutation have been extensively studied. A30P and E46K are associated with late disease onset (ranging from 54–76 years) with mild dementia and visual hallucinations, respectively (Kruger et al., 2001; Zarranz et al., 2004). Carriers of the A53T mutation have the earliest-disease onset (ranging from 36–58 years) leading to cognitive decline and dementia (Athanassiadou et al., 1999). The clinical phenotype associated with *SNCA* multiplications depends on dose: triplication carriers (4 copies) develop typical PD features with severe dementia, whereas duplication carriers (3 copies) may manifest a variety of Lewy body spectrum disorders (e.g. Dementia with Lewy bodies) (Ross et al., 2008).

Alpha-synuclein (aSYN, *SNCA* protein) is unique in that it contains a highly hydrophobic central region containing a non-amyloid-β component, and has a tendency to self-aggregate and form protofibrils (Giasson et al., 2001). This tendency to self-aggregate is what makes aSYN important to PD pathology, as this protein is the main component of LBs (Kubo et al., 2005). Structurally, aSYN consists of three domains (Appendix A. Figure 1.5), and is highly expressed in the presynaptic nerve terminals, which may suggest a role in synaptic transmission (Kahle et al., 2000; Nuscher et al., 2004).

1.3.2.2 *LRRK2 / PARK8*

In 2004, two independent groups simultaneously reported the discovery of mutations in *LRRK2* as the second causal link to ADPD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). Six mutations were originally found in numerous families worldwide, these include R1441C, R1441G, R1441H, Y1699C, N1437H, and the I2020T mutation (Funayama et al., 2002; Paisán-Ruíz et al., 2004; Zimprich et al., 2004). With the exception of the R1441G mutation which was found in more than 8% of familial PD patients from the Basque population(González-Fernández et al., 2007), all the other mutations mentioned above are rare. By contrast, the LRRK2 G2019S mutation is the most common PD-associated mutation (Di Fonzo et al., 2005), reported in at least three different founder populations (Klein and Lohmann-Hedrich, 2007). A founder population is generally established by a small number of people with limited genetic variation. As the population grows, any mutations harboured by this population become increasingly prevalent (Neuhausen, 2000). The G2019S mutation has been reported in approximately 41% of sporadic and 37% of familial PD patients from the North African Arab population, in 18.3% of Ashkenazi Jewish PD patients (and 1.3% of controls) (Lesage et al., 2006, Ozelius et al., 2006), and in 1-7% of familial PD populations of European descent (Zabetian et al., 2006). However, it should be noted that G2019S is relatively rare, or completely absent outside of the above mentioned founder populations (Guedes et al., 2010). In addition, G2019S has been shown to have age dependent, incomplete penetrance as revealed by reports of a healthy 91 and 95 year old carrier of the mutation (Gaig et al., 2006; Carmine et al., 2006).

A broad phenotypic spectrum has been reported per LRRK2 mutation, as the clinical phenotype can be more or less severe and the age at onset (AAO) may vary depending on the mutation, albeit with a large overlap (Haugarvoll et al., 2008). Generally, patients present with late disease onset, show good response to L-DOPA therapy, with additional features including amyotrophy and severe dystonia (Hasegawa and Kowa, 1997). Similarly, the neuropathology of patients with *LRRK2* mutations is highly variable. For example, neuropathological findings from 49 patients revealed a-synuclein positive pathology, tau pathology, and the more common LB pathology (Wider et al., 2010).

LRRK2 consists of seven functional domains (Appendix A. Figure 1.6), and is thought to play a role in the regulation of signal transduction cascades through its dual enzymatic activities (i.e. GTPase and Kinase activity) (Cardona et al., 2014).

1.3.2.3 *VPS35 / PARK17*

The D620N mutation in *VPS35* is a more recently identified cause of PD. This mutation was concurrently described in a Swedish PD family by Vilariño-Güell et al. (2011), and in three Austrian families by Zimprich et al. (2011). In addition, D620N has also been shown to be the cause of late-onset ADPD in at least 13 unrelated families of largely Caucasian, or Japanese descent (Ando et al., 2012; Lesage et al., 2012).

The phenotype of these families was similar to that observed in sporadic PD cases, with learning disabilities, dementia and psychosis being described as part of the clinical profile of the original Swiss family (Vilariño-Güell et al., 2011; Zimprich et al., 2011). The average AAO for carriers of the *VPS35* D620N mutation is 52 years (ranging from 37–72 years), with age dependent incomplete penetrance (Zimprich et al., 2011).

VPS35 is part of the retromer complex that is involved in the transport of endosomes back to the trans-Golgi, and in the sorting of receptors for hydrolases, thus affecting vacuole/lysosomal function and biogenesis (Derivery et al., 2009; Harbour et al., 2010).

1.3.2.4 *EIF4G1 / PARK18*

In 2011, Chartier-Harlin and colleagues reported five sequence variants with a possible role PD in *EIF4G1* using genome-wide linkage analysis. However, of these sequence variants, namely: A502V, G686C, S1164R, R1197W and R1205H, only the latter has been shown to be pathogenic (Chartier-Harlin et al., 2011). The co-segregation of *the* R1205H mutation was first described in a French family with late disease onset, and LB pathology. However, several unaffected carriers of this mutation have recently been described, possibly due to incomplete penetrance (Schulte et al., 2012; Nuytemans et al., 2013).

Functionally, EIF4G1 has been implicated in mRNA translation processes (the final step in protein translation) (Chartier-Harlin et al., 2011; Schulte et al., 2012). Based on the small number of patients described so far, the clinical phenotype of R1205H carriers has been described as mild PD with disease onset ranging from 50 to 80 years of age, with diffuse LB pathology (Chartier-Harlin et al., 2011). Similar to the D620N mutation in *VPS35,* the frequency of the R1205H mutation is extremely low, with estimates of 0.02–0.2% of the PD population (Tucci et al., 2012). Further studies are therefore warranted to ascertain the true contribution of this mutation to PD pathogenesis.

1.3.2.5 *Parkin / PARK2*

The most frequently encountered form of ARPD is associated with mutations in *parkin*, encoding an E3 ubiquitin ligase protein. The identification of *parkin* was facilitated by the discovery of a microdeletion in a family with AR juvenile-onset (≤20 years of age) PD (Matsumine et al., 1997). Since the initial discovery, many research groups have identified more than 150 different mutations throughout the genomic region of *parkin, and* these include exonic deletions, insertions, and missense mutations.

Parkin-linked ARPD is characterized by an early AAO (≤50 years), good response to L-DOPA treatment, and L-DOPA-induced dyskinesia with disease progression (Lücking et al., 2000). While carriers of *parkin* mutations present with typical PD features, the presence of LBs has only been reported in a few cases to date (Farrer et al., 2001; Khan et al., 2003). However, there have been reports of LB or tau pathology in carriers of compound-heterozygote mutations (van de Warrenburg et al., 2001; Pramstaller et al., 2005).

Parkin consists of five functional domains (Appendix A. Figure 1.7), and is prominently expressed in both neuronal and glial cells (Shimura et al., 2000). In addition, parkin acts as a substrate-recognition molecule during ubiquitination in the ubiquitin proteasomal system due to its function as an E3 ubiquitin-ligase (Moore, 2006). Loss-of-function mutations in *parkin*, are therefore thought to reduce or abolish ligase activity, and may also facilitate the neurotoxic accumulation of misfolded proteins (Shimura et al., 2000).

1.3.2.6 *PINK1 / PARK6*

Mutations in *PTEN induced kinase 1* (*PINK1*), were first identified in a large consanguineous family from Sicily (Valente et al., 2001), and subsequently in several European families (Valente et al., 2002). Mutations in *PINK1* have been identified in numerous families worldwide, including a variety of missense and nonsense splice mutations (Valente et al., 2001. Estimates of prevalence from European and Asian populations, suggest that *PINK1* mutations are the second most frequent cause of ARPD, with over 40 point mutations and rare instances of large deletions identified to date (Lesage and Brice, 2009).

The clinical phenotype associated with *PINK1* mutations is similar to that observed in patients with *parkin*-related PD. This includes slow disease progression, good response to L-DOPA, and frequent

L-DOPA-induced dyskinesia (Hatano et al., 2004). Additional features may also include sleep benefit, rest dystonia, and psychiatric problems (Hatano et al., 2004; Ibáñez et al., 2006). The first post-mortem report of a PD patient harbouring a *PINK1*compound-heterozygous mutation presented with neuronal loss in the SNpc, LBs formation and aberrant neurites in the reticular nuclei of the brainstem (Samaranch et al., 2010).

PINK1 consists of three domains (one of which is putative) (Appendix A. Figure 1.8), and acts as a serine/threonine kinase which belongs to the Ca^{2+}/calmodulin family (Valente et al., 2001). PINK1 is ubiquitously expressed in the human brain, with prominent localisation in the inner mitochondrial membrane, and is thought to regulate mitochondrial morphology (Silvestri et al., 2005; Rothfuss et al., 2009). Moreover, PINK1 acts upstream of parkin in a common pathway which is thought to maintain mitochondrial function (Kawajiri et al., 2011).

1.3.2.7 *DJ-1 / PARK7*

Using an RT-PCR strategy, Bonifati and colleagues identified a deletion in exons 1 to 5 of *DJ-1* that showed complete cosegregation with the disease allele in a large consanguineous Dutch family with ARPD (Bonifati et al., 2002). The linkage of the PARK7 locus to chromosome 1p36 was subsequently confirmed in another consanguineous family of Italian descent (Bonifati et al., 2003). The DJ-1 protein was absent in the Dutch family, while the protein appeared to be inactive in the Italian family, thus suggesting that mutations in *DJ-1* may cause PD through a loss of function mechanism. Since the initial discovery, *DJ-1* missense, truncating, splice site mutations, and several deletions have been described in different populations worldwide (Abou-Sleiman et al., 2003).

Similar to *parkin and PINK1*-related PD, the clinical phenotype associated with mutations in *DJ-1* includes an early AAO, slow disease progression, and good L-DOPA response (Dekker et al., 2003). However, the frequency of *DJ-1* mutations is significantly lower than that described for *parkin* or *PINK1* mutations (Kumazawa et al., 2008).

DJ-1 consists of a single functional domain (Appendix A. Figure 1.9), consisting of seven exons. DJ-1 is largely cytoplasmic with prominent localization in the mitochondria, and is thought to play a role in protection of dopaminergic neurons in the presence of oxidative stress (Canet-Avilés et al., 2004; Chen et al., 2005).

1.3.2.8 *ATP13A2 / PARK9*

Mutations in *ATP13A2* were first described in a single consanguineous family of Chilean descent, and are associated with a clinical syndrome called Kufor-Rakeb Syndrome (KRS) (Ramirez et al., 2006). A number of pathogenic mutations have been described in both the homozygous and compound-heterozygous state, resulting in truncated forms of the wild-type protein (Ramirez et al., 2006; Ugolino et al., 2011). Mutant ATP13A2 *is* thought to result in the impairment of proteasomal pathways, and the premature degradation of the mutant ATP13A2 mRNA may also contribute to the variable aetiology of KRS (Park et al., 2011; Dehay et al., 2012).

KRS has a broad spectrum of clinical features including widespread neurodegeneration, dementia, dystonia, with early-onset, L-DOPA-responsive parkinsonism (Ramirez et al., 2006). Additional features of pyramidal degeneration include cognitive dysfunction, and generalized brain atrophy (Behrens et al., 2010). There is considerable phenotypic variability even amongst family members, with rare cases of ataxia (Eiberg et al., 2012).

ATP13A2 consists of 10 transmembrane domains (Appendix A. Figure 1.10), is located in lysosomal membranes and highly expressed in the mammalian brain (Ramirez et al., 2006). ATP13A2 is thought to be responsible for cation transport and the regulation of Mn levels, accumulation of which has previously been shown to be a strong risk factor for neurodegeneration (Schultheis et al., 2004).

1.3.3 Genetic susceptibility alleles

Apart from the above discussed PD-causing genes, candidate gene association studies have also implicated a number of other genes thought to confer an increased risk for PD (UK Parkinson's Disease Consortium et al., 2011). Traditional association studies have explored disease risk associated with allelic variants in *SNCA* and *LRRK2*. The *SNCA* promoter region REP-1 polymorphism is associated with a 1.4-fold increased risk of familial PD (Mueller et al., 2005), while two common variants in *LRRK2* (G2385R and R1628P) appear to increase the risk of both sporadic and familial PD by nearly two-fold, particularly in Asian populations (Bonifati, 2007).

One of the more notable PD risk factors, are dominant mutations in *MAPT* (encoding the microtubule-associated protein tau) (Halliday et al., 2011). Meta-analyses studies have shown that

the H1 haplotype at the *MAPT* locus on chromosome 17q21.31, confers a 1.57-fold increased risk of PD (Wider et al., 2010).

Given the presence of parkinsonism and LB pathology in patients with the AR lysosomal storage disorder Gaucher's disease (GD), PD patients are routinely screened for mutations in *glucocerebrosidase* (*GBA*). *GBA* is a lysosomal enzyme that hydrolyses glucocerebroside, thereby releasing ceramide which acts as a second messenger during signal transduction (Tayebi et al., 2003). Homozygous or compound heterozygous mutations in *GBA* cause GD, while heterozygous mutations have been found to increase the risk of PD (Sidransky et al., 2009). The largest sequencing study of *GBA* in a French population by Lesage et al. (2011) definitely confirms that heterozygous *GBA* mutations may increase risk of PD by more than 5-fold, thus making *GBA* one of the strongest risk factors for the disorder.

The list of genetic risk factors identified to date is continuously increasing and currently includes over 20 independent loci. Interestingly, some studies suggest that the risk for PD increases with the number of risk alleles carried by a single individual, which could be indicative of an additive effect (Hill-Burns et al., 2011).

1.3.4 PD and Spinocerebellar ataxias

Among the list of Mendelian mutations that have been causally related to PD, spinocerebellar ataxias (SCAs) are one of the most interesting group of polyglutamine disorders caused by a CAG repeat expansion which codes for an elongated polyglutamine tract within the respective SCA proteins. SCAs are a heterogeneous group of autosomal dominantly inherited ataxia (i.e. lack of movement) disorder which belong to a larger group of polyglutamine disorders including Huntington's disease, and Spinobulbar muscular atrophy (Rüb et al., 2013). SCAs are characterized by an earlier AAO, and increasing severity of clinical phenotypes in subsequent generations (a phenomenon known as anticipation) that correlates with larger repeat lengths (Schöls et al., 2004). Some of the more prominent features of SCA disorders include: muscle weakness, loss of tendon reflexes, slurring of speech, involuntary eye movements with associated symptoms of cardiomyopathy, scoliosis, diabetes mellitus, and cerebellar ataxia (Zoghbi, 1996). SCA consists of thirty-two different gene loci (*SCA1* to *SCA32*), with the most common of which being *SCA1*, 2, 3, and 7 (Schöls et al., 2004). The general pathological hallmark of SCAs is characterized by progressive neuronal loss and gliosis which primarily affects the cerebellum and related structures in the CNS (Schöls et al., 2004; Klockgether, 2011).

For the purposes of the present study, particular emphasis was placed on the study of CAG repeat expansions at the *SCA2* and *SCA7* loci.

The *SCA2* locus, first described by Gispert et al. (1993) in a Cuban population, is the most frequent SCA subtype in various populations worldwide (Cancel et al. 1997). The genetic basis for the disease is a CAG repeat expansion above a certain threshold in exon 1 of *SCA2* (Pulst et al., 1996). In normal individuals the CAG repeat is polymorphic in length, ranging from 14 to 30 repeats; repeats ranging between 30 and 34 are considered to be 'intermediate' (i.e. do not cause the disease phenotype), while the pathogenic *SCA2* repeats consists of a pure, continuous stretch of 34 to 59 CAGs (Giunti et al., 1998). Interestingly, *SCA2* patients have been known to present with a clinical phenotype suggestive of pure parkinsonian without other neurological signs (Gwinn-Hardy et al., 2000; Ragothaman et al., 2004). It therefore remains a challenge to correctly diagnosis *SCA2* patients as the disease has wide phenotypic overlap with various disorders, including PD (Furtado et al., 2004; Wilkins et al., 2004).

The expression of ataxin-2, the *SCA2* protein product, has been previously described in the dopaminergic neurons of the SNpc (Huynh et al., 2007). Additionally, *parkin*, the most common cause of ARPD, has been shown to interact with ataxin-2. This interaction has been previously described in *SCA2* siblings who were shown to exhibit neuronal loss in the SN, with prominent evidence of intranuclear ubiquitin and ataxin-2 associated inclusions (Armstrong et al., 2005; Huynh et al., 2007). Furthermore, *parkin* knockout mice studies by Huynh et al. (2007) have demonstrated that variation in endogenous parkin levels alters the steady-state levels of endogenous ataxin-2, and decreases ataxin-2 induced cytotoxicity in HEK293 cells (Huynh et al., 2007).

Spinocerebellar ataxia type 7 (*SCA7*) is characterized by prominent language impairment, and progressive loss of vision (Rüb et al., 2013). The underlying genetic cause is an expanded CAG repeat at the 5´ end of *SCA7*, which has been shown to co-segregate with the disease in several families (Stevanin et al., 2000). *SCA7* CAG repeat alleles can be divided into the following categories: normal (<19 repeats), intermediate (28 to 33 repeats), reduced penetrance (34 to 35 repeats), and pathogenic expansion (>36 repeats) (Sequeiros et al., 2010).

Bryer and colleagues first described *SCA7* as the second most prevalent SCA subtype, preceded only by *SCA1*, in the South African (SA) population (Bryer et al., 2003). Unlike the *SCA1*, which is the most frequent type of SCA in the SA Coloured, White and Indian populations, *SCA7* occurs

exclusively in individuals of Black African ancestry, due to a common founder haplotype in this population group (Smith et al., 2012). Studies do indeed suggest that Black SA *SCA7* positive patients are descended from a common ancestor. This is based on the observation that more than 50% of these patients are heterozygous for a common single nucleotide polymorphisms (SNP) (rs3774729) in the 3' region of *SCA7* (Greenberg et al., 2006; Scholefield et al., 2009). SNP's are single-nucleotide substitutions of one base for another that occur in more than one percent of the general population, and serve as the most common type of genetic variation among people (Wu and Jiang, 2013).

A recent report by Alves and colleague suggest that the autophagy lysosome pathway (ALP) may be impaired by the selective accumulation of mutant ataxin-7 in the cerebellum and cerebral cortex of *SCA7* patients (Alves et al., 2014). Apart from the ubiquitin-proteasome system (UPS) which is mainly involved in the breakdown of short-lived proteins, the ALP represents the major mechanism by which intracellular long-lived proteins, and cytoplasmic organelles can be degraded (Rubinsztein, 2006). Formation of mutant ataxin-7 aggregates is thought to lead to the production of toxic protein fragments, and impairment of the ALP due to excess protein aggregation (Alves et al., 2014). The removal of ataxin-7 protein aggregates, through the activation of the autophagy pathway has been shown to interfere with the potential toxicity of these aggregates, and thereby alleviate disease progression in mouse models of the disease (Ravikumar et al., 2004; Tanaka et al., 2004).

1.4 Potential mechanisms involved in PD pathogenesis

The study of rare, inherited genes underlying familial forms of PD has provided insight into the molecular mechanisms of disease pathogenesis. Mutations in these genes have been functionally linked to several molecular pathways implicated in neuronal apoptosis in several neurodegenerative disorders, including PD (Figure 1.11). However, only two clearly defined disease mechanisms, namely: mitochondrial dysfunction and impairment of the protein degradation pathways (consisting of the UPS, and the ALP) will be discussed in the following sections, with an emphasis on their normal function, and how their dysfunction/impairment may contribute to disease pathogenesis.

1.4.1 Mitochondrial dysfunction and oxidative stress

A number of proteins associated with familial PD have an important role in maintaining mitochondrial function, In particular, the mitochondrial kinase PINK1, and the cytosolic E3 ubiquitin ligase parkin are thought to act in a common pathway to regulate mitochondrial function (Gegg et al., 2010). Several lines of evidence suggest that mitochondrial dysfunction may be attributed to reduced complex I activity in PD patients, and reduced mitochondrial membrane potential accompanied by increased ROS production in PD cell models (Gegg et al., 2010; Thomas et al., 2011). Mutations in *parkin* and *PINK1* are the most common cause of ARPD, with both proteins being highlighted as key role players in the clearance of damaged mitochondria via autophagy—a process known as mitophagy (Matsuda et al., 2010). A number of studies have provided clues as to how PINK1 may regulate parkin-mediated mitophagy, and how PD-associated *PINK1* and *parkin* mutations result in defective mitophagy. One such study showed that *PINK1* knockdown in SH-SY5Y cells induces mitophagy through effects on oxidative stress and mitochondrial fission, whereas overexpression of *PINK1* stabilised mitochondrial networks (Dagda and Chu, 2009). This observation is interesting because defects in autophagy/mitophagy have been shown to mimic a series of reported PD features such as impaired motor coordination, and the accumulation of protein aggregates in residual neurons (Narendra et al., 2012). Mitochondrial protection in the presence of excess oxidative stress is mediated by the kinase activity of PINK1(Matsuda et al., 2010). *PINK1* mutations are therefore thought to result in increased ROS, which is known to induce neuronal apoptosis (Gandhi et al., 2009). Notably, strong evidence suggests that another AR cause of PD, namely DJ-1, may maintain mitochondrial function in the presence of excess oxidative stress, due to its interaction with mitochondrial protein mortalin, which maintains mitochondrial homeostasis and antagonizes ROS (Burbulla et al., 2010; Thomas et al., 2011).

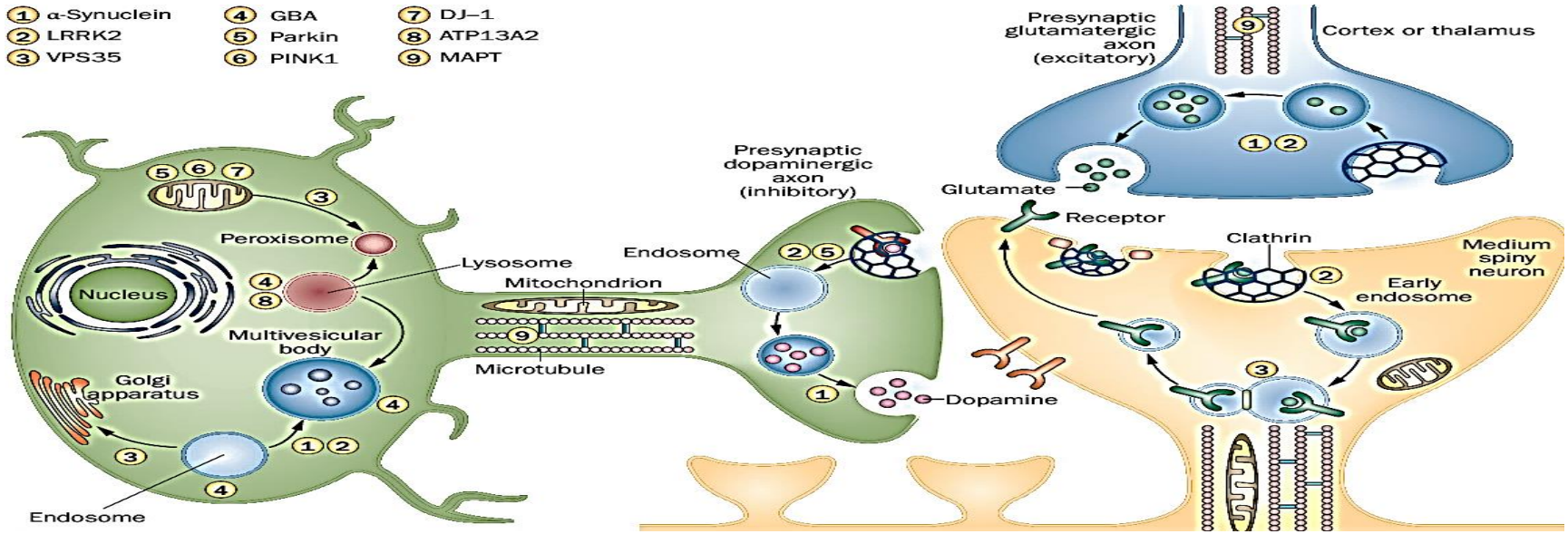


Figure 1.11 Putative mechanisms underlying the pathogenesis of PD. An axon of a presynaptic neuron (blue), a dopaminergic neuron of the SNpc (green), and a dendritic spine of a medium spiny neuron (orange) are illustrated. Under normal cellular conditions, *SNCA* **(1)** promotes exocytosis in presynaptic terminals; *LRRK2* **(2)** facilitates the postsynaptic phosphorylation of endophilin A, and the release of clathrin-coated endocytic vesicles. *VPS35* **(3)** mediates receptor recycling via cargo recognition of early endosomes, which is also required for the normal functioning of lysosomal acid hydrolase *GBA* **(4)**. *Parkin* **(5)** is involved in proteasomal function and mitochondrial maintenance together with *PINK1* **(6)**, while *DJ-1* **(7)** maintains mitochondrial function in the presence of oxidative stress. *ATP13A2* **(8)** has a role in lysosome-mediated autophagy, and *MAPT* (Microtubule-associated protein tau) **(9)** has a role in the regulation of cargo trafficking and delivery [taken from (Trinh and Farrer, 2013)].

1.4.2 Failure of protein degradation systems

Protein degradation systems are primarily involved in the clearance of misfolded proteins, and dysfunctional organelles such as defective mitochondria (Korolchuk et al., 2010). The two major degradation systems are the UPS, and ALS, however, only the UPS will be discussed in this section as it is the principal mechanism through which proteins are degraded in the mammalian cytosol and nucleus (Glickman and Ciechanover, 2002). The UPS consists of proteases located in specialised protein complexes, and proteasomes which form the main machinery for protein degradation. The main function of the proteasomes is to target cytosolic proteins for regulated degradation in a sequential series of reactions catalysed by ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3) which transfer the ubiquitin polypeptide to target proteins as part of the UPS (Figure 1.12). Once a chain of several ubiquitin molecules has been formed, this is then added to the target protein (a process referred to as polyubiquitination), which is recognized by a series of proteases (i.e. the 26S proteasome) and degraded through proteolysis (McNaught et al., 2004). Given the presence of ubiquitinated proteins in LBs, it has been proposed that abnormal protein accumulation, resulting from the impairment of the UPS, may be a key feature of PD pathogenesis. Proteasome inhibitors have been shown to induce selective degeneration of cultured dopamine neurons with the formation of LB-like inclusions in model systems (Zeng et al., 2006). Systemic administration of proteasome inhibitors has also been shown to induce parkinsonism in experimental animals, and elicit selective degeneration of dopaminergic neurons, in which inclusion bodies that stain positively for both aSYN and ubiquitin are formed (McNaught et al., 2004). Loss-of-function mutations in *parkin* are thought to inhibit, or significantly impair the process of ubiquitination, because of its role as an E3 ubiquitin ligase (Shimura et al., 1999).

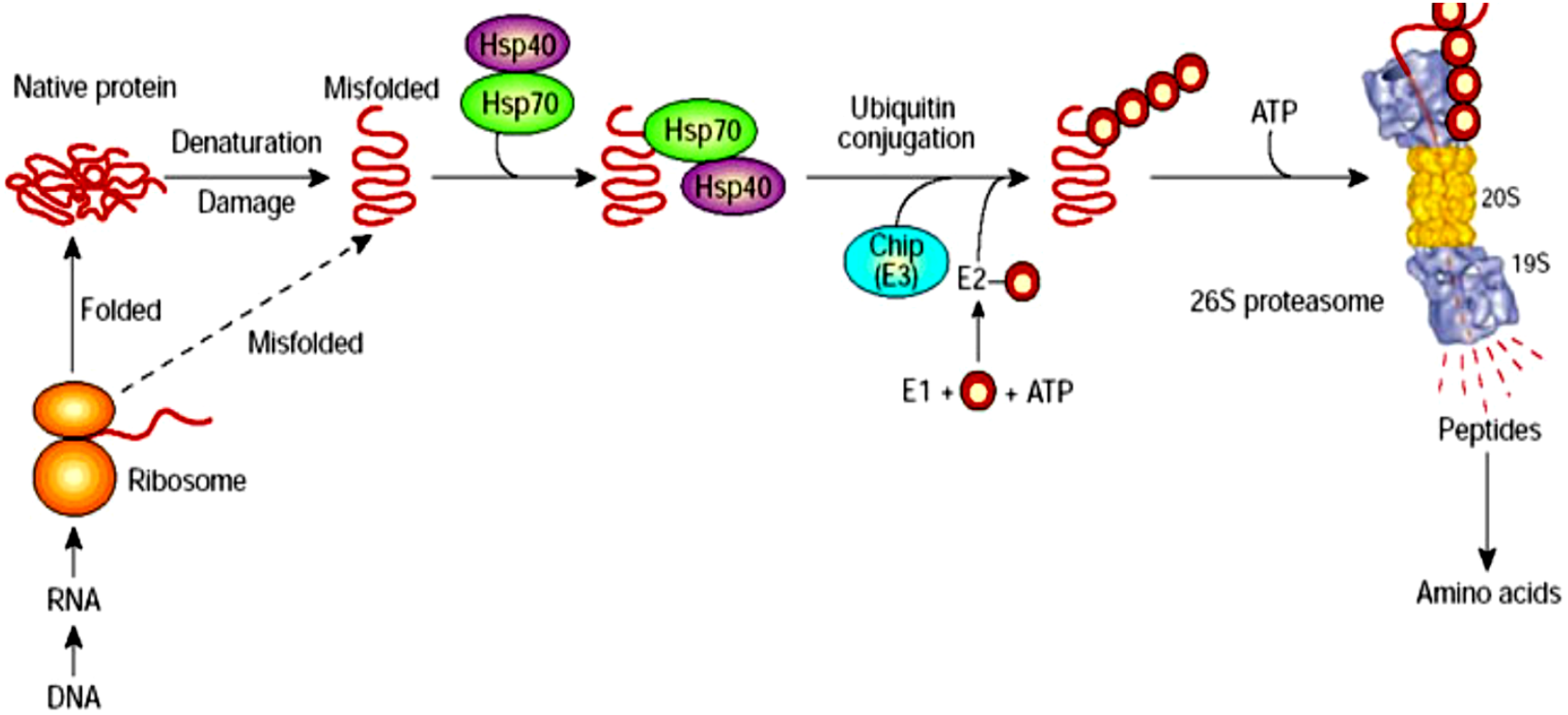


Figure 1.12 The ubiquitin-proteasome system (UPS). The steps by which the UPS clears unwanted proteins: ATP-dependent activation of ubiquitin monomers; labelling of unwanted/damaged proteins with chains of activated ubiquitin molecules by CHIP; transport of ubiquitinated proteins to the proteasome by 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)].

1.5 PD research in sub-Saharan Africa

The genetic aetiology of PD in Black patients from Sub-Saharan Africa (SSA) is poorly characterized, as the disorder is generally perceived to have lower prevalence and incidence because of the relative youthfulness of the SSA population (<5% are over the age of 65), scarcity of published reports, socioeconomic issues and cultural misconceptions which generally view neurologic disorders as being part of normal ageing (Dotchin et al., 2012). To the best of our knowledge, only nine studies have investigated the underlying genetic aetiology of PD in SSA populations (Table 1.2), while no incidence studies have been published to date (Dotchin et al., 2008; Lekoubou et al., 2014). The increasing knowledge gap of PD in SSA populations is further hampered by many challenges. These include the exorbitant cost of medication, the scarcity of movement disorders specialists, a general lack of public awareness of PD, and the negative stigmatization associated with affected individuals, partly due to negative cultural perceptions associated with neurological diseases in general (Dotchin et al., 2007; Mshana et al., 2011). The need for comprehensive research of PD in SSA will become considerably more important given the current demographic transition. A greater proportion of aging populations worldwide, are projected to survive to an older age by 2050, thereby facilitating the increased prevalence of age-related diseases such as PD (Bach et al., 2011; Dotchin et al., 2012). This demographic transition is evident from population estimates which show that in 2005, there was approximately 34 million people aged 60 years and over in SSA. This number is now projected to increase to over 67 million by 2030 (Dotchin et al., 2012). Interestingly, the number of aging populations in SSA is growing more rapidly than that of the developed countries. For example, the prevalence of PD is projected to increase by nearly 200% in Tanzania alone (Dotchin et al., 2012). This figure is significantly greater than the estimated 92% projected increase in the number of people over the age of 60 years in countries such as Europe, the United States and Canada over the same time period (Bach et al., 2011).

Table 1.2 Summary of genetic studies performed on Black PD patients from SSA [(taken from (Blanckenberg et al., 2013)].

Gene	Country	No. of patients	Screened/Methods	Results	Reference
LRRK2	Black Africans (country not specified)	4	Exon 41 (sequencing)	No mutations found	(Lesage et al., 2006)
	South Africa	12	Exons 1, 2, 10, 15, 27, 41 and 49 + G2019S (exon dosage-MLPA)	No mutations found	(Keyser et al., 2010)
	South Africa	16	G2019S (HRM)	No mutations found	(Bardien et al., 2010)
	Ghana	54	Exons 31 and 41 (sequencing)	No mutations found	(Cilia et al., 2012)
	Nigeria	57	Exons 31 and 41 (sequencing)	No mutations found	(Okubadejo et al., 2008)]
	Zambia	17	Exons 29 to 48 (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	22	All 51 exons (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	29	Exons 1, 2, 10, 15, 27, 41 and 49 + G2019S	No mutations found	(Yonova-Doing et al., 2012)
SNCA	South Africa	12	All 6 exons + A30P (exon dosage-MLPA)	No mutations found	Keyser et al., 2010)
	Zambia	29	All 6 exons + A30P (exon dosage-MLPA)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	39	All 6 exons (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
VPS35 and ***EIF4G1***	South Africa	18	D620N mutation (KASP assay), R1205H mutation (KASP assay)	No mutations found	(Blanckenberg et al., 2014)
Parkin	South Africa	17	All 12 exons (SSCP and HRM)	No mutations found	(Bardien et al., 2009; Haylett et al., 2012)
		17	All 12 exons (exon dosage-MLPA)	**Duplication of exon 2 & deletion of exon 9 in one proband and an affected sibling. Heterozygous G430D mutation and deletion of exon 4 in another proband**	(Haylett et al., 2012; Keyser et al., 2010)
	Zambia	12	All 12 exons (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	29	All 12 exons (exon dosage-MLPA)	**Heterozygous deletions of exon 2 and exon 4 in one proband**	(Yonova-Doing et al., 2012)
	Nigeria	57	All 12 exons (sequencing)	No mutations found	(Okubadejo et al., 2008)
PINK1	South Africa	12	All 8 exons (exon dosage-MLPA)	No mutations found	Keyser et al., 2010)
	South Africa	18	All 8 exons (HRM)	No mutations found	Keyser et al., 2010)
	Zambia	12	All 8 exons (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	29	All 8 exons (exon dosage-MLPA)	No mutations found	(Yonova-Doing et al., 2012)
DJ-1	South Africa	12	Exons 1, 3, 5 and 7 (exon dosage-MLPA)	No mutations found	Keyser et al., 2010)
	Zambia	12	All 7 exons (sequencing)	No mutations found	(Yonova-Doing et al., 2012)
	Zambia	29	Exons 1, 3, 5 and 7 (exon dosage-MLPA)	No mutations found	(Yonova-Doing et al., 2012)

HRM, high-resolution melt; MLPA, multiplex ligation-dependent probe amplification. SSCP, single-strand conformation polymorphism.

1.6 PD research in South Africa

Cosnett and Bill (1988) performed one of the earliest studies on PD, which investigated the prevalence of the disorder in over 2000 inpatient neurological consultations in three Durban based hospitals. They found the prevalence of PD to be uncommon in the Black population groups, relative to White and Indian patients from the same population, and of the same age bracket (between 60 and 80 years) (Cosnett and Bill, 1988). Despite research efforts, very little is still known of the molecular aetiology of PD in the unique Black SA population, which may be partly attributed to ascertainment bias. In the context of this population, the term 'Black population' refers to a group of individuals whose ancestry can be directly traced to the African continent. Moreover, this ethnic group is comprised of individuals who speak indigenous African languages including: IsiZulu, Northern and Southern Sesotho, IsiXhosa, IsiNdebele, Sepedi, Tsonga, Tshivenda, and Setswana. To date only six published studies have investigated the underlying genetic factors which may account for PD in selected ethnic groups from the SA population. Based on these studies, mutations in the known PD-causing genes have been found to be a minor cause of the disorder, therefore suggesting that a novel gene may account for the disease onset in the majority of SA Black patients screened thus far (Bardien et al., 2009; Keyser et al., 2010; Haylett et al., 2012; Blanckenberg et al., 2014). This is a highly plausible hypothesis, given that the Black ethnic population harbours one of the oldest, most unique genetic ancestries, with the greatest level of diversity worldwide (Ramsay et al., 2011). Yet, no molecular genetic studies of PD have exclusively focused on the Black SA population group to date. A comprehensive genetic study, primarily of this group, could prove invaluable in the elucidation of novel, yet unexplored disease-causing pathways for PD. This is the focus of the present study.

1.7 Mutation detection techniques

A number of different mutation screening techniques were used in the present study, and the underlying principle of each technique will be discussed in the next section. These include the use of Sanger sequencing and high-resolution melt (HRM) for the detection of single base pair changes and small insertions and deletions, and multiplex-ligation dependent probe amplification assay (MLPA) for identification of exonic rearrangements. In addition, a next generation sequencing approach (NGS) was used for comprehensive and systematic screening of all known involved in neurodegenerative disorders.

1.7.1 Sanger sequencing

Sanger sequencing is a first generation sequencing technology which uses dideoxy nucleotide triphosphates (ddNTPs) as DNA chain terminators so that the labelled fragments can be separated according to size using electrophoresis. This sequencing approach allows for elucidation of DNA sequence reads and the detection of point or frameshift mutations using the ABI sequencing machine (e.g. ABI 3130XL Genetic Analyzer), which uses differently coloured fluorescent dyes to label each of the four ddNTPs (A,T,C,G). Amplified single stranded target DNA is used as a template and extended by a DNA polymerase, separated by capillary electrophoresis and is detected by laser excitation as the dye-labelled fragments migrate through a transparent window of the capillary array to yield a sequencing result (Dianzani et al., 1993).

1.7.2 High-resolution melt

HRM is a rapid approach for SNP genotyping, and cheaper alternative to Sanger sequencing with regards to mutation screening. The HRM technique is based on a melting (dissociation) curve analysis, and can be considered to be a next-generation application of amplicon melting analysis (Vossen et al., 2009). HRM allows for the simple characterization of DNA samples based on their disassociation/melt profile behaviour, used to detect single base pair differences between samples. DNA sequences are discriminated according to their GC content, strand complementarity and sequence length. HRM is essentially a post PCR technique performed on high-throughput instrumentation, and dedicated analysis software, which facilitate the detection of small changes in sequence variation using specialized double-stranded DNA (dsDNA) intercalating dyes (e.g. SYBR® Green I, or EvaGreen®) (Li et al. 2012). These dyes are highly

fluorescent when bound to dsDNA, and become gradually less fluorescent when bound to single stranded DNA (i.e. ssDNA). As the strands separate, the dye is released, causing a decrease in fluorescence with the increasing temperature. HRM instrumentation collects and analyses fluorescent signals in real time, thereby characterizing the different DNA fragments, such that the fluorescence (y-axis) is plotted against temperature (x-axis) (Figure 1.13). Since different DNA sequences melt at slightly different rates, they can be analysed and compared to a wild-type sample using the respective melt curves. Any PCR products which exhibit an altered melt profile, distinct to that of the wild-type control sample, must be confirmed using Sanger sequencing to identify and confirm the sequence variation.

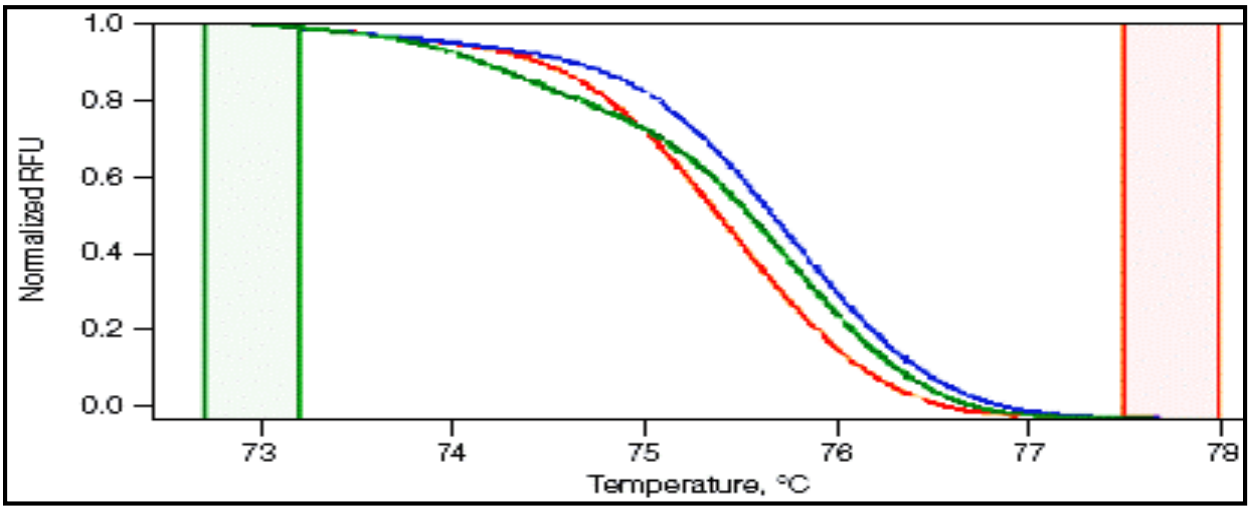


Figure 1.13 Illustration of high-resolution melt curve analysis. The green and red boxes indicate the pre- and post-melt regions, used for data normalization. RFU denotes relative fluorescence units (taken from http://www.bio-rad.com/en-za/applications-technologies/high-resolution-melting).

1.7.3 Multiplex ligation-dependent probe amplification assay

MLPA is the most widely used approach for the detection of exonic rearrangements [i.e. copy number variation (CNV)]. MLPA can be divided into five steps as illustrated in Figure 1.14: (1) DNA denaturation and hybridization of probes, (2) ligation reaction, (3) PCR amplification, and (4) separation of PCR products using capillary electrophoresis. Once the PCR products have been separated, the peak patterns are than compared to reference samples. A normal sample has a peak ratio of 1 (range of 0.7-1.3), a heterozygous deletion has a peak ratio of 0.5 (range 0.3

– 0.7), while a homozygous deletion has a peak ratio of zero. Heterozygous duplications have a peak ratio of 1.5 (range 1.3 – 1.7), and homozygous duplications have peak ratio of 2 (range 1.7 – 2.3) (Schouten et al. 2002). Possible CNVs must be validated by Sanger sequencing and quantitative-PCR (qPCR).

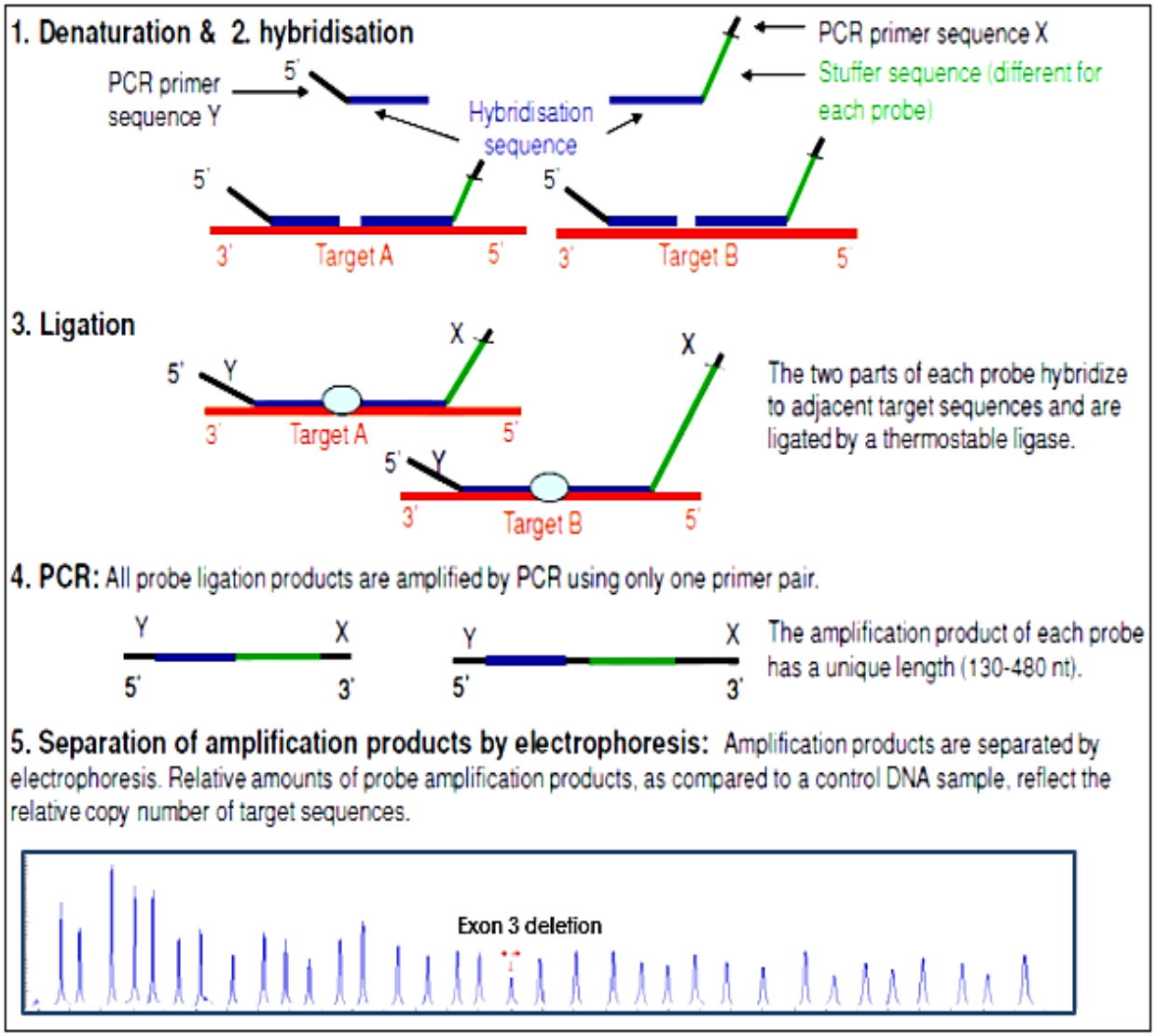


Figure 1.14 Outline of the Multiplex ligation-dependent probe amplification reaction**.** Each amplicon generates a unique fluorescent peak and each peak represents a specific exon. Peak height differences reflect CNV detected by the MLPA probes [taken from (Schouten et al. 2002)].

1.7.3.1 *Data interpretation and presentation of results using qPCR*

Possible CNVs detected using MLPA can be graphically represented as follows: after the 30th cycle of the DNA melting process using the LightCycler® 96 Real-time PCR system, the wild type samples has a characteristic peak height and pattern corresponding to both the reference amplicon and the analysed amplicon (Figure 1.15 a, green curve). In the case of samples with CNVs, the peak pattern is distorted. When one of the exons of the analysed gene undergoes a deletion, its peak decreases and, at the same time, the peak of the reference amplicon increases (Figure 1.15 a, red curve). The situation is reversed in the case of exon amplification, with the peak of the reference amplicon decreasing and the peak of the analysed amplicon increasing (Figure 1.15 a, blue curve). In order to observe these differences, the results are compared with DNA samples with an unchanged copy number of templates for each of the amplified fragments. These results are subsequently represented in a bar graph format (Figure 1.15 b), such that the highest peak values are determined, and the ratio thereof is then compared to that of the reference peak. The peak height ratio should be 0.5X for samples with a deletion (*n* copies) and 1.5X for samples with a duplication (3n copies), whereas the peak height ratio for a wild type sample should be 1X (2*n* copies) (Borun et al., 2014).

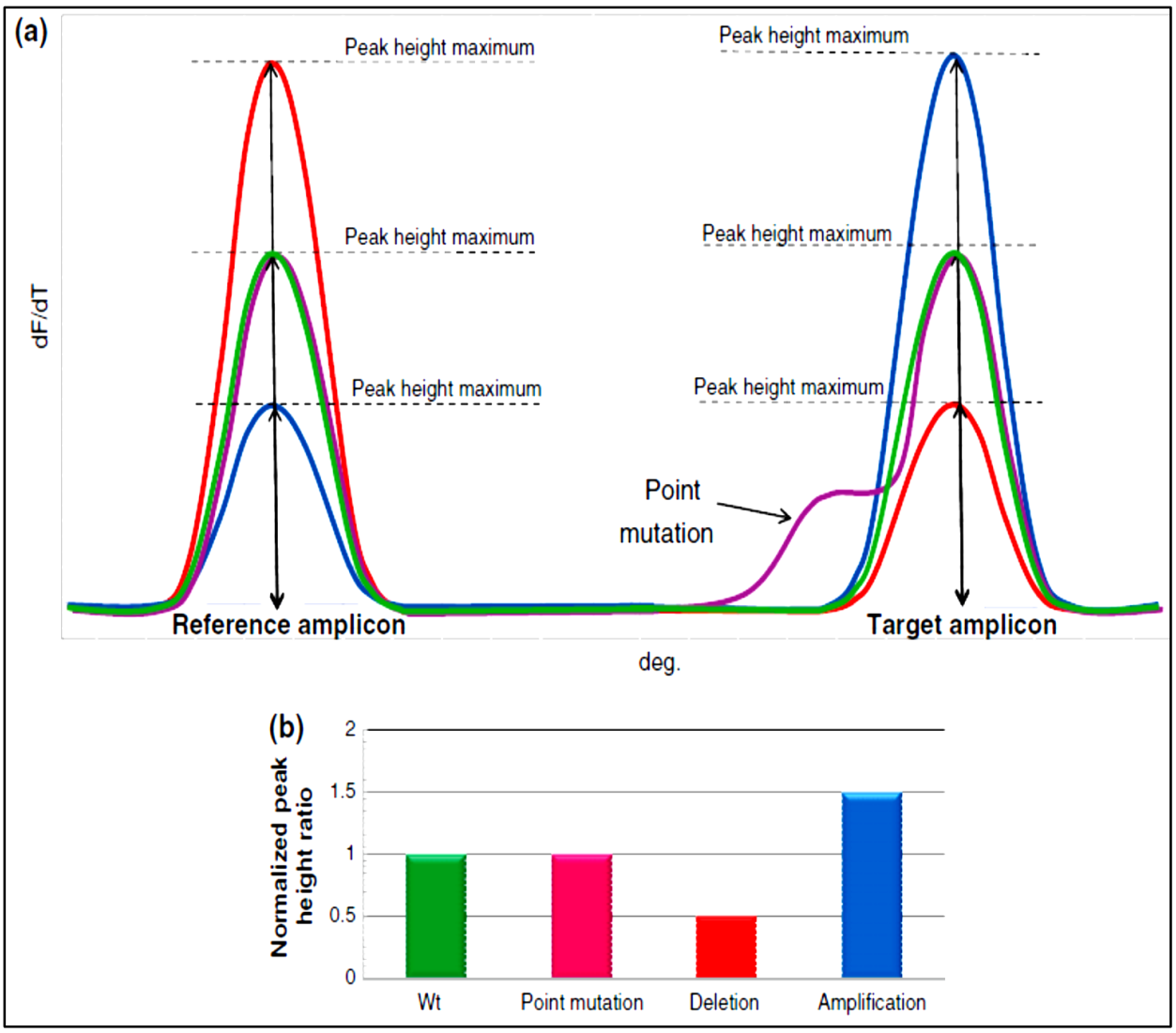


Figure 1.15 Conceptual qPCR graph presenting an outline of the method. (a) Melting profile containing examples of possible genotypes. Green curve represents wild type sample, blue curve represents sample with possible exonic amplification of the target amplicon, red curve represents a sample with possible deletion of the target amplicon, and the purple curve represents a sample with point mutation in the target amplicon. (b) Bar graph results. Columns represent normalised peak height ratio of the target amplicon to the reference amplicon calculated for each [taken from (Borun et al., 2014)].

1.7.4 Next generation sequencing

Sanger sequencing is considered to be a 'first-generation' sequencing technique, while more recent sequencing technologies are referred to as next-generation sequencing (NGS). NGS technologies comprise of various strategies that rely on a combination of template preparation, sequencing and imaging, genome alignment, assembly methods, and data analysis to produce thousands to millions of sequencing reactions at an unparalleled speed (van Dijk et al., 2014). The unique combination of specific protocols distinguishes one NGS technology from another, and determines the type of data produced from each platform. The general workflow is performed as follows: firstly, the source nucleic acid material is converted into a sequencing library. A wide variety of NGS library preparation protocols exist, but they all generally fuse fragments of DNA with platform-specific adapters. Long DNA molecules are first fragmented into a suitable size (~50–500 nucleotides) followed by the addition of specialized adapters. A size selection step is then performed to enrich further for molecules of the desired size and to eliminate free adapters. Lastly, PCR is performed to select for molecules containing adapters at both ends and to generate sufficient quantities for sequencing (Figure 1.16).

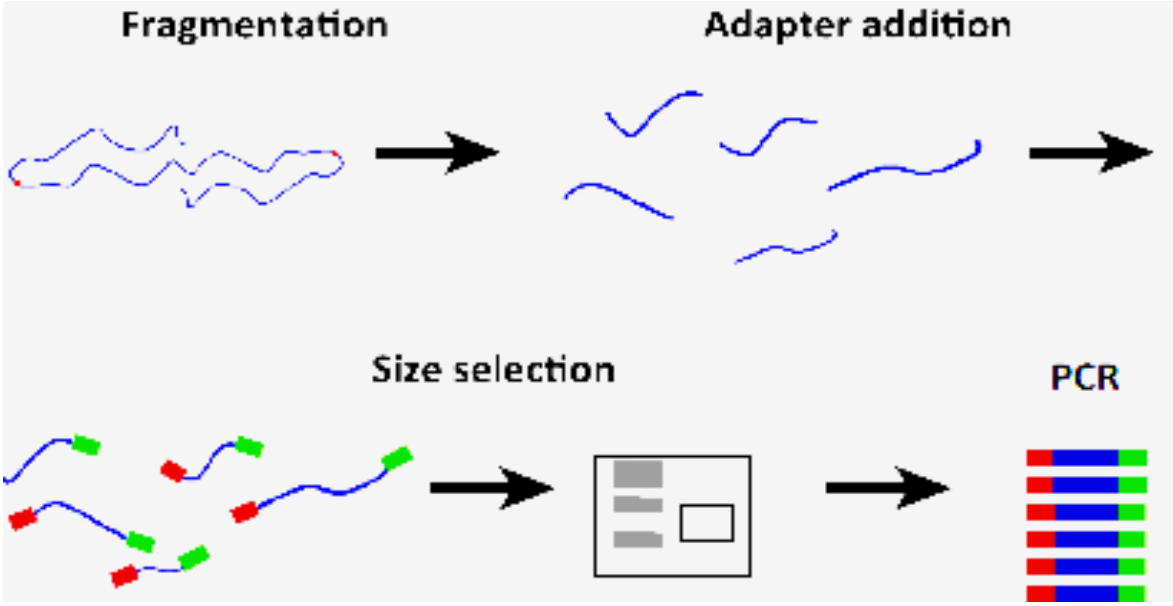


Figure 1.16 Simplified representation of a typical NGS library preparation workflow [taken from (van Dijk et al., 2014)].

Using a variety of imaging techniques and bioinformatics software, a large volume of sequence data is processed and subjected to specified analytical software tools to identify putative sequence variants in individual DNA samples. However, given the technical limitations of NGS technologies, sequencing artefacts are sometimes reported (Metzker, 2010). It is therefore necessary to always confirm NGS variants by Sanger sequencing.

1.7.5 Nomenclature

In this thesis the nomenclature system for the description of changes in DNA sequences is based on that previously described by Antonarakis and the Nomenclature Working Group (http://www.dmd.nl/mutnomen.html) (Antonarakis, 1998; den Dunnen and Antonarakis, 2001). The term 'sequence variation' or 'variant' is used to describe sequence variations/changes of unknown or unconfirmed protein function. The term 'polymorphism' refers to variants found at a frequency of ≥1% in the general population. The term 'mutation' is used to describe sequence changes with confirmed clinical significance or phenotypic consequence. This term is generally assigned to variants when there is strong evidence in support of a disease-causing role (e.g. evidence of segregation with disease in large pedigrees, and/or laboratory based evidence in support of a functional effect on a protein level).

1.7.6 Online PD databases

PD databases containing information about genes and genetic variants associated with the disease were used extensively in parts of this project. The Mutation Database for Parkinson's disease (MDPD, http://datam.i2r.a-star.edu.sg/mdpd/) (Tang et al., 2009), and the Parkinson Disease Mutation database (PDmutDB, http://www.molgen.ua.ac.be/PDmutDB/) were used for researching known PD mutations and genetic variants during analysis of sequence data (Nuytemans et al., 2010). These databases contain entries from over 1000 of the most recent PD publications.

1. 8 Aims and objectives of the present study

Given that PD in Black SA patients has been understudied, the aim of the present study was to investigate whether these patients have a different genetic aetiology to that reported for other PD patients worldwide. To this end, the objectives of the study were to:

1. Determine whether there are real diagnostic or clinical differences between Black PD patients and that reported for other SA subpopulation groups.

2. Screen the known PD genes (i.e. *parkin, PINK1, DJ-1, ATP13A2, SNCA, LRRK2, VPS35,* and *EIF4G1*) for disease-causing mutations in this group of patients using Sanger sequencing, high-resolution melt, multiplex ligation-dependent probe amplification assay and next generation sequencing techniques.

3. Determine whether Black patients diagnosed with PD harbour pathogenic repeat expansions at the spinocerebellar ataxia - type 2 (*SCA2*) and –type 7 (*SCA7*) loci.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study participants

This study was approved by the Human Research Ethics Committee at the University of Stellenbosch (Protocol number 2002/C059). A total of 147 ethnically-matched control samples were recruited from healthy unrelated blood donors from the Blood Donation clinic of the SA Western Province Blood Transfusion Service. A total of 47 unrelated PD patients, from the Black SA ethnic group, were recruited from the Movement Disorders clinic at Tygerberg Academic Hospital in Cape Town (*n*=32), as well as through a collaboration with a research group from the University of Pretoria (*n*=15). The patients had been examined by movement disorder specialists and met the UK Parkinson's Disease Society Brain Bank Research criteria for diagnosis of PD (Gibb and Lees 1988). The average AAO of the study group was 55.3 years (SD 11.2, ranging from 30 to 77 years). Fifteen patients (32%) had disease onset before or at the age of 50 years-old. Twenty-four patients (51%) were male. When possible, pedigrees were constructed for selected PD cases (Appendix B). Thirty patients (64%) had sporadic PD, 12 (26%) presented with a familial form of the disease (i.e. reported a first-, or second-degree relative with PD or PD-related features), and 5 patients (11%) had an unknown family history of the disease. The control samples were not clinically assessed for any signs of PD and a family history was not taken. A total 57% of the control samples were male. As the samples had been 'de-identified', no information was available on the ages of these controls.

After obtaining written informed consent from the study participants, peripheral blood samples were collected and genomic DNA was extracted. The procedure for the phenol-chloroform DNA extraction method is as described by Sambrook and Russell (2006).

The 47 probands were screened for disease-causing mutations in all the known PD genes using various techniques as outlined in Table 2.1. Notably, varying numbers of patients were screened with different methods due to varying availability of DNA samples at the time of screening. Initially Sanger sequencing was used for mutation screening, but due to the relatively high cost of this method, HRM was later used. MLPA was used for detection of CNV changes in all known PD genes, and NGS was used towards the end of the study through collaboration with Professor Matthew J. Farrer from the University of British Columbia, Canada.

Table 2.1 PD probands screened for disease-causing mutations using different mutation screening techniques.

Mutation-screening techniques										
Patient ID	**Sanger sequencing**					**HRM**			**MLPA**	**NGS**
	parkin	***DJ-1***	***VPS35***	***GBA***	***EIF4G1***	***parkin***	***LRRK2***	***SNCA***	**8 known PD genes**	**All known PD genes**
37.12	√	√	√	√	√	**ND**	**ND**	**ND**	√	**ND**
42.03	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
42.04	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
42.56	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
43.59	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
52.23	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
54.74	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
55.52	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
55.65	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
60.39	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
60.43	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
61.79	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
61.81	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
63.42	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
63.73	√	**ND**	√	√	√	**ND**	**ND**	**ND**	√	√
78.74	√	**ND**	√	√	√	**ND**	**ND**	**ND**	√	√
84.52	√	√	√	√	√	**ND**	**ND**	**ND**	√	√
11.893	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

Table 2.1 PD probands screened for disease-causing mutations using different mutation screening techniques. Continued

Mutation-screening techniques										
Patient ID	**Sanger sequencing**					**HRM**			**MLPA**	**NGS**
	parkin	***DJ-1***	***VPS35***	***GBA***	***EIF4G1***	***parkin***	***LRRK2***	***SNCA***	**8 known PD genes**	**All known PD genes**
96.87	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	√
10.307	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	√
10.308	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	√
10.309	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	√
10.310	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	√
10.314	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	**ND**
10.317	√	√	**ND**	√	**ND**	**ND**	**ND**	**ND**	√	**ND**
11.894	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	**ND**
11.781	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	**ND**
11.793	**ND**	**ND**	√	√	√	√	√	√	√	**ND**
11.794	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.795	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.796	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.895	**ND**	**ND**	√	√	√	√	√	√	√	**ND**
11.798	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.799	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.800	**ND**	**ND**	√	√	√	√	√	√	√	**ND**
11.801	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.802	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**

Table 2.1 PD probands screened for disease-causing mutations using different mutation screening techniques. Continued

Mutation-screening techniques										
Patient ID	**Sanger sequencing**					**HRM**			**MLPA**	**NGS**
	parkin	***DJ-1***	***VPS35***	***GBA***	***EIF4G1***	***parkin***	***LRRK2***	***SNCA***	**8 known PD genes**	**All known PD genes**
11.803	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.804	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.805	**ND**	**ND**	√	√	√	√	√	√	√	**ND**
11.806	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.808	**ND**	**ND**	√	√	√	√	√	√	**ND**	**ND**
11.830	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	√
11.832	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	√
11.833	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	√
11.834	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	√
11.835	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	√	√

ND = not done/ screened as DNA sample was not available at the time of screening, or sample precluded on basis of AAO (some genes are a rare cause of PD and limited to young AAO (e.g. *GBA*), while others such as *VPS35* and *EIF4G1* are limited to late AAO cases). √ = DNA sample was screened using the specified mutation screening technique.

2.2 Polymerase chain reaction (PCR) and Sanger sequencing

Polymerase chain reaction (PCR) primers were designed using Primer3 software for each of the specified exons of the different PD genes. Primers selected and reaction conditions are detailed in Appendix C Table 2.2. PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, USA). Each 25µl reaction contained 50ng template genomic DNA, 0.4µM of each primer, 75µM dNTPs (Promega, USA), 1.5mM $MgCl_2$, 1X NH_4 buffer (Bioline, UK), 5% formamide (for selected primer sets) and 0.01U BIOTAQ DNA polymerase (Bioline, UK). PCR conditions comprised of an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at different temperatures according to each primer set for 30 s, extension at 72°C for 45 s, and a final extension step of 72°C for 5 min. All PCR reactions performed included a negative control to which no DNA template was included (to exclude possible PCR contamination).

Prior to sequencing, PCR products were cleaned by adding 5U of Exonuclease I (Promega, USA) and 0.5U SAP (Shrimp Alkaline Phosphatase) (Cleveland, Ohio, USA) to 8.0µl PCR product. This cocktail was then incubated at an initial temperature of 37°C for 15 min followed by 15 min at 80°C (to denature the enzymes) in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA).

The sequencing reaction was performed at the Central Analytical Facility (CAF), Stellenbosch University using the Big Dye Terminator Sequence Ready Reaction Kit version 3.1 (Applied Biosystems, Foster City, USA). The reactions were electrophoresed and analysed on a 3130 x1 Genetic Analyser (Applied Biosystems, Foster City, USA). The sequence chromatographs were analysed using Sequencher DNA Sequencing Software version 5.1 (http://www.genecodes.com/). All putative sequence variants were compared against known mutations and SNPs found on dbSNP and the Parkinson's disease mutation databases (http://www.thepi.org/parkinson-s-disease-mutation-database/; http://www.pdgene.org/).

2.3 GBA screening

Due to the presence of a highly homologous pseudogene (*PSGBA*), a customised PCR program was used to selectively amplify the functional gene (*GBA*). *GBA* is longer than *PSGBA* because of several *Alu* insertions (i.e. defective transposons, lacking the necessary enzyme functions to produce a DNA copy of itself) in the intronic tracts of *GBA,* and a 55-bp deletion in exon 9 of *PSGBA*. However, despite the length difference, *PSGBA* maintains a 96% sequence identity with the functional *GBA* gene (Figure 2.1). The high degree of sequence identity, and the physical

proximity between *PSGBA* and *GBA,* facilitates the transcription of the pseudogene, hence the need to use a customised PCR program to selectively amplify the functional gene. To this end, *GBA* was amplified as 3 large genomic DNA fragments (a 2972 bp fragment encompassing exons 1–5; a 2049 bp fragment encompassing exons 6–7 and a 1682 bp fragment encompassing exons 8–11), using primer sequences provided in Appendix C Table 2.3, and a customized 64°C to 54°C touch-down PCR program, summarized in Appendix C Table 2.4. Both exonic and flanking intronic regions of *GBA* were sequenced. A 25µl PCR reaction was performed using 50 ng genomic DNA together with 0.4µM forward and reverse primers, 75µM dNTPs, 0.04U/µl DreamTaq™ Green Taq polymerase (Fermentas, UK) and 1X Tampon buffer (Bioline, UK). PCR products were purified, sequenced and subsequently analysed as described in section 2.2.

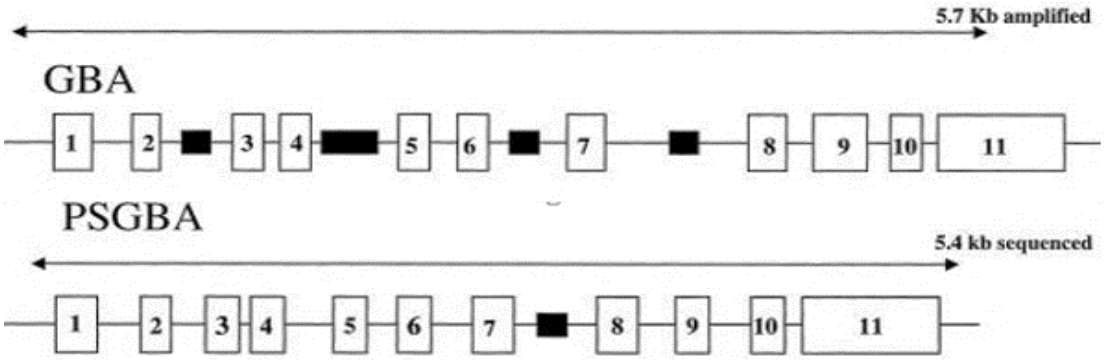


Figure 2.1 Schematic representation of *GBA* and its homologous pseudogene *PSGBA.* Exons are depicted as boxes with identification numbers inside. *Alu* sequences are represented as black boxes [taken from (Sorge et al., 1990; Imai et al., 1993)].

2.4 High-resolution melt

Each 25µl PCR reaction contained 50ng template DNA, 0.4µM of each primer, 75µM dNTPs (Promega, USA), 1.5mM $MgCl_2$, 1X NH_4 buffer (Bioline, UK), 5% formamide (for selected primer sets) and 0.01U BIOTAQ DNA polymerase (Bioline, UK) and 2µM SYTO9 fluorescent dye (Invitrogen, USA). Apart from slight deviations in annealing temperature to optimize he amplification of selected exons, standard PCR conditions comprised of an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension step of 72°C for 7 min. HRM analysis was performed from 75 to 95°C, with each

successive step rising by 0.1°C using the RotorGene 6000 machine (Corbett Life Science, Australia). A negative control was included in each run and samples which exhibited an altered melt profile were validated using Sanger sequencing. Where possible, samples with known variants were included as positive controls.

2.5 Multiplex ligation-dependent probe amplification technique (MLPA)

The MLPA technique was used to detect CNV in the known PD genes. Two commercially available probe kits, SALSA P051-C3 and P052-C2 Parkinson MLPA kits (MRC Holland, Amsterdam, The Netherlands; http://www.mlpa.com) were used for this assay. Together, the kits consisted of probes for all exons of *SNCA*, *parkin*, and *PINK1*, and specific exons of *DJ-1* (exons 1, 3, 5, 7), *LRRK2* (exons 1, 2, 10, 15, 27, 41, 49), *UCH-L1* (exons 1, 4, 5, 9), and *ATP13A2* (exons 2, 9). In addition, several probes for *CAV1* (exon 3), *CAV2* (exon 3), *GCH1* (exons 1, 2, 3, 5, 6) and two-point mutations (A30P in *SNCA* and G2019S in *LRRK2*) were also added as part of the probe kits. Each probe is composed of two half-probes (5' and 3' half-probes), consisting of a target-specific sequence and a universal primer sequence allowing the simultaneous multiplex PCR amplification of all probes (Schouten et al. 2002). All exons of parkin were included in both kits, and the probe sequences of the same exon were different between the two kits. The assay was performed for all the samples as outlined in Table 2.1, using both kits according to the manufacturer's protocol.

Initially, 35ng of genomic DNA was denatured at 98°C for 5 min and then allowed to hybridize to the MLPA probe set P051 and P052 (in independent reactions for each probe set). Thereafter, ligation was performed at 54°C for 15 min using a Ligase-65 enzyme. PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) for 35 cycles (95°C for 30 s; 60°C for 30 s; 72°C for 1 min) and a final 20 min step at 72°C. The primers used for the PCR reactions were the SALSA PCR forward primer (labeled) 5'-FAM-GGG TTC CCT AAG GGT TGG A-3' and SALSA PCR reverse primer (unlabeled) 5'-GTG CCA GCA AGATCC AAT CTA GA-3'.The PCR products were analysed using a 3130x1 Genetic Analyzer (Applied Biosystems) with GeneScan™-500LIZ™ size standard (Applied Biosystems). The height or area of the PCR derived fluorescence peaks was measured, thereby quantifying the amount of PCR product after normalization. This measurement was then compared with that of the control DNA samples in order to determine the relative amount of target DNA sequence in the input DNA sample. ABI electopherographs were analysed using Coffalyser software version 9.4 (MRC Holland, Amsterdam, The Netherlands; http://www.mlpa.com). Possible CNVs were validated using qPCR, and Sanger sequencing.

2.5.1 *Verification of MLPA using qPCR*

Possible CNVs (including exonic deletions or multiplications) detected using Coffalyser software version 9.4 (MRC Holland, Amsterdam, The Netherlands; http://www.mlpa.com) were verified by performing qPCR using the LightCycler® 96 Real-time PCR system (Roche Diagnostics Corporation, Indianapolis, USA), and the LightCycler® FastStart DNA Master PLUS SYBR Green I kit (Roche Diagnostics Corporation). qPCR was performed in triplicate for each sample. Each 10µl reaction consisted of the following reagents: 2µl Hybridization FastStart Mix (Roche Diagnostics), 0.5µM of each primer and 30ng genomic DNA. The PCR conditions were: 95°C for 10 min, 95°C for 10 s, 55°C for 10 s, 72°C for 15 s (45 cycles). The β-globin gene (HBB) was used as the reference gene (i.e. internal standard) for subsequent calculations (e.g. normalization of results). Results were analysed using the LightCycler® 96 Genetic Variation Analysis Software version 1.5 (Roche Diagnostics; Randburg; South Africa; www.roche-diagnostics).

2.6 Targeted resequencing using next generation sequencing

A customized target-capture panel covering the exonic regions of 168 major genes which were either linked or associated with PD, dementia, and related neurodegenerative conditions was designed by our collaborator Professor Matthew J. Farrer from the University of British Columbia, Canada (Appendix D. Table 2.5). Genomic DNA was sheared to 150bp using the Covaris® E220 sonicator and enriched for exonic regions of interest according to the TargetSeq™ Custom Enrichment Kit protocol (Life Technologies, Carlsbad, CA, USA). Quantification and quality control was performed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and a Qubit Fluorometer (Life Technologies). Captured DNA was then amplified by bead emulsion PCR with the EZ Bead system (Life Technologies) and sequenced on a SOLiD 5500xl platform (Life Technologies).

Mapping, sequence alignment, duplicate removal, SNP calling and indel detection were performed by Lifescope version 2.5.1 (Life Technologies). Annotation was performed with ANNOVAR (Wang, Li, and Hakonarson 2010) using hg19 as the reference genome. The latter includes a normalized conservation score from the UCSC Genome Browser (Karolchik et al., 2014), PHAST PhyloP (http://compgen.bscb.cornell.edu/phast/), as well as prediction scores from sequence homology–based programs Sorting Intolerant from Tolerant (SIFT) (Sim et al., 2012), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org/). Ethnically-matched control data was extracted from the 1000

Genomes Project (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/release/20110521/) and the NHLBI Exome Sequencing Project (ESP; http://evs.gs.washington.edu/EVS/) and then compared. The frequency of genetic variants observed was subsequently compared with Black control subjects (n = 48). Sanger sequencing was used to validate the NGS results, and customized primers were designed for this purpose (Appendix E. Table 2.6).

2.6.1 *in silico functional predictions programs*

In silico predictive methods were used to predict the potential of each amino acid substitution to impact protein structure and activity. Three predictive programs, namely: SIFT, PolyPhen-2, and MutationTaster were used concurrently, to compensate for the unique limitations of each program. The basis of these respective programs is sequence conservation over evolutionary time, the physical and chemical properties of the exchanged residues, and the protein structural domain information. It is not uncommon to get different functional predictions for the same variant when using these programs, as each uses a unique algorithm which forms an integral part of its predictive score. Functional predictions of the variants identified using NGS and validated using Sanger sequencing, were determined using the tolerance scores summarized in Appendix F Table 2.7.

2.7 Screening for repeat expansions at the *SCA2* and *SCA7* loci

Automated fluorescent genotyping of the SCA loci was performed using the ABI PRISM 3100*xl* Genetic Analyzer (Applied Biosystems). This approach was used due to the limitations of conventional capillary electrophoresis (CE). A definitive repeat expansion cannot be determined using CE due to the existence of polymorphisms within the SCA primer-binding sites which may result in the preferential amplification of a single allele which may either represent two normal alleles of the same size (homozygous), or a single normal allele with a second allele expanded beyond the range of detection. To circumvent these limitations, the fluorescent tandem repeat-primed PCR (TP-PCR) assay was used to specifically amplify large repeat expansions at the SCA loci. The TP-PCR assay was first described by Warner et al. (1996) to replace Southern blotting as a means of detecting hyperexpansions of trinucleotide repeat sequences. TP-PCR usually consists of three primers (Figure 2.2): a forward primer that is fluorescently labelled and flanks the repeat region (TPF), a repeat primer that is complementary to the targeted repeat but carries a

nonspecific tail sequence (TP-R1), and a third primer that is identical to the nonspecific tail sequence (TP-R2). The tail primer is designed to prevent progressive shortening of the amplicons during PCR cycling, thereby producing a characteristic peak profile of increasing length. This profile enables the rapid identification of large pathogenic CAG repeats that cannot be amplified using primers that flank the repeat region due to size limitations with conventional PCR. However, the main drawback of TP-PCR is that it does not enable the repeat number to be accurately determined.

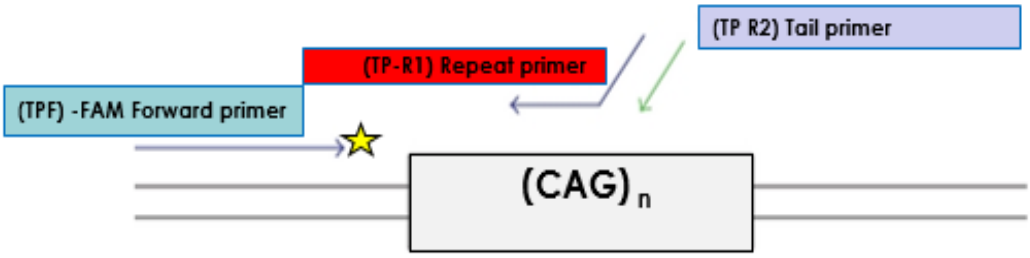


Figure 2.2 Positions of three primers used in the TP-PCR assay. This assay facilitated the rapid detection of CAG expansions in exon 3 of *SCA7*. The asterisk indicates the fluorescently-labelled forward primer.

TP-PCR was performed according to a protocol kindly provided by Ms Danielle Smith (Division Human Genetics, University of Cape Town), which was adapted from Majounie et al. (2007). Primer sequences used for SCA7- TP-PCR were as follows:
Locus-specific primer (Forward primer) 5'-FAM-CCCAGCATCACTTCAGGACT-3', repeat-specific primer 5'-TACGCATCCCAGTTTGAGACGCAGCAGCAGCAGCAGCAGCAG-3', and a tail primer 5'-TACGCATCCCAGTTTGAGACG-3'. TP-PCR assay was performed in a reaction volume of 25 µl containing 50 ng genomic DNA, 0.01U GoTaq in 1X GoTaq buffer (Promega), 75µM dNTPs (Promega, USA), 5% DMSO, 5M Betaine (Promega, USA), 0.4µM of each primer (TPF, TP-R1, and TP-R2). A *SCA7* positive control, consisting of 57 CAG repeats was included in the assay. The reactions were subjected to 30 PCR cycles consisting of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by a 10 minute extension at 72°C. All PCR products were analysed by capillary electrophoresis on the ABI 3130x1 Genetic Analyzer. The GeneScan™ Rox500 size standard was used for HEX-labelled PCR products, and the GeneScan™ LIZ250 size standard was used for FAM-labelled PCR products (Applied Biosystems). Results were analysed using GeneMapper® software, version 4.1 (Applied Biosystems). Positive control samples were included with each assay (*SCA7*-positive control with 54 CAG repeats).

In order to size the CAG repeat expansions in exon 1 at the *SCA2* loci, the following forward and reverse primers were used: 5'-56-FAM-AGGAGACCGAGGACGAGGAC-3' and 5'-GCGTGCGAGCCGGTGTAT-3 respectively. Primers flanking the *SCA2* CAG repeat were designed for this purpose (Figure 2.3).

Figure 2.3 Positions of primers flanking the SCA2 CAG repeat. These primers facilitated the detection of CAG expansions in exon 1 of *SCA2*, and the sizing of the repeat expansion. The asterisk indicates the fluorescently-labelled forward primer.

The PCR products were amplified using the following PCR program: 96°C for 5min; followed by 30 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4min, with 5% formamide as an additive in the PCR reaction. All PCR products were analysed by capillary electrophoresis on the ABI 3130x1 Genetic Analyzer as described for SCA7. Result were analysed as described for *SCA7*.

2. 8 Agarose gel electrophoresis

In order to confirm successful amplification of the appropriate DNA target, PCR products were run on agarose gels containing ethidium bromide (EtBr), which intercalates DNA molecules and allows for visualisation under UV light. For a 2% agarose gel, 2g of agarose powder was dissolved in 100ml 1X SB buffer and heated in a 950 Watts microwave at maximum heat for 2.5 min. Following a short cooling phase, 0.35µg/ml EtBr was added to liquid agarose, which was subsequently left to set in a gel mould for 20 minutes. 2µl PCR product was mixed with 2µl Cresol® loading dye. 2µl of the Gene Ruler 100bp DNA Ladder (Fermentas International Inc., Canada) was loaded into the first well. The gel was run at 120 Volts until the loading dye had reached the bottom of its respective column. Following electrophoresis, the gel was visualized under UV-light, using the UVIPro Gel Documentation System (UVITec, UK). A PCR product was considered to have been successfully amplified if only one well defined band was visible. A negative control, consisting of standard PCR mix and sterilised water instead of template DNA, was included in each electrophoretic run.

CHAPTER 3: RESULTS

3.1 Clinical characteristics of patients

Of the total 47 Black PD patients recruited for this study, fifteen were referred from the University of Pretoria where they were diagnosed by Professor Clara Schutte and Dr. Celia Mahne. The remaining thirty-two patients were recruited from the Movement Disorders Clinic at the Tygerberg Academic Hospital in Cape Town, where they were diagnosed by consulting Movement disorder specialists Professor Jonathan Carr and Dr. Percy Kumire. The clinical examination of these patients included a standardized questionnaire for demographic data collection and detailed neurological examination. The definition of the syndrome of PD was based on UK Parkinson's Disease Society Brain Bank Research criteria for diagnosis of PD, as described by Gibb and Lees (1988).

Overall, no real diagnostic or clinical differences were reported between Black PD patients, and that reported for other SA subpopulation groups. These observations were based on a comprehensive clinical evaluation of each patient, and each patient's response to L-DOPA treatment. Due to cost-constraints, neuroimaging (brain computerised tomographic scan and magnetic resonance imaging) was only available for two patients, neither of whom were considered to be remarkable or distinctly different from other PD patients from different SA ethnic groups. It can therefore be suggested that Black PD patients cannot be distinguished from other ethnic populations solely based on their clinical profile.

3.2 Screening of *parkin*, *DJ-1*, *GBA*, *VPS35*, *EIF4G1*, *SNCA* and *LRRK2* using Sanger sequencing and HRM

In this study all exons of *parkin* (exons 1-12), *DJ-1* (exons 1-7) and *GBA* (exons 1-11) were screened. Only selective exons were screened in the following genes: *VPS35* (exon 15), *EIF4G1* (exon 23), SNCA (exon 2, and 3), and LRRK2 (exon 32, and 41), as common disease-causing mutations have only been previously identified in these specific exons.

The previously described G430D heterozygous mutation in exon 12 of *parkin* (Haylett et al., 2012) was verified in the present study (Figure 3.1). No additional mutations in *parkin* were identified, but a number of polymorphisms were found (Table 3.1). Notably, all the sequences were aligned using the NCBI Human Reference Genome NCBI36/hg18.

Patient 37.12 (G>A)

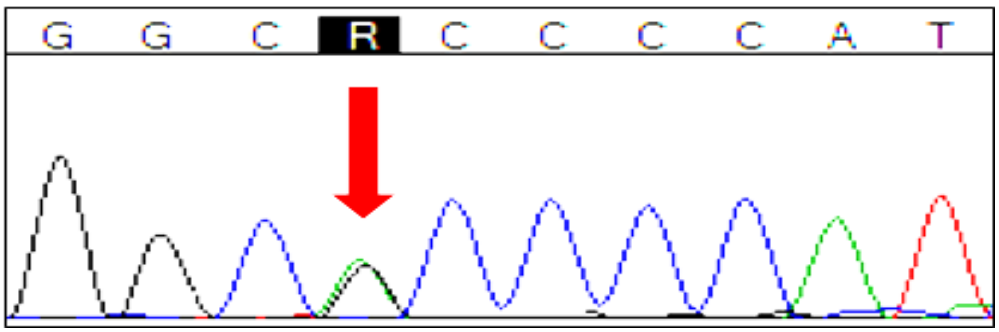


Figure 3.1 Sequence chromatogram indicating the position of the heterozygous G430D (1429 G>A) mutation in p*arkin* exon 12.

The heterozygous G430D mutation was found in a female proband (78.74) with AAO of 56 years. This mutation is found in the *parkin* RING2 domain which is known to mediate the enzymatic catalytic activity.

To assess the structural change resulting from the glycine to aspartic acid change at position 430 (G430D), the structural information was obtained from the Protein Data Bank –file 4I1H (http://pdb.rcsb.org/pdb/explore/explore.do?structureId=4I1H), and annotations about this protein were obtained from Uniprot-entry PRKN2_HUMAN (http://www.uniprot.org/uniprot/O60260). Figure 3.2 illustrates the amino acid structural change from a wild-type to a mutant amino acid. The mutant residue introduces a charge in a buried residue which may lead to alterations in protein folding.

Figure 3.2 Predicted structural change resulting from the heterozygous G430D (glycine to aspartic acid) mutation in p*arkin* exon 12.

The mutant and wild-type amino acid residues are distinctly different. Predictive algorithms such as PolyPhen-2 have predicted this amino acid change to be 'possibly damaging' on a functional level, because this change occurs in the RING2 domain, a region that is evolutionarily conserved across a number of species.

Common polymorphisms were classified as sequence variants if they were found at a frequency of ≥1% in the general population, and these were identified using various public databases including The UCSC Genome browser (http://genome.ucsc.edu), the Mutation Database for Parkinson's disease (http://datam.i2r.a-star.edu.sg/mdpd/index.php), the international HapMap project (http://pngu.mgh.harvard.edu/~purcell/plink/res.shtml#hapmap), and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

Table 3.1 Sequence variants found throughout the genomic region of parkin.

Patient ID	Sequence variants
37.12	IVS2+ 20 C>T, IVS8 -21_-17del
42.03	IVS8+48C>T
42.04	IVS2+ 20 C>T, IVS4+75T>A
42.56	IVS2+ 20 C>T, R402C
43.59	IVS8+48C>T, V380L, IVS4+91C>A
52.23	IVS2+ 20 C>T
55.52	IVS2+ 20 C>T, L261L, V380L, IVS4+91C>A
55.65	IVS2+ 20 C>T, IVS4+ 75T>A, IVS8 -21_-17de
60.39	IVS2+ 20 C>T, P37P
60.43	V380L, D394N, IVS2-18 T>A
61.79	VS2+ 20 C>T, V380L
61.81	M192L, V380L
63.42	IVS2+ 20 C>T, P37P, IVS4+91 C>A
63.73	IVS2+ 20 C>T, P37P, C238C, IVS+91 C>A
78.74	G430D
96.87	IVS2+ 20 C>T, P37P, C238C, 3'UTR+16G>A
10.307	IVS8+48C>T, IVS4+ 75T>A
10.308	M192L, IVS8+48C>T
10.309	C238C , L261L, M192L Homo C , IVS4+ 75T>A
10.310	IVS4+ 75T>A
10.314	IVS8+48C>T, V380L, IVS4+ 75T>A
10.317	IVS2+ 20 C>T, P37P, C238C, IVS4+ 75T>A

Previously described mutations are highlighted in red. Common polymorphisms are highlighted in blue. Novel sequence variants identified in the present study are highlighted in black.

Similarly, no pathogenic mutations were identified in *DJ-1, VPS35, EIF4G1* and *GBA* (Table 3.2).

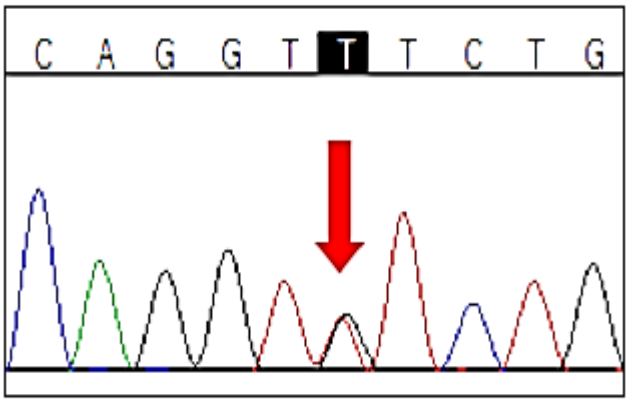
Table 3.2 Sequence variants identified in DJ-1, GBA, and selective exons of VPS35 and EIF4G1.

Sequence variants				
Patient ID	***DJ-1***	***GBA***	***VPS35***	***EIF4G1***
37.12	A56T	-	H579H	-
42.04	IVS1+84C>T, IVS4-98 G>A	-	-	-
42.56	G78G , IVS2-109 C>T	-	-	-
43.59	G78G	T369M	-	-
52.23	IVS2-109 C>T	-	-	-
54.74	IVS2-109C>T	-	-	-
55.52	IVS4-98 G>A, IVS6+32A>G	-	-	-
55.65	IVS5-31C>T, IVS6+32A>G	-	-	-
60.39	IVS4-98 G>A	T369M	-	-
60.43	IVS5-31C>T, IVS6+32A>G		-	-
61.79	IVS5-31C>T, IVS4-98 G>A	-	-	-
63.42	IVS1+37G>A, G78G	-	-	-
84.52	IVS4-98 G>A	-	-	-
11.793	ND	ND	H579H	-
11.794	ND	ND	H579H	-
11.795	ND	ND	H579H	-
11.796	ND	ND	H579H	-
11.895	ND	ND	H579H	-
11.798	ND	ND	H579H	-
11.799	ND	ND	H579H	-
11.800	ND	ND	H579H	-
11.801	ND	ND	H579H	-
11.802	ND	ND	H579H	-
11.803	ND	ND	-	R1223H
11.804	ND	ND	H579H	-
11. 806	ND	ND	H579H	-
11.808	ND	ND	H579H	-

ND= not done/screened as the DNA sample was unavailable at the time of screening. Common variants are highlighted in blue. Novel sequence variants identified in the present study are highlighted in black. (-)= no variants were found in the specified patient.

Common polymorphisms in *DJ-1* (A56T), and *GBA* (T369M), were identified in three unrelated patients (Figure 3.3).

Patient 37.12 (A56T)



Patient 43.59, and 60.39 (T369M)

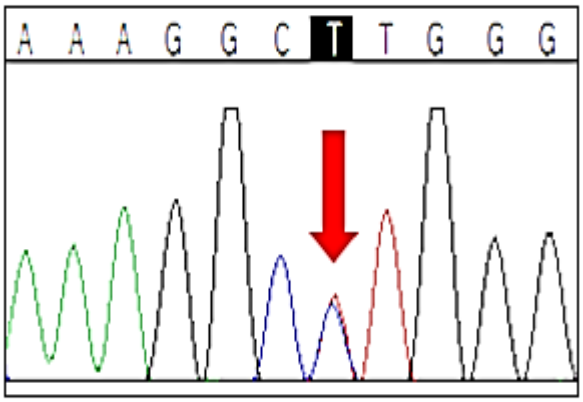


Figure 3.3 Sequence chromatographs indicating the position of the common polymorphisms found in exon 5 of *DJ-1* (529 G>T) (37.12), and exon 2 of *GBA* (745 C>T) (43.59, and 60.39).

Due to the high cost of Sanger sequencing, HRM was used as an alternative screening method. Figure 3.4 illustrates the use of HRM technique as a mutation screening method. The inclusion of positive control samples harbouring known mutations proved to be successful in distinguishing between wild-type samples and mutation-positive samples.

Using HRM to screen exons 2 and 3 of *SNCA,* and exons 31and 41 of *LRRK2*, no pathogenic mutations, or polymorphisms were detected.

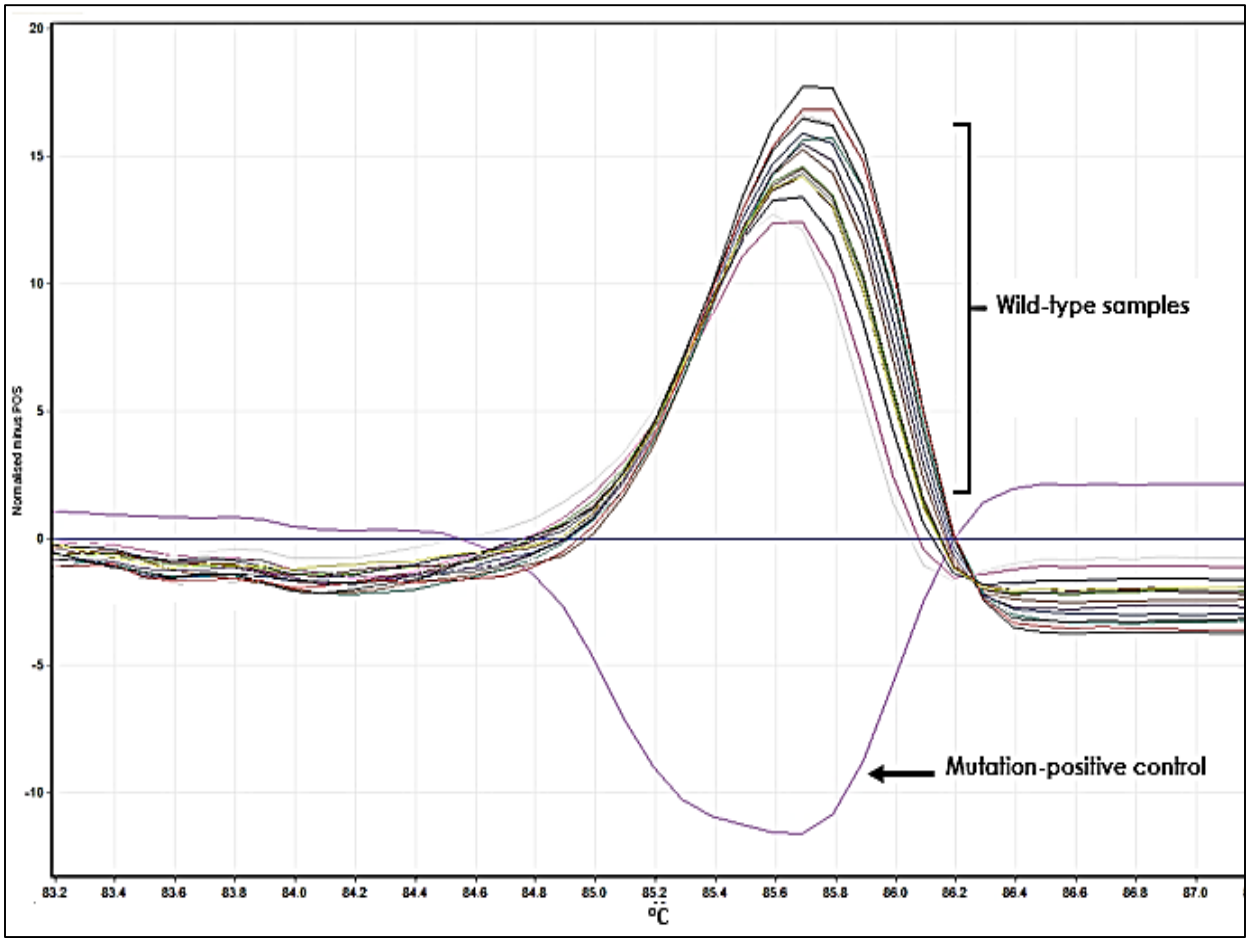


Figure 3.4 HRM difference graph indicating the G2019S variant in *LRRK2* exon 41 (used as a positive control), distinguished from the patient samples which are all wild-type. Normalized fluorescence subtracted from a reference sample is plotted on the y-axis and temperature in degrees Celsius is plotted on the x-axis.

3.3 Screening for copy number variation

CNVs were identified in two of the six known PD genes included in the MLPA assay, these results are summarized in Table 3.3.

Table 3.3 Potential heterozygous CNVs identified in MLPA probe mixes.

Patient ID	Mix 1 (P051-C3)	Mix 2 (P052-C2)	Verified using qPCR
37.12	**PARK2 ex2 Dup, PARK2 ex9 Del**	**-**	✓
61.81	**PARK2 ex5 Del**	**-**	×
10.307	**PINK1 ex1 Del; SNCA ex5 Del**	**-**	×
10.308	**PARK2 ex5 Del**	**-**	×
10.309	**SNCA, ex4,5,6,7 Dup**	**PARK2 ex4, ex9 Dup**	×

Previously described CNV highlighted in blue. Dup = duplication, Del = deletion, ex = exon. Novel CNVs identified in the present study are highlighted in black. ✓ indicates that the CNV was confirmed using qPCR, and **X** indicates that the CNV could not be confirmed as 'real' using qPCR.

No CNV was detected in the following PD causing genes included the MLPA assay: *PINK1, DJ-1, LRRK2*, and *ATP13A2*. The putative CNVs found in *SNCA* could not be verified using qPCR, because the amplification curves of the wild-type controls were not consistent across several runs, resulting in off-target amplification. The PCR products were run on an agarose gel, and revealed non-specific binding, which resulted in primer dimer formation. Further optimisation is required and a *SNCA* specific qPCR primer assay will need to be designed, and a variety of chemical additives (e.g. DMSO, formamide, or Betaine) will also be used as these may significantly minimize or eliminate non-specific amplification when performing a qPCR assay.

Figure 3.5 Graphical representation of previously described *parkin* exon 2 duplication, and exon 9 deletion identified in patient 37.12 by Keyser et al. (2010). Notably, probes for all exons of *parkin* are distributed across the two probmixes (i.e. Mix 1 P051-C3 and Mix 2 P052-C2), the same CNV should therefore be identified in both mixes to be considered for further assessment of potential pathogenicity on a functional level.

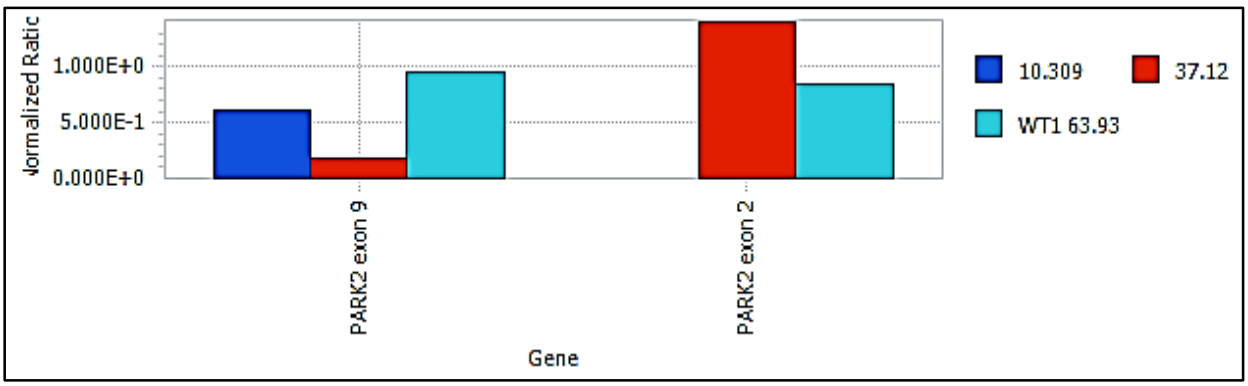


Figure 3.5 Graphical representing of possible CNVs in two unrelated patients. The light blue columns represents wild type sample, dark blue column represents the patient sample with a possible *parkin* exon 9 duplication, red columns represents the patient sample with a possible parkin exon 2 duplication, and exon 9 deletion. Columns represent the normalised peak height ratio of the target amplicon to the reference amplicon as calculated for each sample. The normalized ratio is plotted on the y-axis. The gene of interest and exonic region is plotted on the x-axis.

When one of the exons of the analysed gene undergoes a deletion, its peak height decreases and, at the same time, the peak height of the reference amplicon increases (as seen for 37.12, exon 9 deletion, red column). The situation is reversed in the case of duplication, with the peak of the reference amplicon decreasing and the peak of the analysed amplicon increasing (as seen for 37.12, exon 2 duplication, red column). In order to observe these differences, the results are compared with control DNA samples. The CNV identified in patient 10.309 was found to be a false-positive using qPCR, it was therefore concluded that this patient did not harbour the parkin exon 9 duplication.

The *parkin* exon 5 deletion could not be confirmed using qPCR. This was expected, as this possible CNV is generally considered to be a false positive, due to the presence of a M192L polymorphism in exon 5 which results in a single-base change that alters the binding site of the probes for exon 5 of *parkin in* MLPA kit P051-C3. This false positive CNV was identified in two unrelated patients, 61.81 and 10.308. Sequencing of the region to which the MLPA probes bound, did indeed confirm that both these patients harbour the M192L polymorphism (Figure 3.6), which affects the binding of the exon 5 probes for MLPA kit P051-C3.

Patients 10.308 and 61.81 (A>C)

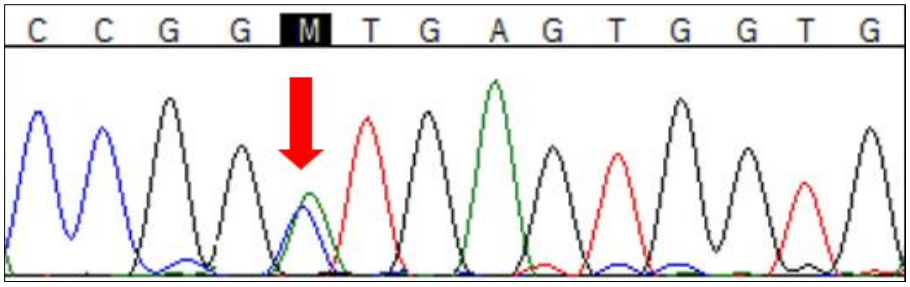


Figure 3.6 Sequence chromatograph confirming the presence of the M192L polymorphism found in the two patients with the false positive deletion in *parkin* exon 5.

3.4 NGS Analysis

NGS was used to screen all known PD-causing genes as well as 160 other genes involved in neurodegenerative diseases in 22 patients. A stepwise filtering approach was used to prioritize the list of variants, and these variants were then compared to those found in various public databases. Selective variants were confirmed using Sanger sequencing, and the frequency thereof was subsequently determined by screening a group of ethnic matched controls (*n* = 99). The following criteria were used to prioritize variants generated from the NGS panel:

1. Synonymous variants and common polymorphisms found in public databases such as dbSNP, 1000 Genomes Project and the Exome Sequencing Project (ESP) were excluded.
2. Only variants identified in patients, and not in controls were prioritized.
3. Only variants implicated in PD or Dementia were included. Dementia is a common feature of PD with disease progression.
4. Variants with a minor allele frequency (MAF) greater than 0.03% were excluded. The cut-off frequency was based on estimates of the global PD incidence, ranging between 3 and 22 per 100,000 person-years for all age groups (Muangpaisan et al. 2011).

Using the above criteria, a prioritized list of 15 sequence variants in 10 genes was compiled (Table 3.4). These were selected from an initial list of 98 sequence variants in 35 genes in both patients and controls. Using Sanger sequencing, 13 variants were validated, while the variants found in *MAPT* and *ATP13A2* were found to be false-positives.

Table 3.4 List of prioritised sequence variants identified using high-throughput targeted resequencing.

Patient ID	rsID	Gene	UCSC Exon	AA Change	Disease	dbSNP	1kG MAF	ESP EA MAF	ESP AA MAF	Validated by SS
61.81	novel	ATP13A2	Exon 22	S811R	Kufor-Rakeb, PD	NA	0	NA	NA	x
55.65	rs112725508	DCTN1	Exon 8	P209R	ALS, FTD, PD	NA	0	NA	NA	√
60.43	rs112868112	DNAJC13	Exon 44	E1740D	Dementia, PD	NA	0	NA	NA	√
96.87	rs121908301	GBA	Exon 12	G517R	Dementia, PD	NA	0	NA	NA	√
54.74	novel	LRRK2	Exon 16	I610T	PD	NA	0	NA	NA	√
61.81	novel	LRRK2	Exon 36	H1758P	PD	NA	0	NA	NA	√
84.52	novel	LRRK2	Exon 44	N2133S	PD	NA	0	NA	NA	√
52.23	novel	LRRK2	Exon 49	T2423S	PD	NA	0	NA	NA	√
10.308	novel	MAPT	Exon 11	L550P	FTD, PD	NA	0	NA	NA	x
78.74	rs191486604	PARK2	Exon 12	G430D	PD	0	0	0.000349	0	√
54.74	rs137853055	PARK2	Exon 8	Q311K	PD	0	0	NA	NA	√
10.308,10.309, 61.81	rs115477764	PINK1	Exon 7	E476K	PD	0.00011	0.009	0	0.042896	√
10.307	rs63751071	PSEN1	Exon 5	I143M	Dementia	NA	0	NA	0	√
61.81	rs112451138	PSEN1	Exon 7	V191A	Dementia	NA	0	NA	0	√
10.310	Not assigned	PSEN2	Exon 6	V139M	Dementia	NA	0	0.000116	0	√

Variants highlight in red = previously reported pathogenic mutations, blue = common variants, black = sequence variants not previously described in literature. 1kG MAF= 1000 Genomes project minor allele frequency of variants, ESP EA MAF = minor allele frequency of variants in European American populations, ESP AA MAF = minor allele frequency of variants in African American populations. AA = amino acid change. √ indicates that variants were validated using Sanger sequencing (SS)

Three *in silico* functional predictive programs, namely SIFT, PolyPhen-2, and MutationTaster were concurrently used to predict the potential impact each amino acid substitution may potentially have on the function of the encoded protein. These functional predictions are summarized in Table 3.5.

Table 3.5 *in silico* functional predictions, based on amino acid substitutions of variants found in the respective genes.

Gene	Exonic region	AA change	SIFT prediction	PolyPhen 2 prediction	MutationTaster prediction	Concordance across all three programs
DCTN1	Exon 8	P209R	Tolerated	Benign	Polymorphism	Yes
DNAJC13	Exon 44	E1740D	Intolerant (LC)	Benign*	Polymorphism	No
GBA	Exon 12	G517R	Tolerant	Benign	Polymorphism	Yes
LRRK2	Exon 16	I610T	NV	NV	Polymorphism	No
LRRK2	Exon 36	H1758P	NV	NV	Polymorphism	No
LRRK2	Exon 44	N2133S	NV	NV	Disease causing	No
LRRK2	Exon 49	T2423S	NV	NV	Polymorphism	No
PARK2	Exon 12	G430D	Intolerant	Probably damaging	Disease causing	Yes
PARK2	Exon 8	Q311K	Intolerant	Probably damaging	Disease causing	Yes
PINK1	Exon 7	E476K	Tolerated	Benign	Polymorphism	Yes
PSEN1	Exon 5	I143M	Intolerant	Probably damaging	Disease causing	Yes
PSEN1	Exon 7	V191A	Intolerant	Probably damaging	Disease causing	Yes
PSEN2	Exon 6	V139M	Intolerant (LC)	Benign*	Polymorphism	Yes

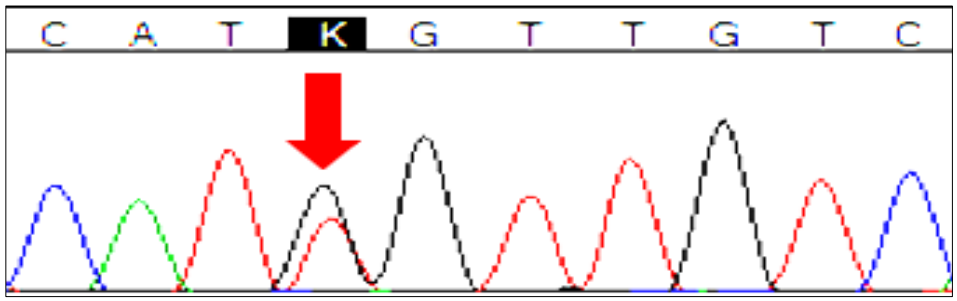
PolyPhen-2 predictions with an asterisk should be interpreted with caution, as the protein alignment did not have enough sequence diversity for the algorithm to give a confident score. SIFT (LC) predictions= low confidence (i.e. the protein alignment does not have enough sequence diversity). NV (not valid) = transcript has several partial isoforms.

Only classifications which were consistent across all three predictive programs were considered to be of high confidence. Consistent predictions could not be made for the 4 *LRRK2* novel variants using SIFT and PolyPhen-2, possibly because annotation of these variants was performed with ANNOVAR using GRCh37/hg19 as the reference genome (performed by Professor Matthew Farrer), whereas the publically available predictive programs currently are still using the NCBI36/hg18 reference genome from March 2006 (available at http://genome.ucsc.edu).

3.4.1 Known pathogenic mutations

Of the 13 variants confirmed using Sanger sequencing, I143M, and V191A (Figure 3.7 and Figure 3.8) were of interest given that these variants are listed as 2 of more than 150 mutations shown to be causal for early-onset familial Alzheimer's disease (AD) (http://www.molgen.vib-ua.be/ADMutations/). These mutations are found in exon 5 and 7 respectively, in the gene encoding for presenilin-1 (*PSEN1)*. The I143M mutation is thought to alter the way in which the amyloid precursor protein is cleaved, this alteration results in aggregation of the protein in the brain, leading to nigral damage, and the development of AD. In the present study, the I143M mutation was found in one male proband with AAO of 44 years (10.307), and V191A mutation was found in another male proband with AAO of 55 years (61.81). These variants were found to be evolutionarily-conserved across different species, and predicted to be pathogenic by all three functional predictive algorithms (Figure 3.7 and Figure 3.8). However, to the best of our knowledge, neither of these patients have been clinically diagnosed with AD, nor do they have a known family history of the disease (personal communication, Dr. Kumire).

A



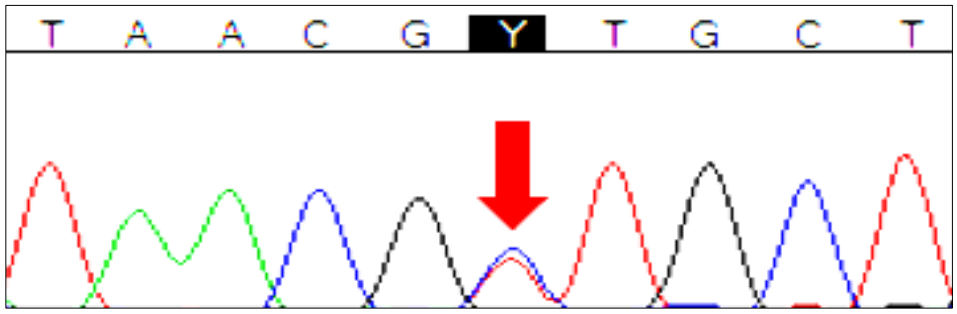
I143M variant

B

```
Zebrafish      PFREDTETVGQRALHSMLNAIIMISVIVVMTLVLVVLYKYRCYKVIQAWLFFSNLLLLFF
AC frog        PFTEDTESVGQRALNSILNATIMISVIIVMTILLVVLYKYRCYKVIHGWLIISSLLLLFF
Cattle         PFTEDTETVAQRALHSILNAVIMISVIVIMTILLVVLYKYRCYKVIHAWLIVSSLLLLFF
Human          PFTEDTETVGQRALHSILNAAIMISVIVVMTILLVVLYKYRCYKVIHAWLIISSLLLLFF
Mouse          PFTEDTETVGQRALHSILNAAIMISVIVIMTILLVVLYKYRCYKVIHAWLIISSLLLLFF
               ** ****:*.****.*:*** ******::**::**************:.**:.*.******
```

Figure 3.7 (**A**) Sequence chromatograph of a proband with the I143M (425 G>T), variant in exon 5 of *PSEN1* (**B**) Sequence alignments of I143M in Zebrafish (NP_571099.1), African clawed (AC) frog (AAH77762.1), cattle (AAI51459.1), human (NP_000012.1), and mouse (AAH30409.1). The highlighted amino acids indicate the position of the I143M variant. The conserved amino acids are indicated by asterisk.

A



V191A variant

B

```
Zebrafish       FSLIYLGEVFKTYNVAMDYFTLALIIWNFGVVGMICIHWKGPLRLQQAYLIMISALMALV
AC frog         FSYIYLGEVFKTYNVAVDYITLALLIWNFGVVGMICIHWKGPLLLQQAYLIMISALMALV
Cattle          FSFIYLGEVFKTYNVAMDYISVALLIWNFGVVGMIAIHWKGPLRLQQAYLIMISALMALV
Human           FSFIYLGEVFKTYNVAVDYITVALLIWNFGVVGMISIHWKGPLRLQQAYLIMISALMALV
Mouse           FSFIYLGEVFKTYNVAVDYVTVALLIWNFGVVGMIAIHWKGPLRLQQAYLIMISALMALV
                ** *************:**.::**:**********.******* ****************
```

Figure 3.8 (**A**) Sequence chromatograph of a proband with the V191A (571 C>T) variant in exon 7 of *PSEN1.* (**B**) Sequence alignments of V191A in Zebrafish (NP_571099.1), African clawed (AC) frog (AAH77762.1), cattle (AAI51459.1), human (NP_000012.1), and mouse (AAH30409.1). The highlighted amino acids indicate the position of the V191A variant. The conserved amino acids are indicated by asterisk.

3.4.2 *HRM screening in ethnically-matched controls*

The frequency of the prioritized variants was determined in 99 Black SA controls. Only the *DNAJC13* E1740D variant was found in a control at a frequency of 1.0%. The variant was easily distinguished using HRM (Figure 3.9). As observed from the normalized melt curve, the sample with the E1740D variant has a different melt curve profile to that of the wild-type control samples. However, the melt profile of the positive control is identical to that of the E1740D-positive sample.

The presence of the E1740D variant was verified in one control sample using Sanger sequencing (Figure 3.10).

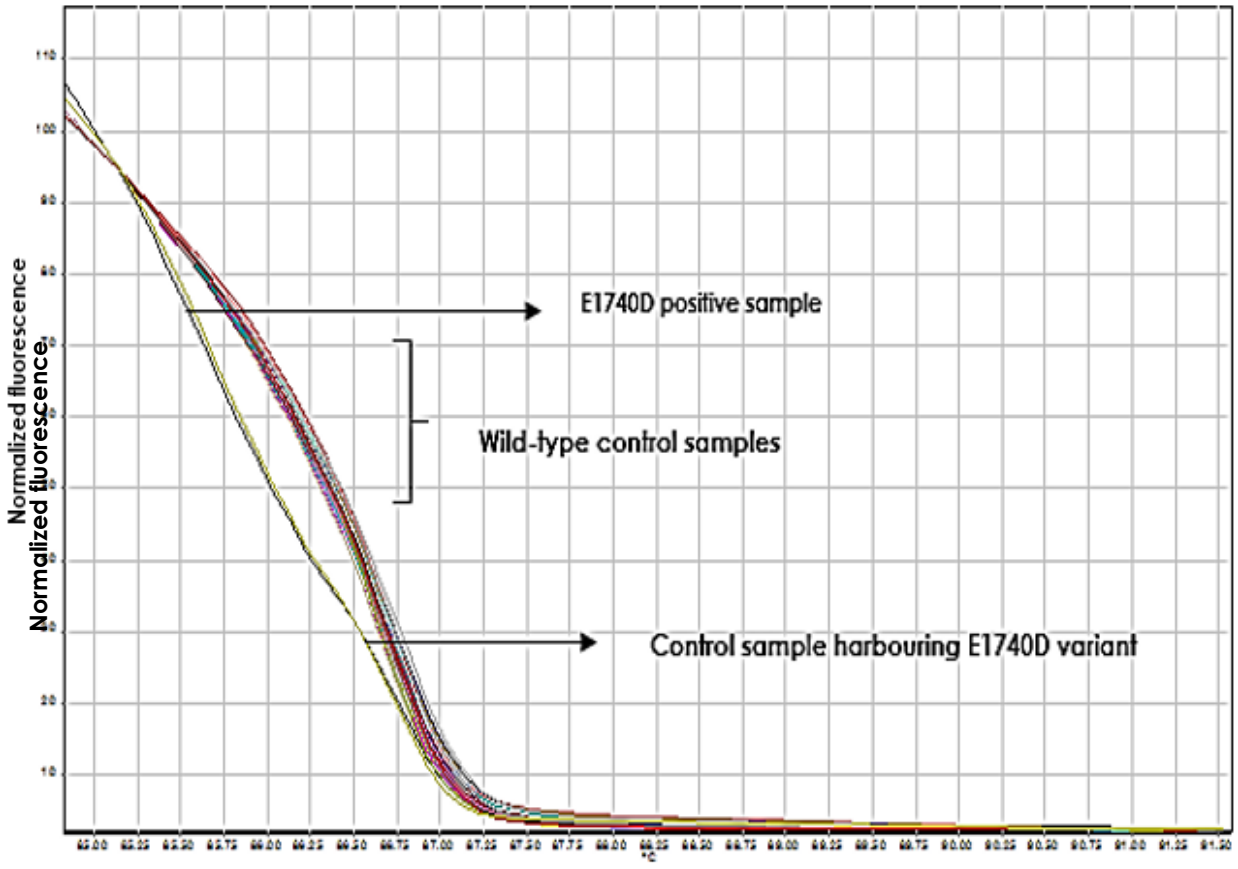


Figure 3.9 HRM normalized graph illustration of the E1740D variant indicating that the sample harbouring the variant can be distinguished from the wild-type. Normalized fluorescence subtracted from a reference sample is plotted on the y-axis and temperature in degrees Celsius is plotted on the x-axis.

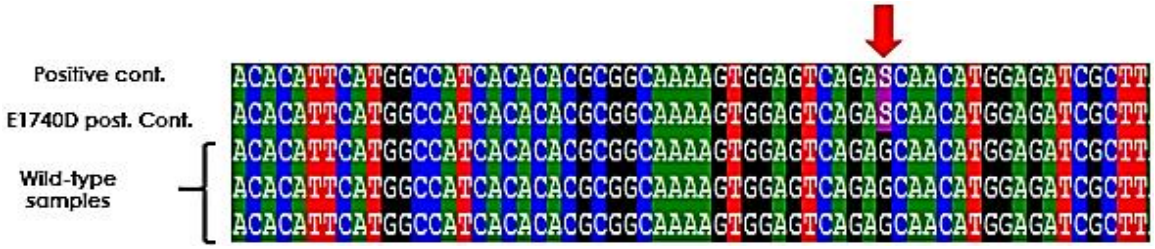


Figure 3.10 Sequence alignment generated using BioEdit. Sequence alignment of the positive sample, a control sample found to be positive for the E1740D variant in exon 44 of DNAJC13, and three WT samples. The position of the E1740D variant is indicated by the red arrow.

3.5 Repeat expansions at the SCA7 and SCA2 loci.

3.5.1 *SCA7*

To genotype the CAG repeats at the *SCA7* locus, the DNA samples of all 47 Black PD patients were successfully amplified using TP-PCR (Figure 3.11).

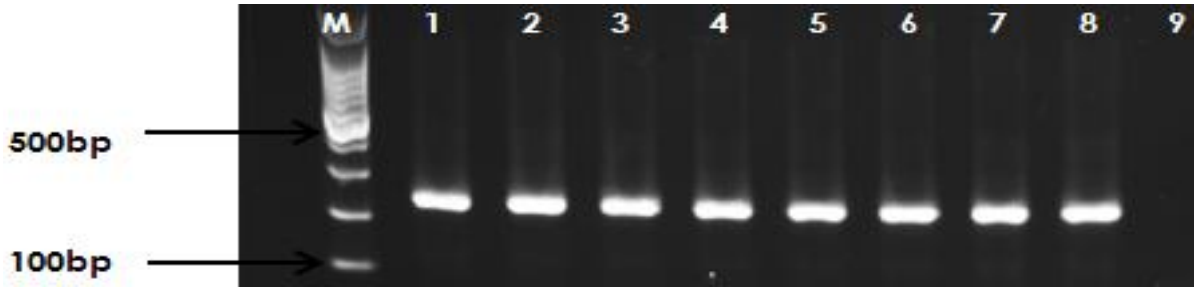
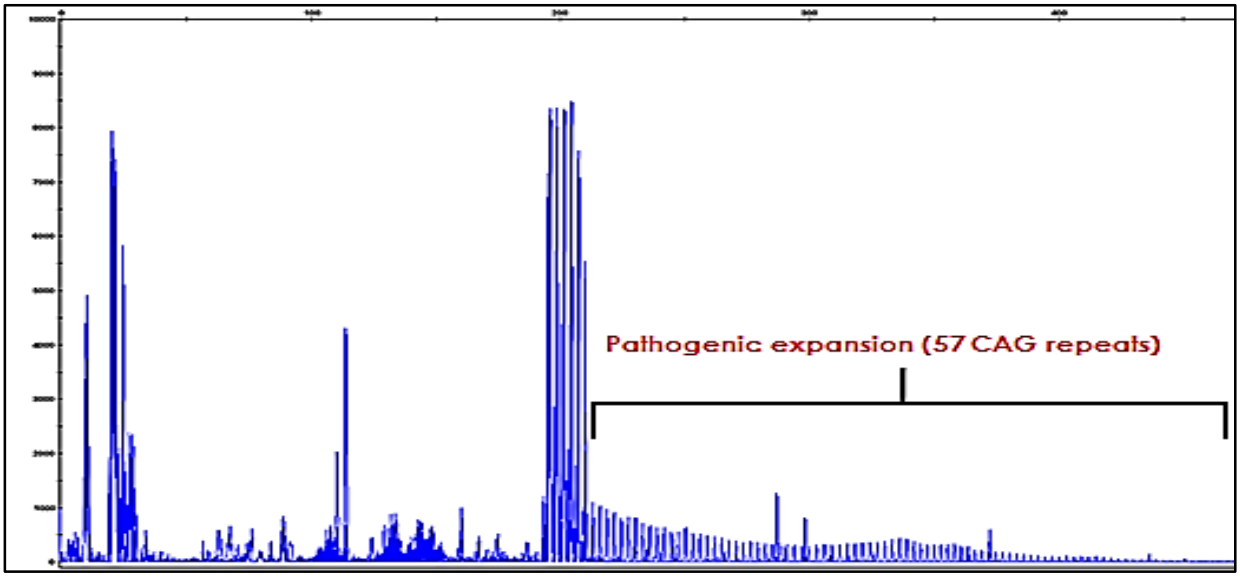


Figure 3.11 *SCA7* exon 3 fragments amplified by PCR. The PCR products were loaded on a 1.5% agarose gel, resolved for 1 hour at 100V in 1X SB buffer and stained with 0.35µg/ml EtBr. The photographs were taken after exposure to UV light. A 100bp molecular weight marker was loaded in the first lane (M) of each gel, with the 100bp and 500bp band indicated. Lanes 1 to 8 represents the different patient samples, with the negative control loaded in lane 9.

Genotyping of the PCR products was subsequently performed using the ABI 3130xl Genetic Analyzer, which facilitated distinction between patients with a normal *SCA7* profile (<19 CAG repeats), and those harbouring CAG repeats, which fall within the pathogenic expanded range (>33 repeats). The size standard GeneScan™ LIZ250 was used to analyse the electropherogram peaks using GeneMapper software. All samples were genotyped on at least two separate occasions. A positive control with 57 CAG repeats was also included in the ABI analysis for comparative purposes (Figure 3.12 A). Of the 47 patients screened, 45 had a *SCA7* profile which was similar to that of a wild-type sample (Figure 3.12 B).

A. Positive control with a pathogenic peak profile



B. Patient with a normal repeat peak profile

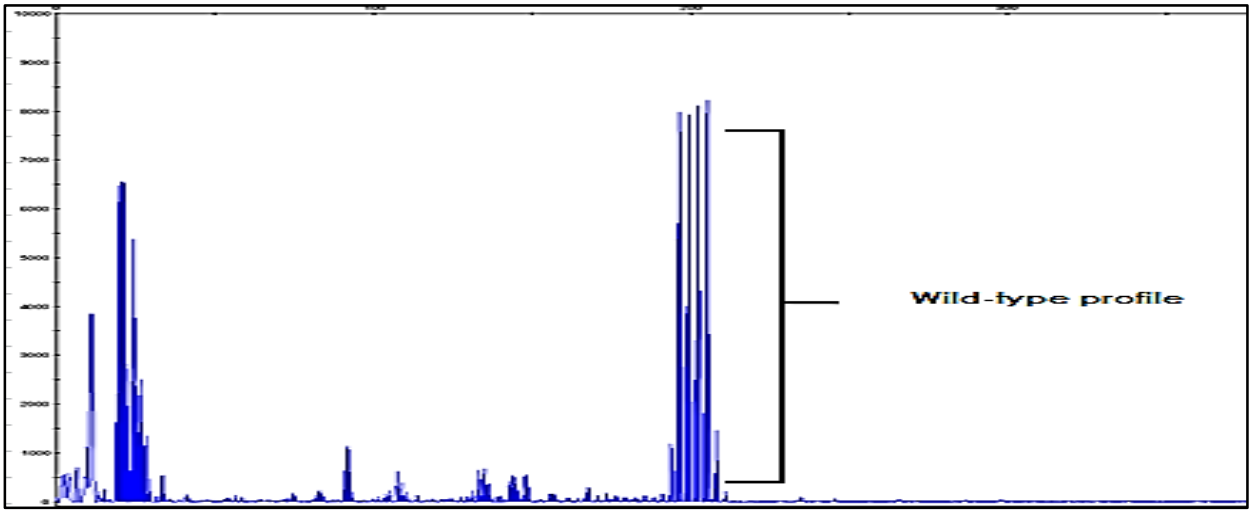
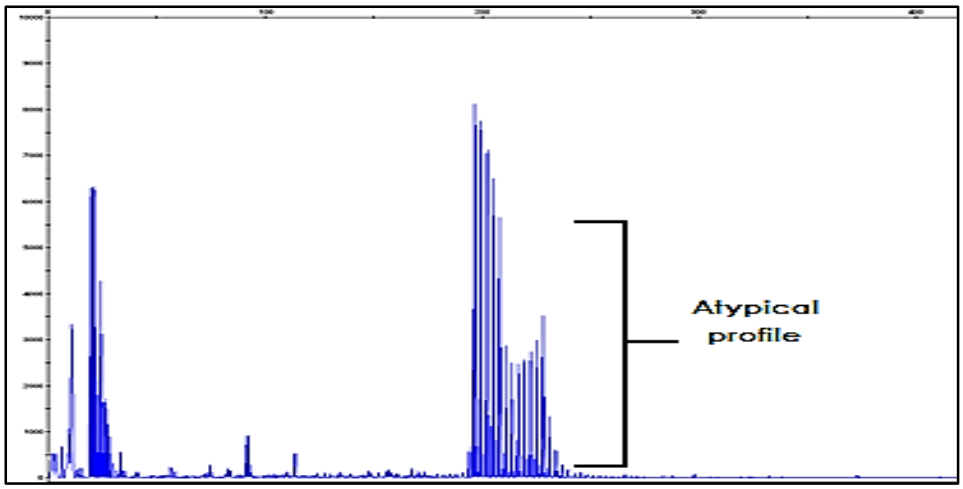


Figure 3.12 Electropherograms of *SCA7* profiles. (**A**) The peak profiles show a gradually descending array of peaks, characteristic of a positive control with a repeat expansion in the pathogenic range. (**B**) Characteristic peak profile of an individual with a repeat expansion in the normal range (*SCA7* negative). Numerical values on the x-axis denote the product size (in bp), and the y-axis denotes arbitrary units of fluorescence intensity.

The SCA7 profile for two unrelated male patients was observed to be atypical (Figure 3.13), and may be indicative of a CAG repeat expansion which falls within the intermediate range (i.e. 28-35 CAG repeats).

A. Patient 11.798



B. Patient 11.799

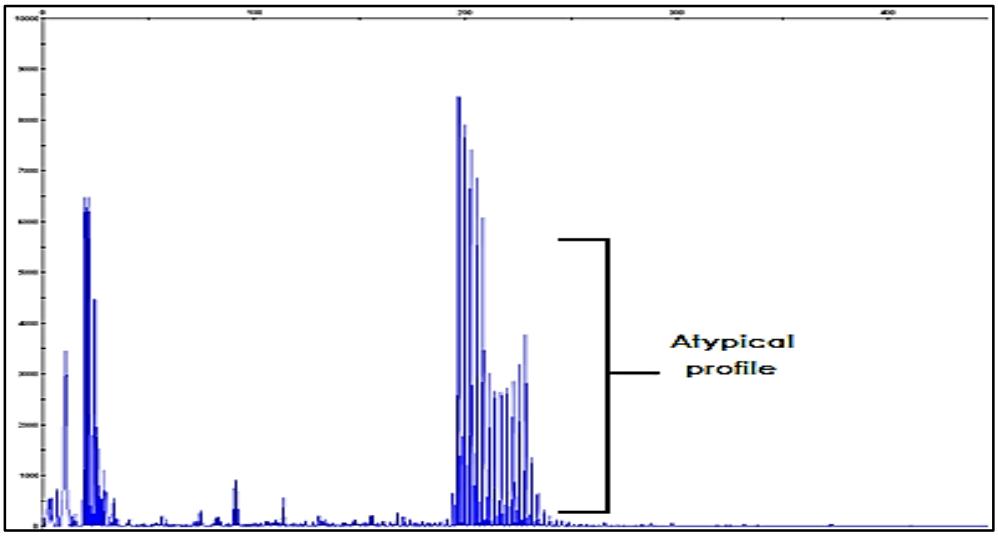


Figure 3.13 Electropherograms of two patients with an atypical *SCA7* profile.

Further genetic analysis of the two patients were undertaken in order to clarify their *SCA7* status.

Figure 3.14 illustrates that the HRM technique could be effectively utilized to distinguish the positive control sample harbouring the 57 CAG repeats (pathogenic range) from the patients who were shown to have the atypical *SCA7* peak profile (Figure 3.13 A and B), and the wild-type samples. From the HRM melt curve profile, it can be deduced that patients 11.799 and 11.798 do not harbour *SCA7* repeats which fall within the pathogenic range, as their melt profiles were clearly distinguishable from that of the positive control.

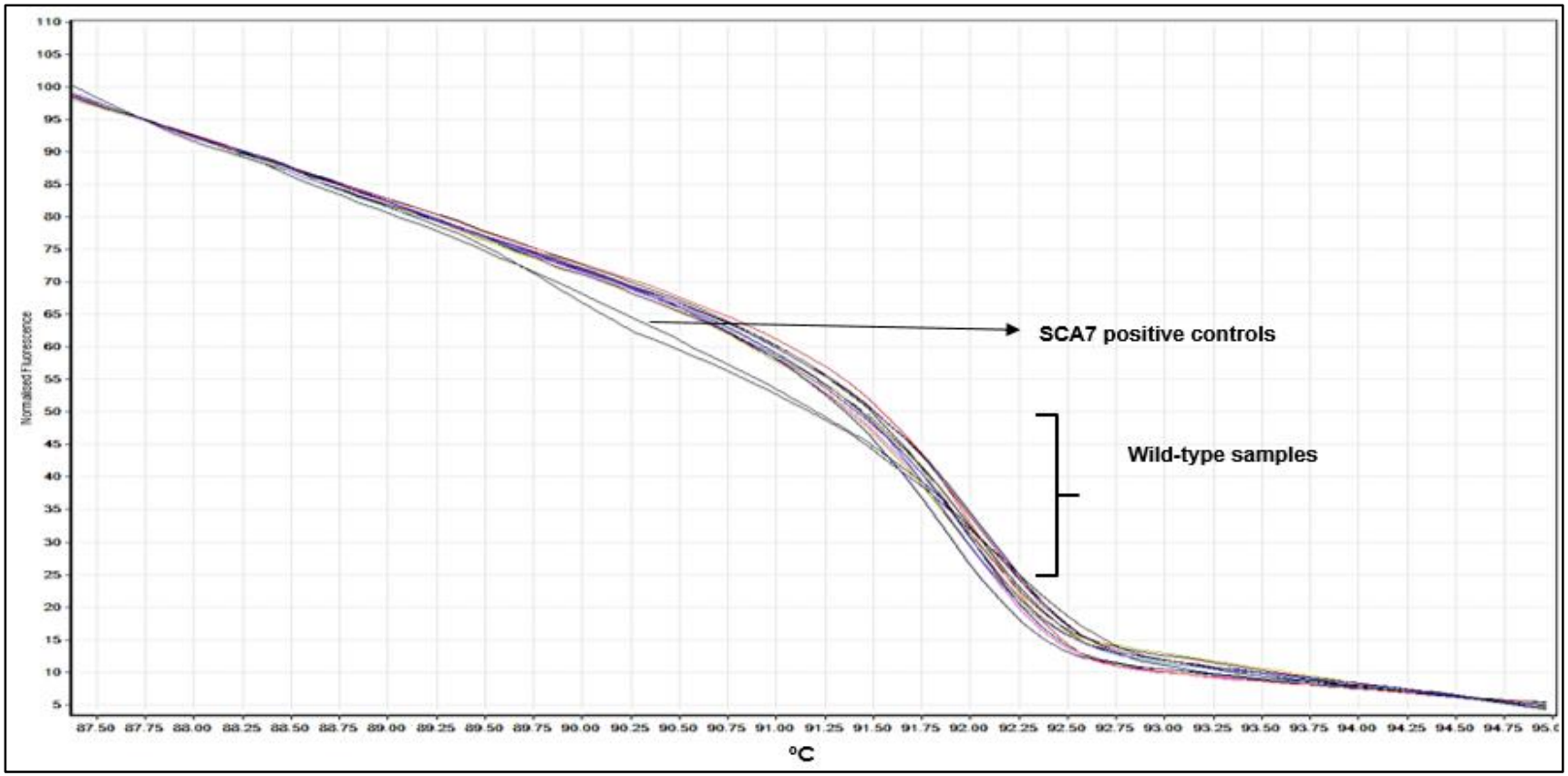


Figure 3.14 HRM curve exhibiting the melt profile of the positive control, from that of patient 11.799, 11.798 and samples which are all wild-type. Normalized fluorescence subtracted from the reference sample is plotted on the y-axis, and the temperature in degree celsius is plotted on the x-axis.

3.5.2 *SCA2*

Exon 1 of the SCA2 locus was successfully amplified in all 47 Black patients by PCR (Figure 3.15)

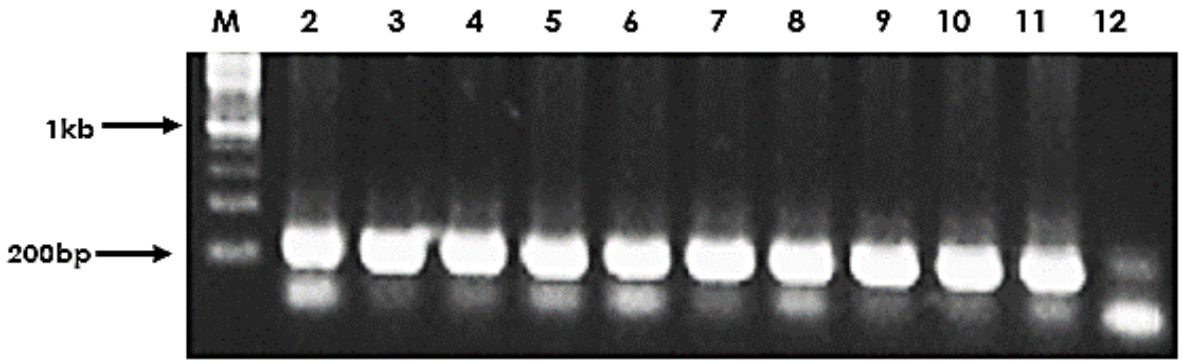
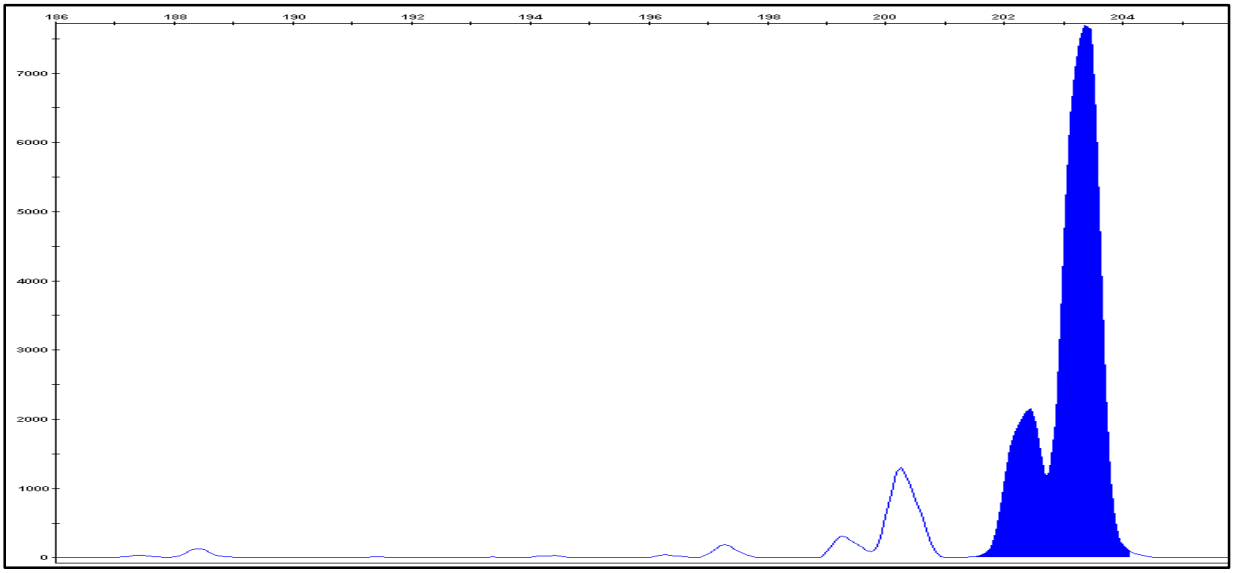


Figure 3.15 *SCA2* exon 1 fragment amplified by PCR. The PCR products were loaded on a 1.0% agarose gel, resolved for 30 minutes at 120V in 1X SB buffer and stained with 0.35µg/ml EtBr. The gel photographs were taken after exposure to UV light. A 200 bp molecular weight marker was loaded in the first lane (M), with the 200bp and 1kb band indicated. Lanes 2 to 11 represents the different patient samples, with the negative control loaded in lane 12.

Genotyping of the PCR products was subsequently performed using the ABI 3130xl Genetic Analyzer, which facilitated distinction between alleles in the normal range (14 to 30 repeats) and those in the pathogenic expanded range (34 to 77 repeats) using the the size standard GeneScan™ LIZ250. All samples were genotyped on at least two separate occasions.

Figure 3.16 (A) shows the ABI profile of a patient who is homozygous for 22 CAG repeats, and Figure 3.16 (B) shows the ABI profile of a patient who is heterozygous for the same number of repeats at the *SCA2* locus.

A. Patient 60.39



B. Patient 55.65

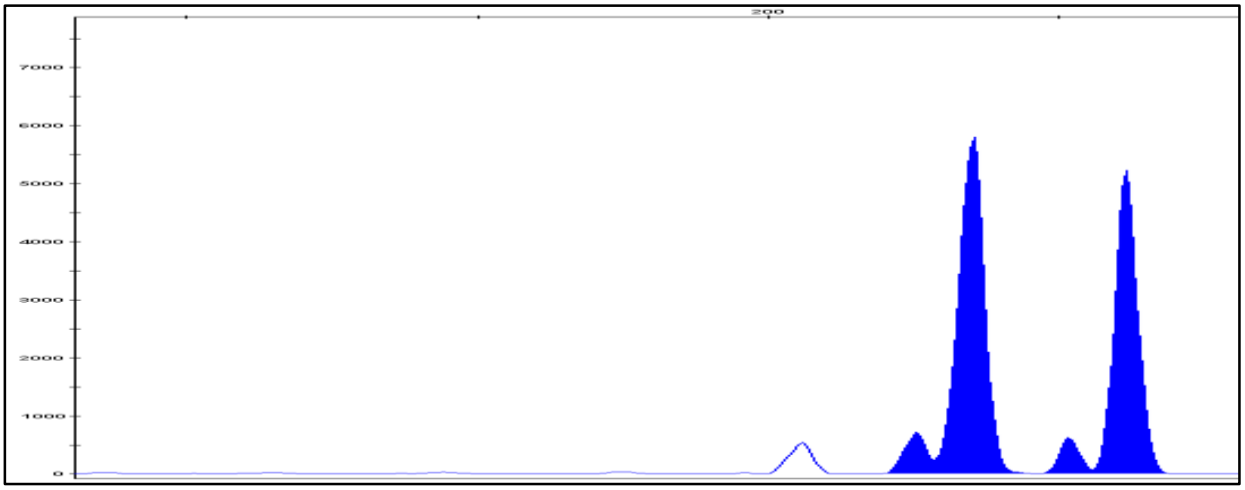


Figure 3.16 ABI *SCA2* genotypic profiles. Peak profiles could be distinctly classified as (**A**) homozygous or (**B**), heterozygous based on each individuals electopherograph profile.

In order to accurately size the CAG repeats at the *SCA2* locus, two patients with homozygous profiles were selected for sequencing, the PCR products were amplified using an unlabelled primer pair identical to that previously used to genotype the repeats using the ABI Genetic Analyzer.

The sequence chromatograph of one individual (who was found to harbour 22 repeats at the *SCA2* locus, Figure 3.17) was then used as a basis to calculate the repeat length for all the other patients using GeneMapper® software.

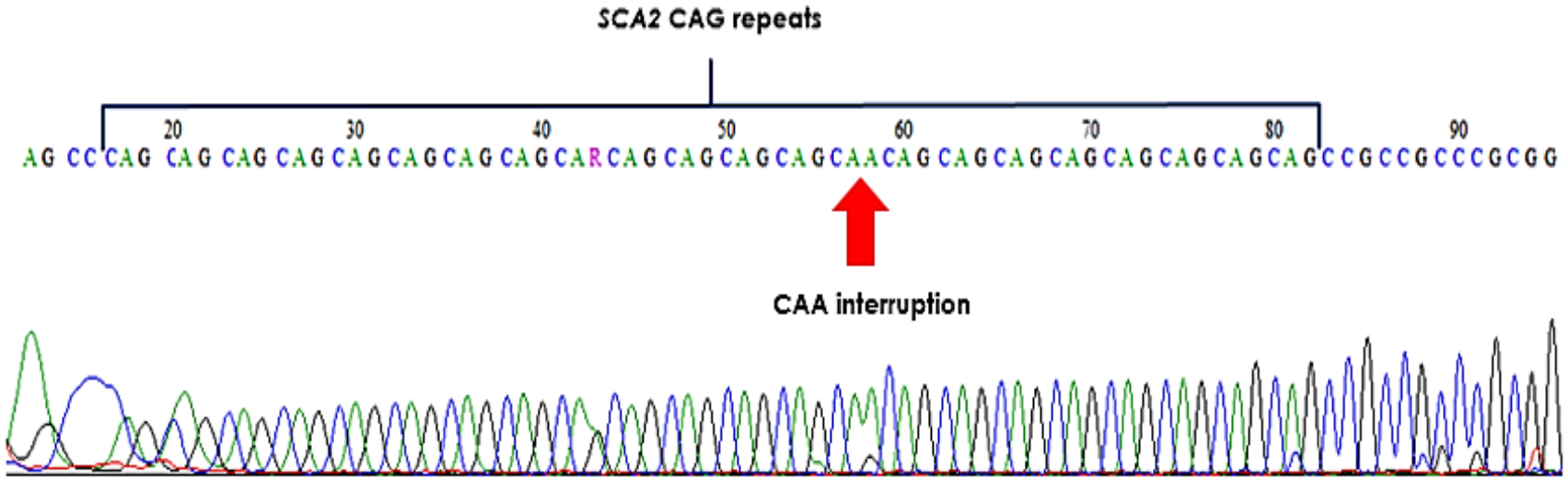


Figure 3.17 Sequence chromatogram of a patient harbouring 22 CAG repeats, which falls within the normal range of repeats in exon1 of the *SCA2* locus.

None of the patients harboured a pathogenic repeat expansion at the *SCA2* locus. All 47 patients screened were found to have a *SCA2* repeat length which falls within the normal range (i.e. 14-30 CAG repeats). It was determined that 22 repeats were the most common number of repeats among the 94 chromosomes screened as illustrated in Figure 3.18. Twenty-two repeats were present at a frequency of 68% (64/94).

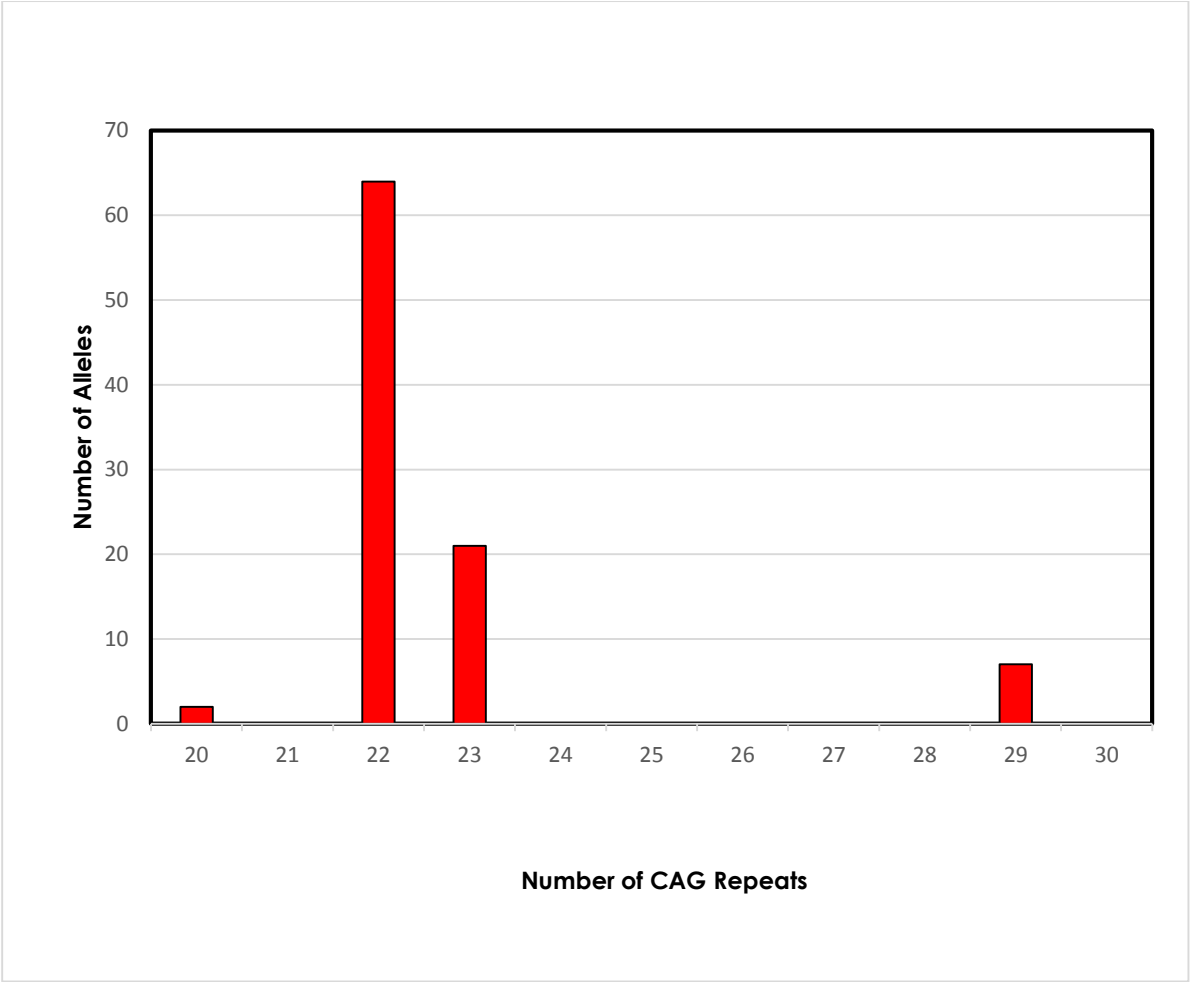


Figure 3.18 Graphic representation of the number of repeats in the *SCA2* gene in the 47 Black PD patients.

Twenty-one chromosomes were found to have 23 repeats at the *SCA2* locus (22.3%), 7 chromosomes had 29 repeats (7.4%), which is the largest number of repeats identified in this group of patients, while 20 repeats were found at the lowest frequency (2.1%), with only two chromosomes having this number of repeats at the *SCA2* locus.

CHAPTER 4: DISCUSSION

4.1 General discussion

A growing body of studies thus far have comprehensively reported on the genetic aetiology of PD. However, most have targeted the European, North-American and East-Asian populations (Dahodwala et al., 2009). Genetic studies on the SSA populations, especially those of Black African ancestry remain almost totally underrepresented, or unexplored in some countries (Blanckenberg et al., 2013; Pringsheim et al., 2014). This is a significant shortcoming given that there is strong evidence to suggest that SSA populations have the highest degree of genetic diversity, compared to other populations, and it is thought that this genotypic and phenotypic diversity may even exist within the same population group (Tishkoff et al., 2009).

To the best of our knowledge, the present study is the first report on the molecular genetic basis of PD in the Black SA ethnic group. Moreover, this is the first comprehensive analysis of all known PD genes: *SNCA, LRRK2, EIF4G1, VPS35, parkin, PINK1, DJ-1,* and *ATP13A2* in this population to date. Common disease-causing mutations were not detected using a variety of mutation screening techniques. It is therefore possible that other, still unknown genetic determinants or novel genes for PD may account for the disorder in this population group. Two patients carrying previously described *parkin* mutations were verified in the present study. However, no mutations were found in the remaining PD causing genes in any of the other patients screened. Given the unique ancestry of the Black SSA population, it is possible that PD disease-causing genes found in other ethnic populations worldwide, may not contribute to the disease aetiology in this group of patients. Although this study represents the largest Black SA PD cohort screened to date (n=47), it is necessary to screen a larger cohort in order to obtain a more representative, and accurate estimate of the frequency of PD mutations which may be attributable to the Black SA population.

The genetic aetiology of PD is thought to be distinctly different across the various ethnic population groups worldwide (Van Den Eeden et al., 2003; Wright Willis et al., 2010). A case-in-point is the *LRRK2* G2019S mutation in exon 41 which has been found in 30–40% of familial and sporadic PD patients from North Africa, and 10–30% of Ashkenazi Jews (Lesage et al., 2006; Ozelius et al., 2006). About 0-2% of PD patients in other countries carry this mutation, but G2019S has never been reported in a single Black patient to date (Guedes et al., 2010; Bardien et al., 2011). Okubadejo and colleagues performed a study in which fifty-seven Nigerian patients with sporadic PD were screened, this included sequencing of *LRRK2* exon 31 and exon 41, all *parkin* exons, as well as repeat expansions at the *SCA3* locus (Okubadejo et al., 2008). No pathogenic mutations were identified in any of these genes. Another study also sequenced *LRRK2* exon 31 and exon 41 in a Black Ghanaian PD cohort consisting of fifty-four patients, once again, no pathogenic mutations were detected (Cilia et al., 2012). Similar results have also been found in a study by Yonova-Doing et al. (2012) in which thirty-eight Zambian patients were screened

for all exons of *SNCA*, *LRRK2*, *parkin*, *PINK1*, and *DJ-1* using Sanger sequencing, and MLPA analysis of *parkin*, *PINK1*, *DJ-1*, *LRRK2*, and *SNCA* was also performed. A common limitation encountered in all of these studies is that of a small patient sample size. Nevertheless, studies performed thus far, concur that further genetic screening of Black PD patients is warranted, given that the majority of cases screened to date cannot be attributed to known PD genes.

Relatively few epidemiologic studies on PD in African countries have been published so far, thus the real prevalence of this disorder in this region is still unclear. However, populations are undergoing a demographic transition which is characterized by an increase in mean life expectancy (Dotchin et al., 2012). Population ageing is a common trend in developed countries where the largest proportion of elderly people currently resides (Sanderson and Scherbov, 2014). However, this trend has only recently been described in SSA populations, with the latest projections suggesting that the gap in life expectancy between SSA and the world average will drop to 10 years by 2050, from nearly 20 years in 2010 (Dorsey et al., 2007; Sanderson and Scherbov, 2014). As life expectancy continues to increase, age-related disorders such as PD are expected to become increasingly common, particularly given the current lack of preventative therapy, predictive markers, or a cure for the disease. Given the scarcity of genetic studies on PD in SSA populations and the expected rise in the incidence of this disease in these populations, the aim of the present study was to determine the genetic aetiology of PD in a group of Black SA patients.

In the first part of the study, a description of the current clinical profile of thirty-two individuals was documented as provided by consulting Neurologist Professor Jonathan Carr and Dr. Percy Kumire from the Movement Disorders Clinic at Tygerberg Academic Hospital. A systemic analysis was performed to assess if there is any appreciable clinical variation between these and other SA PD patients of different ethnicities. Overall, the clinical profile of PD in Black SA patients was considered to be similar to that of other population groups, although young-onset cases were less commonly encountered, and the frequency of a positive family history was lower than that reported for White SA PD groups. However, such differences could be attributed to ascertainment biases.

In the genetic part of the study, the patients were screened for known PD mutations in *parkin*, *DJ-1*, *EIF4G1*, *VPS35, LRRK2*, *SNCA*, and *GBA* using Sanger sequencing and HRM. The Sanger sequencing biochemistry is considered 'gold-standard' as a mutation screening technique given its ability to achieve read-lengths of up to 1,000 bp with a potential detection accuracy of 99.999% (van Dijk et al., 2014). The heterozygous G430D mutation which was found in one of our patients was initially described by Sun et al. (2006), and later described by Haylett et al. (2012) in a separate study by our group. In the latter study, the G430D mutation was found in two unrelated patients of different ethnic backgrounds (i.e. Black and White) with an AAO of

56 and 27 years respectively, each harbouring the G430D mutation, in addition to an exon 4 deletion in *parkin* (Haylett et al., 2012). Sequence variants were classified as polymorphisms if the variant had a minor allele frequency of ≥ 1% across various public databases without known association to disease, was located in a non-coding region, or resulted in a synonymous change. All of the polymorphisms identified in the present study, have been described previously in the SA population in separate studies by our group (Bardien et al., 2009; Keyser et al., 2010; Haylett et al., 2012). These include: six non-synonymous variants (M192L, V380L, R402C, G235V, S167N and D394N), three synonymous variants (P37P, L261L, and C238C), and two intronic variants (IVS2 +25T>C, IVS8+48C>T) found throughout the *parkin* genomic region. In addition, five novel intronic variants were found in the present study (IVS4+75T>A, IVS4+91C>A, IVS4 +75T>A, IVS4 +100C>A, IVS4 +101G>C). Taken together, we did not find *parkin* mutations to confer a major contribution to disease in the current patient group; only 1 out of the 24 patients screened using Sanger sequencing were found to have pathogenic mutations. In contrast, previous studies of numerous PD families of different ethnic backgrounds worldwide have demonstrated a considerable contribution of *parkin* mutations to early-onset (≤50 years) PD forms (Lücking et al., 2000; Hedrich et al., 2004). This difference may possibly be attributed to the AAO of our patient cohort. Of the patients screened in the present study using Sanger sequencing, 38% (9/24) had an AAO of less than 50 years (range 30-49). It is possible, as demonstrated in several studies, that the frequency of *parkin* mutations decreases significantly with increasing age at disease onset (i.e. occurs in nearly 80% of individuals with AAO before age 20, and <25% in those with onset after 50 years of age) (Periquet et al., 2003; Hedrich et al., 2004). The low frequency of *parkin* mutations in our study cohort could therefore be partially attributed to the late age of disease onset for the majority of the patients screened (i.e. 62.5%, 15/24), who had an AAO of between 50 and 74 years, or due to the fact that these patients harboured mutations in other genes.

Mutations in *DJ-1* are the least common of all known causes of ARPD forms (<1% of early-onset PD) (Sironi et al., 2013). It was therefore not surprising that no novel or known pathogenic mutations were identified in *DJ-1*. Furthermore, six intronic variants, one non-synonymous (A56T), and one synonymous (G78G) polymorphism were found throughout the genomic region of *DJ-1* in the 22 patients screened in this part of the study.

Two patients were found to harbour a non-synonymous polymorphism in *GBA* (T369M). This polymorphism was previously classified as a mutation by Beutler et al. (1996), but later found to be a common polymorphism in 1.1% of 126 PD patients, and in 1.5% of 210 controls screened (Walker et al., 2003). This polymorphism has also been shown to be a minor risk factor for PD in a multicentre study which screened PD patients from North America, Europe, and Australia (Nalls et al., 2013). The allelic frequency of the T369M polymorphism was found to be marginally higher in controls than in PD patients [(odds ratio) OR = 6.97, 95% (confidence interval) CI 0.93–

52.02; *P* = 0.03], which was consistent with findings from other multicentre studies (Lesage et al., 2011; Pankratz et al., 2012).

No pathogenic mutations or sequence variants were found in *EIF4G1* or *VPS35*. It is however possible that some mutations could have been missed as only the two previously described R1205H and D620N mutations in *EIF4G1* and *VPS35,* respectively, were screened for, while the entire gene was screened in the case of *parkin*, *DJ-1* and *GBA*. However, to date no other mutations have been reported in the other exons of these two genes.

No mutations or novel variants were found in the patients who were screened for exons 2 and 3 of *SNCA*, and exons 31 and 41 of *LRRK2*. Screening the entire *LRRK2* gene, consisting of 51 exons, was not cost-effective given the fact that the most frequent pathogenic mutations have only been found in these two exons. Nearly 80 *LRRK2* variants have been reported worldwide, but proven pathogenicity has only been demonstrated for seven mutations (Ross et al., 2011). Four of these mutations are found in exon 31 (N1437H, R1441G, R1441G, and R1441GH), one in exon 35 (Y1699C, has only been found in one family from the United Kingdom), and two are found in exon 41 (G2019S, and I2020T) (Paisán-Ruíz et al., 2004). Similarly, patients were only screened for mutations in two exons of *SNCA*, as only four mutations in *SNCA* have been shown to contribute to disease pathogenesis: one in exon 2 (A53T), and three in exon 3 (A30P, E46K and G51D) (Golbe et al., 1990; Papadimitriou et al., 1999). The pathogenicity of the most recently identified H50Q mutation (Proukakis et al., 2013) in exon 4 of *SNCA* had not been proven at the time of screening, this exon had thus been excluded for mutation screening. Notably, the lack of mutations found cannot be attributed to the mutation screening technique, as positive controls were included in the HRM analysis, and this approach has been successful in detection of a number of variants across patient and control samples in previous studies by our group (Keyser et al., 2010; Glanzmann et al., 2014).

In the present study, one patient was found to harbour CNV mutations: a *parkin* gene deletion of exon 9 and a duplication of exon 2. This had previously been reported by another study by our group (Keyser et al., 2010). However, these CNV variants were independently confirmed in the present study using qPCR (Figure 3.5). Moreover, the *parkin* exon 5 deletion identified in two patients using MLPA was shown to be a false positive result using qPCR and Sanger sequencing. These patients (i.e. 61.81, and 10.308) were shown to have the M192L polymorphism (Figure 3.6) which is located at the annealing site of the MLPA P051-C3 kit's probes for *parkin* exon 5, the presence of which resulted in the false positive signal compatible with a heterozygous deletion of *parkin* exon 5. Notably, no other CNVs were detected in the remaining PD genes included in the MLPA kits (i.e. *SNCA, PINK1, DJ-1, LRRK2*, and *ATP13A2*).

Using a targeted resequencing approach, 22 patients were screened using a custom-made gene panel covering the exonic regions of 168 major genes (Appendix C Table 2.5) which are either linked or associated with PD and related neurodegenerative conditions. Targeted resequencing, is a high-throughput mutation screening approach which falls under the second-generation arm of next generation sequencing technologies (van Dijk et al., 2014). To avoid the challenges associated with whole genome sequencing, a more focused targeted resequencing approach was used as this technique offers a powerful, cost-effective alternative which facilitates in-depth interrogation of the genomic regions of interest (Chasman and Adams, 2001; Sim et al., 2012). Additionally, targeted resequencing allows for increased focus of the genomic regions most likely to provide relevant data, thereby increasing the chances of finding biologically relevant variants or mutations (Li et al., 2014). The mutation-screening efficiency of the targeted resequencing approach has previously been demonstrated in numerous studies (Hodges et al., 2007; Rivas et al., 2011; Lin et al., 2012).

In the present study, targeted resequencing yielded a prioritised list of 15 non-synonymous sequence variants in 10 genes (Table 3.4), which were selected for further analysis. The validity of 13 out of 15 variants was confirmed using Sanger sequencing, while two were shown to be false-positives. Three unrelated patients each harboured an identical *PINK1* polymorphism (E476K), which has been shown to decrease mitochondrial membrane potential under oxidative stress conditions (Rogaeva et al., 2004; Kawajiri et al., 2011), but further studies are warranted to prove its pathogenicity. For the other sequence variants identified; the potential functional effect was assessed using computational predictive tools SIFT, PolyPhen-2, and MutationTaster as summarized in Table 3.5. *In silico* methods were utilised in an effort to predict the potential impact the sequence variants could potentially have on protein function with the hope of using this knowledge to select candidate genes for future functional studies. The ability of these *in silico* tools to predict the potential of amino acid substitutions to impact protein structure and activity relies on factors which are important to protein function, including but not limited to, sequence similarity and homology, conservation over evolutionary time, secondary structure, solvent accessibility, polarity, side chain volume, and surface area accessibility (Chasman and Adams, 2001; Sim et al., 2012). Three different programs were used, as each one uses a different predictive algorithm, which highlights certain protein characteristics, while omitting others. For example, SIFT predictive scores are based on the sequence homology among related genes (Ng and Henikoff, 2001), while PolyPhen-2 incorporates the sequence conservation, the molecular characteristics of the amino acid residues involved, and the secondary structural features of the protein using the annotated protein database, SwissProt (Sunyaev et al., 2001). MutationTaster is considered to be comparatively more reliable given that SIFT and PolyPhen-2 predictions are restricted to homologous sequences that are available in the protein database, while MutationTaster uses a naïve Bayes classifier which enables the software to give predictions for novel variants, and

genes which are not fully annotated or available in protein databases (Wu and Jiang, 2013). Of the 13 confirmed sequence variants, tolerance predictions for 8 (61.5%) variants were consistent across the three programs Table 3.5. Functional predictions for the 4 novel variants found in *LRRK2* could only be obtained using MutationTaster, and predictive scores were inconsistent for the *DNAJC13* variant E1740D, which may be attributed to the existence of several partial isoforms of the gene. Previously described *parkin* mutations, Q311K and G430D were accurately classified as disease-causing across the three programs. The Q311K mutation which is located between the first really interesting new gene (RING1) finger domain and the in-between-RING (IBR) domain, has been shown to cause reduced ubiquitin modification, while the G430D mutation (located in the second RING finger domain, RING2), has been shown to alter the solubility and localization of the protein in a functional study performed in SH-SY5Y cells (Sriram et al., 2005). However, it should be noted that for the individual harbouring the Q311K variant, a second pathogenic mutation in parkin was not found. *Parkin*-related PD is generally thought to be early-onset, but interestingly, both mutations in the present study (i.e. G430D, and Q311K) were found in two unrelated patients both of whom had late onset PD (56 and 63 years of age, respectively). However, it is possible that these patients may have developed the disease at an earlier age, but were clinically diagnosed only at a much later stage. This is indeed conceivable given that these patients reside in the rural Eastern Cape, with little or no access to a Movement disorder specialist.

The I143M variant in *PSEN1* was consistently predicted to be disease-causing across the three programs. This variant has previously been shown to co-segregate with disease in a large SA family of Black African ancestry, with early AD (Heckmann et al., 2004). All of the mutations in the presenilin genes (i.e. *PSEN1* and *PSEN2*) are dominantly inherited (i.e. one mutant allele is sufficient to cause the carrier to develop AD). However, the consulting clinicians in this study have stated that the patient harbouring the *PSEN1* I143M mutation does not have AD, but may suffer from severe dementia. Such a case is however possible, as there has indeed been reports of disease-causing mutations that show incomplete penetrance, even for Mendelian diseases. A case in point is the age-dependent penetrance of the *LRRK2* G2019S mutation which varies considerably in different PD populations. In a study of 126 at-risk family members of probands from Cantabria Spain, the G2019S mutation was shown to have a penetrance of 28% at age 59 years, 51% at age 69 years, and 74% at age 79 years (Sierra et al., 2011). This reduced penetrance is considerable even in PD patients from Cantabria, who have amongst the highest reported frequency of G2019S mutations reported so far after North African Arabs and Ashkenazi Jews (Guedes et al., 2010; Sierra et al., 2011). One may thus suggest that unique modifier genes or other features of the genetic background may strongly influence the phenotypic expression of disease-causing mutations.

The novel *LRRK2* variant N2133S was classified as 'disease-causing', while the other three (H1758P, T2423S and I610T) were classified as 'polymorphisms' using MutationTaster. These variants were found in four separate individuals with AAO ranging from 50 to 63 years, which is typical of the late PD AAO (50 years and older) usually associated with *LRRK2*. Using the SMART Protein Analyzer (http://smart.embl-heidelberg.de/), it was determined that these variants are found in the COOH-terminal WD40 domain (N2133S), the kinase domain (T2423S), the COR (COOH terminal of Roc) domain (H1758P), and between the ARM (Armadillo) and ANK (ankyrin repeat) domain (I610T). The functional significance of these sequence variants remains to be established.

The use of targeted resequencing allowed for the simultaneous selection of many target regions across multiple samples, and provided an effective solution for maximizing high-throughput mutation-screening. The frequency of the 13 prioritised variants was obtained from controls which were extracted from the 1000 Genomes Project (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/release/20110521/), the NHLBI Exome Sequencing Project (ESP; http://evs.gs.washington.edu/EVS/), and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Furthermore, the frequency of these variants was screened in 147 (99 with HRM and 48 with NGS) ethnically-matched SA Black controls. Only 1 out of the 13 sequence variants was found in 1 control (Figure 3. 9), which was confirmed using Sanger sequencing (Figure 3. 10). This *DNAJC13* E1740D sequence variant was classified as a 'polymorphism' by MutationTaster, with conflicting predictions from SIFT and PolyPhen-2 (Table 3.5). This discordance across the three *in silico* programs may be because the gene has not been fully annotated, and several isoforms currently exist of its protein domains. Therefore, the functional significance of the E1740D sequence variant remains to be established.

The high-throughput targeted resequencing approach has facilitated the screening of the entire coding regions of all known PD genes as well as 160 other genes associated with related neurological conditions (i.e. Alzheimer's disease, Kufor-Rakeb, and Dementia). At the time of the study 22 of the patients were analysed using this approach, the remaining 25 patients will be screened in the coming months.

SCA7 represents the most common SCA subtype in the SA population, and is due to a pathogenic CAG expansion in the ataxin-7 protein (Zander et al., 2001). It has since became clear from a study by Scholefield et al. (2009) that a common founder haplotype surrounding the *SCA7* region explains the exclusive detection of SCA7 in the Black SA population group. Given the exclusive existence of this founder event in this population group, and the similar pathogenic mechanism utilised by ataxin-7 and a-synuclein to impair the ALP in SCA7 and PD, respectively, it was of interest to determine if our study group had pathogenic repeat expansions at the *SCA7* locus.

Using the repeat-primed PCR with fluorescently-labelled primers, the wild-type *SCA7* profile was easily distinguishable from that of the positive control with a repeat expansion (Figure 3.12 A). However, due to the limitations of this approach, the accurate size of the *SCA7* repeat length in each patient could not be determined. Using this approach, an atypical expansion profile was detected in two PD patients (Figure 3.13, A and B). These patients have a profile which is neither typical of the wild-type or the positive control which harbours 57 CAG repeats. It is possible that these patients (11.798 and 11.799) contain a CAG repeat length which falls within the reduced penetrance range (i.e. 34 to 35 repeats). However, using HRM these two patients' melt profiles were more similar to the wild-type than the positive control (Figure 3.14) indicating that they probably do not harbour pathogenic expansions. All of the other patients had repeat profiles that looked like that of a wild-type sample.

Pure PD symptoms (i.e. resting tremor, bradykinesia, rigidity), with excellent response to L-DOPA therapy have previously been described in patients, harbouring pathogenic repeat expansions at the *SCA2* locus (Gwinn-Hardy et al., 2000). Misdiagnosis of *SCA2* patients with PD is not uncommon given the number of overlapping phenotypic features shared by the two movement disorders (Ragothaman et al., 2004; Socal et al., 2009). What is more, parkin (the most frequent cause of ARPD) has been shown to regulate the intracellular levels of both wild-type and mutant ataxin-2 (Huynh et al., 2007). Given the links between *SCA2* and PD, the length of the CAG repeat expansions at the *SCA2* locus was determined in the current PD group and compared with that described for other PD patients worldwide. Moreover, this was a unique opportunity to assess whether *SCA2* repeat expansions could potentially form part of the genetic aetiology of this group of patients.

The CAG repeat length at the *SCA2* locus was determined in all 47 patients. Analysis using GeneMapper® software revealed that all these patients had a repeat length which fell within the normal range (i.e. 14 to 30 repeats). Twenty-two was found to be the most frequent number of repeats in 64 chromosomes (68%), while 23 repeats were found at a frequency of 22.3% in the 94 chromosomes screened (Figure 3.18). Our findings are in agreement with previous findings in various ethnic populations worldwide, including: French (Imbert et al., 1996), Japanese (Mizushima et al., 1999), Indian (Choudhry et al., 2001), and Polish (Sobczak, 2004) populations, where 22 CAG repeats was found to be the most common repeat length in the majority of PD patients screened. Twenty-nine (7 chromosomes) and 20 repeats (2 chromosomes) were the highest and lowest number of CAG repeats, respectively found in the present study. However, 29 repeats is the most frequent repeat length in European and Japanese populations (Takano et al., 1998). Notably, the sequence chromatographs of two patients who were homozygous for 22 CAG repeats revealed the presence of a single CAA interruption (Figure 3.17). This is not uncommon, patients with PD, or parkinsonism often have shorter CAG repeat lengths, in addition to one or more CAA interruptions which are thought to

stabilise the repeat, thereby limiting further repeat expansion (Furtado et al., 2002; Kim et al., 2007). Moreover, the CAA interruption is thought to stabilise the mutant ataxin-2 protein in selective neurons (Lu et al., 2004). Taken together, results from the present study may suggest that repeat expansions at the *SCA2* locus do not confer a genetic contribution to PD in this group of patients, nor is it a modifier for the disorder. However, a larger patient group is necessary to substantiate the aforementioned results in a more representative sample size.

4.2 Study limitations

4.2.1 *Study participants*

Limitations of the present study included the rather small number of study participants (*n* = 47) from the Black ethnic group. We initially started the screening analysis with a total group of 24 patients; it was only towards the latter part of the study that we were able to secure 47 patients for mutation screening. However, despite extensive efforts, additional patients could not be recruited for this study. This may be due to ascertainment biases, the diagnostic criteria applied, different population aging dynamics, and a general lack of awareness of the disease owing to the scarcity of published literature, particularly in the native languages spoken by the millions of South Africans residing in the rural areas where access to education is frequently inadequate (Mshana et al., 2011; Dotchin and Walker, 2012).

It was also difficult to follow-up on most of our patients (i.e. to get further information, or rebleeds when necessary), as many were from the rural regions of the Eastern Cape, and could therefore not be reached. A further compounding limitation in this study, was the relatively few number of patients who had one or more affected family members (*n* = 5, 11 %). It was therefore difficult to determine the pathogenicity of novel variants as co-segregation with disease within a family could not be assessed. Whether this is reflective of a true difference in PD susceptibility in these populations, the unique genetic diversity of this ethnic group or due to ascertainment bias is still unclear.

4.2.2 Limitations of mutation screening techniques

4.2.2.1 *High-resolution melt*

Although heterozygote detection using HRM is relatively good in our experience, data from two independent studies suggest that the misclassification risk is about 10% (Montgomery et al., 2007; Wittwer, 2009). HRM accuracy is highly dependent on the PCR product length, with more errors or misclassification being evident as the length increases above 400 bp (Reed and Wittwer, 2004; Erali and Wittwer, 2010). Moreover, different saturating DNA dyes are variably effective. For example, LC Green® Plus is thought to detect heterozygotes better than SYTO® 9 (which was used in the present study) which detect heterozygotes better than EvaGreen®, which is much better than SYBR® Green I (Lyon and Wittwer, 2009). Although most single base pair changes can be successfully genotyped using HRM, the homozygous genotype of selective variants, resulting from a single base pair change, can be difficult to detect at times, even under the best instrumental and experimental conditions (Montgomery et al., 2007; Gundry et al., 2008).

4.2.2.2 *Multiplex-ligation dependent probe amplification*

MLPA analysis was used in this study to screen for large deletions/duplications in the known PD genes, because of its effectiveness in detecting homo- and heterozygous CNV in a multiplex manner. The MLPA technique is potentially prone to false positive results as seen with the M192L polymorphism in MLPA probe mix P051-C3 (Scarciolla et al., 2007). Therefore, confirmation of all exonic deletions/duplications should be performed using techniques such qPCR, southern blotting, long range PCR, fluorescence in situ hybridization (FISH), or Sanger sequencing to exclude false-positives. The MLPA technique is also very sensitive to contaminants (e.g. high salt concentrations, and PCR inhibitors such as small remnants of phenol), which can result in aberrant peak profiles. Although MLPA results are reported to be reliable with only 20ng DNA, in our experience we found that 35–50 ng is required for reliable and reproducible results. MLPA is also more sensitive to the method used for DNA extraction (e.g. phenol–chloroform extraction, ethanol precipitation, or spin column-based nucleic acid), and also to DNA degradation than conventional PCR (Scarciolla et al., 2007; Keyser et al., 2010).

4.2.2.3 *High-throughput targeted resequencing*

The advantages of second-generation sequencing technologies are currently offset by several disadvantages. The most prominent of which includes the read-length (which is currently much shorter than conventional sequencing for all of the new platforms), and the raw accuracy (on average, base-calls generated by the new platforms are nearly tenfold less accurate than base-calls generated by Sanger sequencing) (Liu et al., 2012). The 22 patients screened by NGS in the present study were screened using the SOLiD 5500xl platform which has certain limitations to its high-throughput capacity. Although the SOLiD sequencing platform is widely claimed to have 99.94% accuracy (Liu et al., 2012), it has the shortest reads (maximum of 75 nucleotides) of all the sequencing platforms, and relatively long run times (van Dijk et al., 2014). Moreover, the SOLiD platforms panel of sample preparation kits and services is less well developed and less widely used than the Illumina MiSeq, Ion Torrent, and Pacific Biosciences sequencing platforms. As a comparison, sequencing datasets generated using Illumina MiSeq, Ion Torrent, SOLID platform, and Pacific Biosciences sequencing platforms were shown to demonstrate error rates of <0.4%, 1.78%, 3–5%, and 13%, respectively (Bansal, 2010; Quail et al., 2012). Therefore, all the sequence variants identified using these NGS technologies must be confirmed by Sanger sequencing, because of the tendency of these methods to detect false positives.

4.2.2.4 *in silico functional predictive tools*

Computational tools, being *in silico* methods, are not 100% accurate. Flanagan and colleagues assessed the utility of various *in silico* bioinformatic tools in a diagnostic setting, and found that the sensitivity of SIFT and PolyPhen-2 was reasonably high (69% and 68%, respectively), but their specificity was low (13% and 16%) (Flanagan et al., 2010). Moreover, both programs were found to be significantly better at predicting loss-of-function mutations than gain-of-function mutations (Flanagan et al., 2010). In a separate study in 2013, Frousios et al evaluated the utility of SIFT, PolyPhen-2, and MutationTaster compared to nine other *in silico* programs and found MutationTaster to have the highest sensitivity and specificity rate (88% and 92%, respectively) (Frousios et al., 2013). The predictions of these tools should thus be interpreted with caution and further evidence to support/refute pathogenicity should be sought before reporting novel changes. Three important criteria have to be fulfilled in order to accurately infer the pathogenicity of a sequencing variant, namely: (i) co-segregation of the variant with the disease, (ii) absence of the variation in healthy age and ethnic matched group, and (iii) evidence of a biological role of the mutated protein either using *in vitro* and/or *in vivo* models (Meeus et a., 2010).

4.3 Future work

The continued recruitment of Black PD patients will be instrumental for future studies, preferably those with young AAO as they are more likely to have a genetic predisposition. However, patients with late-age of disease onset will not be excluded as mutations in *LRRK2* have been found in late onset PD cases, with both familial and sporadic forms of the disease (Bonifati, 2007; Nuytemans et al., 2010). Additionally, mutations in *VPS35, and EIF4G1*have only been found in late onset PD cases (Trinh et al., 2014). The present study identified a number of known SNPs and novel variants from the comprehensive screening of all known PD genes. Apart from the previously identified *parkin* CNVs (duplication of exon 2 and deletion of exon 9) in one Black PD patient (Keyser et al., 2010), the *parkin* mutations in another patient (G430D and deletion of exon 4) (Haylett et al., 2012), and the *parkin* exon 8 mutation (Q311K) identified in the present study, no pathogenic mutations were identified in any of the remaining seven known PD genes screened. It can therefore be deduced that the causative genes in the remaining 44 patients may either be due to a novel mutation in a known PD gene, or a new PD-causing gene. For this reason, from the list of 13 confirmed variants, one novel variant in a known PD gene could be selected as a possible candidate for future functional studies, possibly the N2133S variant found in exon 44 of *LRRK2*. This variant may be of interest, because it is located in the WD40 domain, disruption of which has been shown to result in abolished dimer formation, impaired kinase activity, and aberrant protein localization (Jorgensen et al., 2009). Several variants have been described in this domain, two of which have been identified as risk factors for sporadic PD (T2356I and G2385R) (Cardona et al., 2014). Results from a study by Rudenko and colleagues suggests that the *LRRK2* WD40 domain plays an important role in maintaining the enzymatic function of the protein (Rudenko et al., 2012). Therefore, the N2133S variant should be investigated further, using functional experimental approaches to gain better insights into the functionality of this variant. One may attempt to demonstrate changes in gene/protein expression, or kinase activity relative to the wild-type protein using a cell model in SH-SY5Y neuroblastoma cells.

The *DNAJC13* E1740D variant would also be an ideal candidate for future functional studies, because it was previously identified in one individual from a Northern Kalahari Bushman population (African region not specified, dbSNP version 141, rs112868112), and it was found in only one control in the present study. However, DNAJC13 has several incomplete transcripts, and its protein functional domains have not been fully characterized, further research is thus still needed to fully annotate, and prove the putative role of this gene in PD pathogenesis.

For *SCA2* genotyping, all 47 patients should be Sanger sequenced in order to accurately determine whether or not the CAA interruption found in the two patients sequenced in this

study is common to the entire group, and also to determine whether the position of this interruption varies between patients.

Efforts should be made to design PCR primers spanning the *SCA7* repeat region in order to accurately determine the number of repeats in each individual, similar to what was done for the *SCA2* locus. However, conventional PCR for *SCA7* has had limited success (personal communication, Danielle Smith, UCT). Southern blotting is currently one of the available sizing strategies that can be used for this purpose. However, this approach is technically demanding, and may prove costly for the purpose of screening the 47 patients. Another sizing approach, which specifically facilitates the screening of expanded CAG repeats is small pool PCR (SP-PCR), as described by Nakamura et al. (2001). This is a highly sensitive PCR assay used for the detection of low levels of repeat expansions. This approach may be ideal given that all 47 patients in our cohort were shown to not have an expanded repeat profile using the ABI Genetic Analyzer and HRM.

Furthermore, genome-wide SNP microarrays should be used to detect regions of homozygosity (ROH) throughout the genome of the 47 PD patients. Depending on the genomic context, ROH may indicate ancestral homozygosity, uniparental disomy (i.e. when an individual receives two copies of a chromosome, or part of a chromosome from one parent and no copies from the other parent), or parental consanguinity (i.e. a union between two individuals who are related as second cousins or closer) (Alkuraya, 2010; Kearney et al., 2011). Short ROH (less than 5Mb) are considered to be ancestral markers of an outbred population (i.e. when offspring from crosses between individuals from different populations have lower fitness than offspring from crosses between individuals from the same population) (Gibson et al., 2006). The presence of a single, or several large ROH on the same chromosome most likely indicates uniparental disomy, especially if the ROH is telomeric (i.e. located near the telomere region) (Papenhausen et al., 2011). Multiple large ROH spread across different chromosomes is representative of a parental blood relationship (McQuillan et al., 2008). This approach may help to identify a common novel disease-causing mutation for PD shared by the 47 patients, and may further suggest descent from a common ancestor (i.e. that the patients are ancestrally related).

Whole-exome sequencing (WES) is another plausible strategy to pursue. WES is an NGS technology that exclusively looks at the "exome", which consists of all the genome's exons (i.e. the coding portions of genes - these are the regions that get translated, or expressed as proteins). Two families (8 and 15, Appendix B) who were found to have no known PD causing mutations would be ideal candidates for WES because the DNA of at least two affected individuals is available for mutation screening, per family. Moreover, WES would be instrumental in identifying a novel PD gene which may account for the disorder in these families. WES is

essentially an abridged version of the more complete, but extremely costly, whole genome sequencing. Instead of targeting 3 billion base pairs of the human genome, exome sequencing focuses on the DNA segments that code for proteins (i.e. exons, approximately 1.2%), which have previously been shown to harbour over 85% of all disease-mutations and many disease-predisposing SNPs throughout the genome (Botstein and Risch, 2003). Once a patient's DNA is extracted and the exons sequenced, computer programs identify sequence variants in the patients DNA, in comparison to a reference sequence of the human genome. It is envisaged that identification, and subsequent verification of these variants may pin point a rare, or novel genetic variant which may be causative for PD in families 8 and 15.

4.4 Conclusions

This study is the first mutation screening report of all known PD genes, exclusively in the Black SA population. No pathogenic mutations were found in the gene most commonly known to cause PD in European populations (e.g. *LRRK2* G2019S). However, given the rather small size of the cohort studied, definite conclusions regarding variant frequencies cannot be made. However, this study represents the first comprehensive screening of all known PD genes in a sub-Saharan African population, which in itself is a noteworthy achievement. We report here that two previously described *parkin* mutations (i.e. G430D, and Q311K) were found to be a negligible cause of PD in the current cohort, while no mutations were found in any of the remaining known PD genes. It is therefore likely given the differences in the genetic background, and the rich genetic diversity within this population, that novel genes may account for the disease in this group of patients. Although all 47 patients recruited for the study were self-identified as Black, the majority of these patients were from the IsiXhosa Black subpopulation group, and originated from the rural Eastern Cape region. It is therefore important that Black PD patients from other regions of SA be recruited as these patients may not represent the full spectrum of mutations across the different Black ethnic groups in the SA population. There is indeed long standing evidence to suggest that genetic background may confer a strong influence on the phenotypic expression of disease-causing mutations (Romeo and McKusick, 1994), it can therefore be suggested that unique modifier genes may exist within different subpopulations groups, and a regionally representative group of Black PD patients (i.e. patients with Sesotho, IsiZulu, or Tshivenda, etc. ancestral lineage) would provide an unbiased representation of all Black PD patients in the context of the SA population.

The clinical features of PD were not found to be different in this patient group to that of other population groups. However, it would be of interest to undertake a larger longitudinal study to determine whether in fact there are subtle clinical and neurological differences in Black

patients, and differences in their response to treatment. It would also be of interest to compare data concerning genotype, disease AAO, disease duration, and clinical and pathological features found in patients and controls from similar genetic studies of PD in different African countries (Okubadejo et al., 2008; Atadzhanov, 2010; Yonova-Doing, 2012; Cilia et al., 2012). As a start, it may be possible to infer the frequency of common-disease causing mutations, or lack thereof across several African populations using the collective data of more than 300 Black PD patients studied thus far (Pringsheim et al., 2014).

Throughout the course of the present study, the urgent need to expand the study group by increasing the number of patients has been consistently reiterated, as this would facilitate a better understanding of the unique genetic landscape of the Black population group. Additionally, a larger patient group consisting of patients from various SA regions may provide more relevant data with regards to the frequency of selective variants in the Black SA PD population. Furthermore, the continued search for novel disease-causing genes is warranted, as this will in all probability, shed light on yet unknown biological pathways implicated in PD. To this end, the Black PD population are a unique and powerful resource for future studies on this debilitating disorder.

Chapter 5: References

5.1 Electronic references

Abou-Sleiman P. M., Daniel G. H., et al. 2003. “The Role of Pathogenic DJ-1 Mutations in Parkinson’s Disease.” Annals of Neurology 54 (3): 283–86. Doi:10.1002/ana.10675.

Alkuraya F. S. 2010. “Autozygome Decoded.” Genetics in Medicine: Official Journal of the American College of Medical Genetics 12 (12): 765–71. Doi:10.1097/GIM.0b013e3181fbfcc4.

Alves S., Florence C., Martina M., Thibaut M., et al. 2014. “The autophagy/lysosome pathway is impaired in SCA7 patients and SCA7 knock-in mice.” Acta Neuropathologica 32 (10): 1700–1708. Doi: 10.1007/s00401-014-1289-8.

Amar B. R., Ravi Y., Janardhan R., and Pramod K. 2014. “A Clinical Profile of Patients with Parkinson’s Disease and Psychosis.” Annals of Indian Academy of Neurology 17 (2): 187–92. Doi:10.4103/0972-2327.132625.

Ando M., Funayama Y., Kashihara K., et al. 2012. “VPS35 mutation in Japanese patients with typical Parkinson’s disease.” Movement Disorders: Official Journal of the Movement Disorder Society 27 (11): 1413–17. Doi:10.1002/mds.25145.

Antonarakis S. E. 1998. “Recommendations for a Nomenclature System for Human Gene Mutations. Nomenclature Working Group.” Human Mutation 11 (1): 1–3. Doi: 10.1002/ (SICI) 1098-1004(1998)11:1<1: AID-HUMU1>3.0.CO; 2-O.

Armstrong J., Bonaventura I., Rojo A., et al. 2005. “Spinocerebellar Ataxia Type 2 (SCA2) with White Matter Involvement.” Neuroscience Letters 381 (3): 247–51. Doi:10.1016/j.neulet.2005.02.063.

Ascherio A., Zhang S. M., Hernán M. A., et al. 2001. “Prospective Study of Caffeine Consumption and Risk of Parkinson’s Disease in Men and Women.” Annals of Neurology 50 (1): 56–63.

Atadzhanov M. 2010. “Phenotypic Characteristics of Zambian Patients with Parkinson’s Disease.” Medical Journal of Zambia 37 (3): 160–65. Doi:10.4314/mjz.v37i3.

Athanassiadou A., Voutsinas G., Psiouri L., et al. 1999. “Genetic Analysis of Families with Parkinson Disease that Carry the Ala53Thr Mutation in the Gene Encoding A-Synuclein.” The American Journal of Human Genetics 65 (2): 555–58. Doi: 10.1086/302486

Bach J.-P., Ziegler U., Deuschl G., et al. 2011. “Projected Numbers of People with Movement Disorders in the years 2030 and 2050.” Movement Disorders: Official Journal of the Movement Disorder Society 26 (12): 2286–90. Doi:10.1002/mds.23878.

Balogou A. A., Doh A., and Grunitzky K. E. 2001. “[Neurological disorders and endemic goiter: comparative analysis of 2 provinces in Togo].” Bulletin de la Société de pathologie exotique (1990) 94 (5): 406–10.

Bansal V. 2010. “A Statistical Method for the Detection of Variants from next-Generation Resequencing of DNA Pools.” Bioinformatics 26 (12): i318–24. Doi:10.1093/bioinformatics/btq214.

Barbeau A. 1969. “L-Dopa Therapy in Parkinson’s Disease: A Critical Review of Nine Years’ Experience.” Canadian Medical Association Journal 101 (13): 59–68.

Bardien S., Keyser R., Yako Y., Lombard D., and Carr J. 2009. “Molecular Analysis of the Parkin Gene in South African Patients Diagnosed with Parkinson’s Disease.” Parkinsonism & Related Disorders 15 (2): 116–21. Doi:10.1016/j.parkreldis.2008.04.005.

Bardien S., Lesage S., Brice A., and Carr J. 2011. “Genetic Characteristics of Leucine-Rich Repeat Kinase 2 (LRRK2) Associated Parkinson’s Disease.” Parkinsonism & Related Disorders 17 (7): 501–8. Doi:10.1016/j.parkreldis.2010.11.008.

Bardien S., Marsberg A., Keyser R., et al. 2010. “LRRK2 G2019S Mutation: Frequency and Haplotype Data in South African Parkinson’s Disease Patients.” Journal of Neural Transmission (Vienna, Austria : 1996) 117 (7): 847–53. Doi: 10.1007/s00702-010-0423-6.

Behrens M. I., Brüggemann N., Chana P., et al. 2010. “Clinical Spectrum of Kufor-Rakeb Syndrome in the Chilean Kindred with ATP13A2 Mutations.” Movement Disorders: Official Journal of the Movement Disorder Society 25 (12): 1929–37. Doi:10.1002/mds.22996.

Benito-León J., Bermejo-Pareja F., Rodríguez J., et al. 2003. “Prevalence of PD and Other Types of Parkinsonism in Three Elderly Populations of Central Spain.” Movement Disorders: Official Journal of the Movement Disorder Society 18 (3): 267–74. Doi:10.1002/mds.10362.

Beutler E., Gelbart T., Balicki D., et al. 1996. “Gaucher Disease: Four Families with Previously Undescribed Mutations.” Proceedings of the Association of American Physicians 108 (3): 179–84.

Blanckenberg J., Bardien S., Glanzmann B., et al. 2013. “The Prevalence and Genetics of Parkinson’s Disease in Sub-Saharan Africans.” Journal of the Neurological Sciences, September. Doi:10.1016/j.jns.2013.09.010.

Blanckenberg J., Ntsapi C., Carr J., and Bardien S. 2014. “EIF4G1 R1205H and VPS35 D620N Mutations Are Rare in Parkinson’s Disease from South Africa.” Neurobiology of Aging 35 (2): 445.e1–3. Doi:10.1016/j.neurobiolaging.2013.08.023.

Bonifati V. 2007. “LRRK2 Low-Penetrance Mutations (Gly2019Ser) and Risk Alleles (Gly2385Arg)-Linking Familial and Sporadic Parkinson’s Disease.” Neurochemical Research 32 (10): 1700–1708. Doi: 10.1007/s11064-007-9324-y.

Bonifati V., Rizzu P., van Baren M. J., et al. 2003. “Mutations in the DJ-1 Gene Associated with Autosomal Recessive Early-Onset Parkinsonism.” Science (New York, N.Y.) 299 (5604): 256–59. Doi:10.1126/science.1077209.

Borun P., Kubaszewski L., Banasiewicz T., et al. 2014. “Comparative high-resolution melting: A Novel Method of Simultaneous Screening for Small Mutations and Copy Number Variations.” Human Genetics 133 (5): 535–45. Doi: 10.1007/s00439-013-1393-1.

Botstein D., and Risch N. 2003. “Discovering Genotypes Underlying Human Phenotypes: Past Successes for Mendelian Disease, Future Approaches for Complex Disease.” Nature Genetics 33 Suppl (March): 228–37. Doi: 10.1038/ng1090.

Bower J. H., and Zenebe G. 2005. “Neurologic Services in the Nations of Africa.” Neurology 64 (3): 412–15. Doi:10.1212/01.WNL.0000150894.53961.E2.

Bower J. H., Maraganore D. M, McDonnell S. K., and Rocca W. A. 1999. “Incidence and Distribution of Parkinsonism in Olmsted County, Minnesota, 1976-1990.” Neurology 52 (6): 1214–20.

Brewis M., Poskanzer D. C., Rolland C., and Miller H. 1966. “Neurological Disease in an English City.” Acta Neurologica Scandinavica 42: Suppl 24:1–89.

Bronstein J. M., Tagliati M, Alterman R. L., et al. 2011. “Deep Brain Stimulation for Parkinson Disease: An Expert Consensus and Review of Key Issues.” Archives of Neurology 68 (2): 165–165. Doi:10.1001/archneurol.2010.260.

Brooks A. I., Chadwick C. A., Gelbard H. A., Cory-Slechta D. A., and Federoff H. J. 1999. “Paraquat Elicited Neurobehavioral Syndrome Caused by Dopaminergic Neuron Loss.” Brain Research 823 (1-2): 1–10.

Brooks D. J. 2004. “Neuroimaging in Parkinson’s Disease.” NeuroRx 1 (2): 243–54.

Bryer A., Krause A., Bill P., et al. 2003. “The Hereditary Adult-Onset Ataxias in South Africa.” Journal of the Neurological Sciences 216 (1): 47–54.

Burbulla L. F., Schelling C., Kato H., et al. 2010. “Dissecting the Role of the Mitochondrial Chaperone Mortalin in Parkinson’s Disease: Functional Impact of Disease-Related Variants on Mitochondrial Homeostasis.” Human Molecular Genetics 19 (22): 4437–52. Doi:10.1093/hmg/ddq370.

Camacho A., Francisco J. 2012. “[Charcot and his legacy to medicine].” Gaceta médica de México 148 (3): 321–26.

Cancel G., Dürr A., Didierjean O., Imbert G., Bürk K., et al. 1997. “Molecular and Clinical Correlations in Spinocerebellar Ataxia 2: A Study of 32 Families.” Human Molecular Genetics 6 (5): 709–15.

Canet-Avilés R. M., Wilson M. A., et al. 2004. “The Parkinson’s Disease Protein DJ-1 Is Neuroprotective due to Cysteine-Sulfinic Acid-Driven Mitochondrial Localization.” Proceedings of the National Academy of Sciences of the United States of America 101 (24): 9103–8. Doi:10.1073/pnas.0402959101.

Cannon J. R., and Greenamyre J. T. 2011. “The Role of Environmental Exposures in Neurodegeneration and Neurodegenerative Diseases.” Toxicological Sciences: An Official Journal of the Society of Toxicology 124 (2): 225–50. Doi:10.1093/toxsci/kfr239.

Cardona F., Tormos-Pérez M., and Pérez-Tur J. 2014. “Structural and Functional in Silico Analysis of LRRK2 Missense Substitutions.” Molecular Biology Reports 41 (4): 2529–42. Doi: 10.1007/s11033-014-3111-z.

Carmine A., Westerlund M., Sydow O., et al. 2006. “Leucine-Rich Repeat Kinase 2 (LRRK2) Mutations in a Swedish Parkinson Cohort and a Healthy Nonagenarian.” Movement Disorders: Official Journal of the Movement Disorder Society 21 (10): 1731–34. Doi:10.1002/mds.21016.

Carr J, de la Fuente-Fernández R., Schulzer M., Mak E., Calne S. M., and Calne D. B. 2003. “Familial and Sporadic Parkinson’s Disease Usually Display the Same Clinical Features.” Parkinsonism & Related Disorders 9 (4): 201–4. Doi: 10.1016/S1353-8020(02)00048-2.

Charles P., Camuzat A., Benammar N., et al. 2007. “Are Interrupted SCA2 CAG Repeat Expansions Responsible for Parkinsonism?” Neurology 69 (21): 1970–75. Doi:10.1212/01.wnl.0000269323.21969.db.

Chartier-Harlin M., Dachsel J., et al. 2011. “Translation Initiator EIF4G1 Mutations in Familial Parkinson Disease.” American Journal of Human Genetics 89 (3): 398–406. Doi:10.1016/j.ajhg.2011.08.009.

Chasman D., and Adams R. M. 2001. “Predicting the Functional Consequences of Non-Synonymous Single Nucleotide Polymorphisms: Structure-Based Assessment of Amino Acid Variation.” Journal of Molecular Biology 307 (2): 683–706. Doi:10.1006/jmbi.2001.4510.

Chen L., Cagniard B., Mathews T., et al. 2005. “Age-Dependent Motor Deficits and Dopaminergic Dysfunction in DJ-1 Null Mice.” The Journal of Biological Chemistry 280 (22): 21418–26. Doi:10.1074/jbc.M413955200.

Chinta S. J., and Andersen J. K. 2005. “Dopaminergic Neurons.” The International Journal of Biochemistry & Cell Biology 37 (5): 942–46. Doi:10.1016/j.biocel.2004.09.009.

Choudhry, S., Mukerji M., Srivastava A. K., Jain S., and Brahmachari S. K. 2001. “CAG Repeat Instability at SCA2 Locus: Anchoring CAA Interruptions and Linked Single Nucleotide Polymorphisms.” Human Molecular Genetics 10 (21): 2437–46.

Cilia R., Sironi F., Akpalu A., et al. 2012. “Screening LRRK2 Gene Mutations in Patients with Parkinson’s Disease in Ghana.” Journal of Neurology 259 (3): 569–70. Doi: 10.1007/s00415-011-6210-y.

Collier T. J., Kanaan N. M., and Kordower J. H. 2011. “Ageing as a Primary Risk Factor for Parkinson’s Disease: Evidence from Studies of Non-Human Primates.” Nature Reviews. Neuroscience 12 (6): 359–66. Doi: 10.1038/nrn3039.

Corti O., Lesage S., and Brice A. 2011. “What Genetics tells us about the Causes and Mechanisms of Parkinson’s Disease.” Physiological Reviews 91 (4): 1161–1218. Doi:10.1152/physrev.00022.2010.

Cosnett J. E, and Bill P.L. 1988. “Parkinson’s Disease in Blacks. Observations on Epidemiology in Natal.” South African Medical Journal = Suid-Afrikaanse Tydskrif Vir Geneeskunde 73 (5): 281–83.

Dagda R. K., and Chu C. T. 2009. “Mitochondrial Quality Control: Insights on How Parkinson’s Disease Related Genes PINK1, Parkin, and Omi/HtrA2 Interact to Maintain Mitochondrial Homeostasis.” Journal of Bioenergetics and Biomembranes 41 (6): 473–79. Doi: 10.1007/s10863-009-9255-1.

Dahodwala N., Siderowf A., Xie M., et al. 2009. “Racial Differences in the Diagnosis of Parkinson’s Disease.” Movement Disorders : Official Journal of the Movement Disorder Society 24 (8): 1200–1205. Doi:10.1002/mds.22557.

Dauer W., and Przedborski S. 2003. “Parkinson’s Disease: Mechanisms and Models.” Neuron 39 (6): 889–909.

Dehay B., Martinez-Vicente M., Ramirez A., et al. 2012. “Lysosomal Dysfunction in Parkinson Disease: ATP13A2 Gets into the Groove.” Autophagy 8 (9): 1389–91. Doi:10.4161/auto.21011.

Dekker M., Bonifati V., van Swieten J., et al. 2003. “Clinical Features and Neuroimaging of PARK7-Linked Parkinsonism.” Movement Disorders: Official Journal of the Movement Disorder Society 18 (7): 751–57. Doi:10.1002/mds.10422.

Demers C. H., Bogdan R., and Agrawal A. 2014. “The Genetics, Neurogenetics and Pharmacogenetics of Addiction.” Current Behavioural Neuroscience Reports 1 (1): 33–44. Doi: 10.1007/s40473-013-0004-8.

Den D., and Antonarakis S. E. 2001. “Nomenclature for the Description of Human Sequence Variations.” Human Genetics 109 (1): 121–24.

De Rijk M. C., Breteler M. M., Graveland G. A., et al. 1995. “Prevalence of Parkinson’s Disease in the Elderly: The Rotterdam Study.” Neurology 45 (12): 2143–46.

Derivery E., Sousa C., Gautier J. J., et al. 2009. “The Arp2/3 Activator WASH Controls the Fission of Endosomes through a Large Multi-protein Complex.” Developmental Cell 17 (5): 712–23. Doi:10.1016/j.devcel.2009.09.010.

Dianzani I., Camaschella C., Ponzone A., and Cotton R. G. 1993. “Dilemmas and Progress in Mutation Detection.” Trends in Genetics: TIG 9 (12): 403–5.

Di Fonzo A., Rohé C.F., Ferreira J., et al. 2005. “A Frequent LRRK2 Gene Mutation Associated with Autosomal Dominant Parkinson’s Disease.” Lancet 365 (9457): 412–15. Doi: 10.1016/S0140-6736(05)17829-5.

Dissanayaka N., White E., et al. 2014. “The Clinical Spectrum of Anxiety in Parkinson’s Disease.” Movement Disorders: Official Journal of the Movement Disorder Society 29 (8): 967–75. Doi:10.1002/mds.25937.

Djarmati A., Hedrich K., Svetel M., et al. 2004. “Detection of Parkin (PARK2) and DJ-1 (PARK7) Mutations in Early-Onset Parkinson Disease: Parkin Mutation Frequency Depends on Ethnic Origin of Patients.” Human Mutation 23 (5): 525. Doi:10.1002/humu.9240.

Dorsey E. R., Constantinescu R., Thompson J. P., et al. 2007. “Projected Number of People with Parkinson Disease in the Most Populous Nations, 2005 through 2030.” Neurology 68 (5): 384–86. Doi:10.1212/01.wnl.0000247740.47667.03.

Dotchin C., Jusabani A., Gray W. K., and Walker R. 2012. “Projected Numbers of People with Movement Disorders in the Years 2030 and 2050: Implications for Sub-Saharan Africa, Using Essential Tremor and Parkinson’s Disease in Tanzania as an Example.” Movement Disorders 27 (9): 1204–5. Doi:10.1002/mds.25097.

Dotchin C., Msuya O., Kissima J., et al. 2008. “The Prevalence of Parkinson’s Disease in Rural Tanzania.” Movement Disorders: Official Journal of the Movement Disorder Society 23 (11): 1567–1672. Doi:10.1002/mds.21898.

Dotchin C., and Walker R. 2012. “The Management of Parkinson’s Disease in Sub-Saharan Africa.” Expert Review of Neurotherapeutics 12 (6): 661–66. Doi:10.1586/ern.12.52.

Dotchin C., Msuya O., and Walker R. 2007. “The Challenge of Parkinson’s Disease Management in Africa.” Age and Ageing 36 (2): 122–27. Doi:10.1093/ageing/afl172.

Eiberg H., Hansen L., Korbo L., Nielsen I. M., et al. 2012. “Novel Mutation in ATP13A2 Widens the Spectrum of Kufor-Rakeb Syndrome (PARK9).” Clinical Genetics 82 (3): 256–63. Doi:10.1111/j.1399-0004.2011.01745.x.

Erali M., and Wittwer C.T. 2010. “High-resolution melting Analysis for Gene Scanning.” Methods (San Diego, Calif.) 50 (4): 250–61. Doi:10.1016/j.ymeth.2010.01.013.

Fahn S. 2003. “Description of Parkinson’s Disease as a Clinical Syndrome.” Annals of the New York Academy of Sciences 991 (June): 1–14.

Farrer M. 2006. “Genetics of Parkinson Disease: Paradigm Shifts and Future Prospects.” Nature Reviews. Genetics 7 (4): 306–18. Doi: 10.1038/nrg1831.

Farrer M., Chan R., Chen L., Tan S., et al. 2001. “Lewy Bodies and Parkinsonism in Families with Parkin Mutations.” Annals of Neurology 50 (3): 293–300.

Fisher H. K., Clements J. A., and Wright R. R. 1973. “Enhancement of Oxygen Toxicity by the Herbicide Paraquat.” The American Review of Respiratory Disease 107 (2): 246–52.

Flanagan S., Patch A., and Ellard S., 2010. “Using SIFT and PolyPhen to Predict Loss-of-Function and Gain-of-Function Mutations.” Genetic Testing and Molecular Biomarkers 14 (4): 533–37. Doi:10.1089/gtmb.2010.0036.

Forno L. 1996. “Neuropathology of Parkinson’s Disease.” Journal of Neuropathology and Experimental Neurology 55 (3): 259–72.

Fowler J. S., Volkow N. D., Wang G. J., et al. 1996. “Inhibition of Monoamine Oxidase B in the Brains of Smokers.” Nature 379 (6567): 733–36. Doi: 10.1038/379733a0.

Freire M., and Santos J. 2010. “Parkinson’s Disease: General Features, Effects of Levodopa Treatment and Future Directions.” Frontiers in Neuroanatomy 4: 146. Doi:10.3389/fnana.2010.00146.

Freund H. 2005. “Long-Term Effects of Deep Brain Stimulation in Parkinson’s Disease.” Brain: A Journal of Neurology 128 (Pt 10): 2222–23. Doi:10.1093/brain/awh634.

Frousios K., Costas S., Schlitt T., and Simpson M. 2013. “Predicting the Functional Consequences of Non-Synonymous DNA Sequence Variants — Evaluation of Bioinformatics Tools and Development of a Consensus Strategy.” Genomics 102 (4): 223–28. Doi:10.1016/j.ygeno.2013.06.005.

Fuchs J., Nilsson C., Kachergus J., Munz M., et al. 2007. “Phenotypic Variation in a Large Swedish Pedigree due to SNCA Duplication and Triplication.” Neurology 68 (12): 916–22. Doi:10.1212/01.wnl.0000254458.17630.c5.

Furtado S., Payami H., Lockhart P., et al. 2004. “Profile of Families with Parkinsonism-Predominant Spinocerebellar Ataxia Type 2 (SCA2).” Movement Disorders: Official Journal of the Movement Disorder Society 19 (6): 622–29. Doi:10.1002/mds.20074.

Furtado S., Farrer M., Tsuboi Y., Klimek M., et al. 2002. “SCA-2 Presenting as Parkinsonism in an Alberta Family: Clinical, Genetic, and PET Findings.” Neurology 59 (10): 1625–27.

Gaig C., Ezquerra M., Marti M., et al. 2006. “LRRK2 Mutations in Spanish Patients with Parkinson Disease: Frequency, Clinical Features, and Incomplete Penetrance.” Archives of Neurology 63 (3): 377–82. Doi:10.1001/archneur.63.3.377.

Gancher S. 2002. “Modified Hoehn and Yahr Staging.” Text. http://www.ncbi.nlm.nih.gov/books/NBK27684/.

Gandhi S., Wood-Kaczmar A., Yao Z., et al. 2009. “PINK1-Associated Parkinson’s Disease Is Caused by Neuronal Vulnerability to Calcium-Induced Cell Death.” Molecular Cell 33 (5): 627–38. Doi:10.1016/j.molcel.2009.02.013.

Gegg M., Cooper J., Chau K., et al. 2010. “Mitofusin 1 and Mitofusin 2 Are Ubiquitinated in a PINK1/parkin-Dependent Manner upon Induction of Mitophagy.” Human Molecular Genetics 19 (24): 4861–70. Doi:10.1093/hmg/ddq419.

Gerlach M., Double K., Youdim M., and Riederer P. 2006. “Potential Sources of Increased Iron in the Substantia Nigra of Parkinsonian Patients.” Journal of Neural Transmission. Supplementum, no. 70: 133–42.

Giasson B., Murray I., Trojanowski J., and Lee V. 2001. “A Hydrophobic Stretch of 12 Amino Acid Residues in the Middle of Alpha-Synuclein Is Essential for Filament Assembly.” The Journal of Biological Chemistry 276 (4): 2380–86. Doi:10.1074/jbc.M008919200.

Gibb W., and Lees A. 1988. “A Comparison of Clinical and Pathological Features of Young- and Old-Onset Parkinson’s Disease.” Neurology 38 (9): 1402–6.

Gibson J., Morton N., and Collins A. 2006. “Extended Tracts of Homozygosity in Outbred Human Populations.” Human Molecular Genetics 15 (5): 789–95. Doi:10.1093/hmg/ddi493.

Gispert S., Twells R., Orozco G., et al. 1993. “Chromosomal Assignment of the Second Locus for Autosomal Dominant Cerebellar Ataxia (SCA2) to Chromosome 12q23-24.1.” Nature Genetics 4 (3): 295–99. Doi: 10.1038/ng0793-295.

Giunti P., Sabbadini G., Sweeney M., et al. 1998. “The Role of the SCA2 Trinucleotide Repeat Expansion in 89 Autosomal Dominant Cerebellar Ataxia Families. Frequency, Clinical and Genetic Correlates.” Brain: A Journal of Neurology 121 (Pt 3) (March): 459–67.

Glanzmann B., Lombard D., Carr J., and Bardien S. 2014. “Screening of Two Indel Polymorphisms in the 5’UTR of the DJ-1 Gene in South African Parkinson’s Disease Patients.” Journal of Neural Transmission (Vienna, Austria: 1996) 121 (2): 135–38. Doi: 10.1007/s00702-013-1094-x.

Glickman M., and Ciechanover A. 2002. “The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction.” Physiological Reviews 82 (2): 373–428. Doi:10.1152/physrev.00027.2001.

Goetz C. 1986. “Charcot on Parkinson’s Disease.” Movement Disorders: Official Journal of the Movement Disorder Society 1 (1): 27–32. Doi:10.1002/mds.870010104.

Golbe L., Lazzarini A., Duvoisin R., et al. 1996. “Clinical Genetic Analysis of Parkinson’s Disease in the Contursi Kindred.” Annals of Neurology 40 (5): 767–75. Doi:10.1002/ana.410400513.

Golbe L., Di Iorio G., Bonavita V., Miller D., and Duvoisin R. 1990. “A Large Kindred with Autosomal Dominant Parkinson’s Disease.” Annals of Neurology 27 (3): 276–82. Doi:10.1002/ana.410270309.

Goldberg A. 2003. “Protein Degradation and Protection against Misfolded or Damaged Proteins.” Nature 426 (6968): 895–99. Doi: 10.1038/nature02263.

Gomes M., and Engelhardt E. 2013. “Jean-Martin Charcot, Father of Modern Neurology: An Homage 120 Years after His Death.” Arquivos de Neuro-Psiquiatria 71 (10): 815–17. Doi: 10.1590/0004-282X20130128.

González-Fernández M., Lezcano E., et al. 2007. “Lrrk2-Associated Parkinsonism Is a Major Cause of Disease in Northern Spain.” Parkinsonism & Related Disorders 13 (8): 509–15. Doi:10.1016/j.parkreldis.2007.04.003.

Greenamyre J., and Hastings T. 2004. “Parkinson’s--Divergent Causes, Convergent Mechanisms.” Science 304 (5674): 1120–22. Doi:10.1126/science.1098966.

Greenberg J., Solomon G., Vorster A., Heckmann J., and Bryer A. 2006. “Origin of the SCA7 Gene Mutation in South Africa: Implications for Molecular Diagnostics.” Clinical Genetics 70 (5): 415–17. Doi:10.1111/j.1399-0004.2006.00680.x.

Green D., Galluzzi L., and Kroemer G. 2011. “Mitochondria and the Autophagy-Inflammation-Cell Death Axis in Organismal Aging.” Science (New York, N.Y.) 333 (6046): 1109–12. Doi:10.1126/science.1201940.

Guedes L., Rosa F., Coelho M., Bonifati V., and Sampaio C. 2010. “Worldwide Frequency of G2019S LRRK2 Mutation in Parkinson’s Disease: A Systematic Review.” Parkinsonism & Related Disorders 16 (4): 237–42. Doi:10.1016/j.parkreldis.2009.11.004.

Gundry C., Dobrowolski S., et al. 2008. “Base-Pair Neutral Homozygotes Can Be Discriminated by Calibrated High-Resolution Melting of Small Amplicons.” Nucleic Acids Research 36 (10): 3401–8. Doi:10.1093/nar/gkn204.

Guo L., Wang W., and Chen S. 2006. “Leucine-Rich Repeat Kinase 2: Relevance to Parkinson’s Disease.” The International Journal of Biochemistry & Cell Biology 38 (9): 1469–75. Doi:10.1016/j.biocel.2006.02.009.

Gwinn-Hardy K., Chen J., Liu H., Liu T., Boss M., et al. 2000. “Spinocerebellar Ataxia Type 2 with Parkinsonism in Ethnic Chinese.” Neurology 55 (6): 800–805.

Hague S., Rogaeva E., Hernandez D., et al. 2003. “Early-Onset Parkinson’s Disease Caused by a Compound Heterozygous DJ-1 Mutation.” Annals of Neurology 54 (2): 271–74. Doi:10.1002/ana.10663.

Halliday G., Holton J., Revesz T., and Dickson D. 2011. “Neuropathology Underlying Clinical Variability in patients with Synucleinopathies.” Acta Neuropathologica 122 (2): 187–204. Doi: 10.1007/s00401-011-0852-9.

Harbour M., Breusegem S., Antrobus R., et al. 2010. “The Cargo-Selective Retromer Complex Is a Recruiting Hub for Protein Complexes That Regulate Endosomal Tubule Dynamics.” Journal of Cell Science 123 (Pt 21): 3703–17. Doi:10.1242/jcs.071472.

Hasegawa K., and Kowa H. 1997. “Autosomal Dominant Familial Parkinson Disease: Older Onset of Age, and good Response to Levodopa Therapy.” European Neurology 38 Suppl 1: 39–43.

Hatano Y., Sato K., Elibol B., Yoshino H., Yamamura Y., Bonifati V., et al. 2004. “PARK6-Linked Autosomal Recessive Early-Onset Parkinsonism in Asian Populations.” Neurology 63 (8): 1482–85.

Haugarvoll K., R. Rademakers, J. M. Kachergus, et al. 2008. “Lrrk2 R1441C Parkinsonism Is Clinically Similar to Sporadic Parkinson Disease.” Neurology 70 (16 Pt 2): 1456–60. Doi:10.1212/01.wnl.0000304044.22253.03.

Haylett W., Keyser R., du Plessis M., et al. 2012. “Mutations in the Parkin Gene Are a Minor Cause of Parkinson’s Disease in the South African Population.” Parkinsonism & Related Disorders 18 (1): 89–92. Doi:10.1016/j.parkreldis.2011.09.022.

Heckmann J., Low W., de Villiers C., et al. 2004. “Novel Presenilin 1 Mutation with Profound Neurofibrillary Pathology in an Indigenous Southern African Family with Early-Onset Alzheimer’s Disease.” Brain: A Journal of Neurology 127 (Pt 1): 133–42. Doi:10.1093/brain/awh009.

Hedrich K., Eskelson C., Wilmot B., et al. 2004. “Distribution, Type, and Origin of Parkin Mutations: Review and Case Studies.” Movement Disorders: Official Journal of the Movement Disorder Society 19 (10): 1146–57. Doi:10.1002/mds.20234.

Hely M., Reid W., Adena M., et al. 2008. “The Sydney Multicenter Study of Parkinson’s Disease: The Inevitability of Dementia at 20 Years.” Movement Disorders: Official Journal of the Movement Disorder Society 23 (6): 837–44. Doi:10.1002/mds.21956.

Hernán M., Takkouche B., Caamaño-Isorna F., and Gestal-Otero J. 2002. “A Meta-Analysis of Coffee Drinking, Cigarette Smoking, and the Risk of Parkinson’s Disease.” Annals of Neurology 52 (3): 276–84. Doi:10.1002/ana.10277.

Hill-Burns E., Factor S., Zabetian C., Thomson G., and Payami H. 2011. “Evidence for More than One Parkinson’s Disease-Associated Variant within the HLA Region.” PloS One 6 (11): e27109. Doi:10.1371/journal.pone.0027109.

Hirtz D., Gwinn-Hardy K., Mohamed M., Chaudhuri A., and Zalutsky R. 2007. “How Common Are the ‘Common’ Neurologic Disorders?” Neurology 68 (5): 326–37. Doi:10.1212/01.wnl.0000252807.38124.a3.

Holdorff B. 2002. “Friedrich Heinrich Lewy (1885-1950) and His Work.” Journal of the History of the Neurosciences 11 (1): 19–28. Doi:10.1076/jhin.11.1.19.9106.

Huang N., Marcotte E., and Hurles M. 2010. “Characterising and Predicting Haploinsufficiency in the Human Genome.” PLoS Genet 6 (10): e1001154. Doi:10.1371/journal.pgen.1001154.

Huynh D., Nguyen D., Pulst-Korenberg J., et al. 2007. “Parkin Is an E3 Ubiquitin-Ligase for Normal and Mutant Ataxin-2 and Prevents Ataxin-2-Induced Cell Death.” Experimental Neurology 203 (2): 531–41. Doi:10.1016/j.expneurol.2006.09.009.

Ibáñez P., Lesage S., Lohmann E., et al. 2006. “Mutational Analysis of the PINK1 Gene in Early-Onset Parkinsonism in Europe and North Africa.” Brain: A Journal of Neurology 129 (Pt 3): 686–94. Doi:10.1093/brain/awl005.

Ilett K. F., Stripp B., Menard R.H., Reid W., and Gillette J. 1974. “Studies on the Mechanism of the Lung Toxicity of Paraquat: Comparison of Tissue Distribution and Some Biochemical Parameters in Rats and Rabbits.” Toxicology and Applied Pharmacology 28 (2): 216–26.

Imai K., Nakamura M., Yamada M., Asano A., Yokoyama S., Tsuji S., and Ginns E. 1993. “A Novel Transcript from a Pseudogene for Human Glucocerebrosidase in Non-Gaucher Disease Cells.” Gene 136 (1-2): 365–68.

Imai Y., Soda M., and Takahashi R. 2000. “Parkin Suppresses Unfolded Protein Stress-Induced Cell Death through Its E3 Ubiquitin-Protein Ligase Activity.” The Journal of Biological Chemistry 275 (46): 35661–64. Doi:10.1074/jbc.C000447200.

Imbert G., Saudou F., Yvert G., et al. 1996. “Cloning of the Gene for Spinocerebellar Ataxia 2 Reveals a Locus with High Sensitivity to Expanded CAG/glutamine Repeats.” Nature Genetics 14 (3): 285–91. Doi: 10.1038/ng1196-285.

Jaber, M., Robinson S.W., Missale C., and Caron M. 1996. “Dopamine Receptors and Brain Function.” Neuropharmacology 35 (11): 1503–19.

Jorgensen N., Peng Y., Ho C., et al. 2009. “The WD40 Domain Is Required for LRRK2 Neurotoxicity.” PloS One 4 (12): e8463. Doi:10.1371/journal.pone.0008463.

Kachergus J., Mata I.F., Hulihan M., et al. 2005. “Identification of a Novel LRRK2 Mutation Linked to Autosomal Dominant Parkinsonism: Evidence of a Common Founder across European Populations.” American Journal of Human Genetics 76 (4): 672–80. Doi: 10.1086/429256.

Korolchuk D., Barber G., Casper J., et al. 2014. “The UCSC Genome Browser Database: 2014 Update.” Nucleic Acids Research 42 (Database issue): D764–70. Doi:10.1093/nar/gkt1168.

Kawajiri S., Saiki S., Sato S., and Hattori N. 2011. “Genetic Mutations and Functions of PINK1.” Trends in Pharmacological Sciences 32 (10): 573–80. Doi:10.1016/j.tips.2011.06.001.

Kearney H. M., Kearney J., and Colin L. 2011. “Diagnostic Implications of Excessive Homozygosity Detected by SNP-Based Microarrays: Consanguinity, Uniparental Disomy, and Recessive Single-Gene Mutations.” Clinics in Laboratory Medicine 31 (4): 595–613, ix. Doi:10.1016/j.cll.2011.08.003.

Kempster P., Hurwitz B., and Lees A. 2007. “A New Look at James Parkinson’s Essay on the Shaking Palsy.” Neurology 69 (5): 482–85. Doi:10.1212/01.wnl.0000266639.50620.d1.

Keyser R., Lesage S., Brice A., Carr J., and Bardien S. 2010a. “Assessing the Prevalence of PINK1 Genetic Variants in South African Patients Diagnosed with Early- and Late-Onset Parkinson’s Disease.” Biochemical and Biophysical Research Communications 398 (1): 125–29. Doi:10.1016/j.bbrc.2010.06.049.

Keyser R., Lombard D., Veikondis R., Carr J., and Bardien S. 2010b. “Analysis of Exon Dosage Using MLPA in South African Parkinson’s Disease Patients.” Neurogenetics 11 (3): 305–12. Doi: 10.1007/s10048-009-02296.

Khalid M., Aoun R. A., and Mathews T. 2011. “Altered Striatal Dopamine Release Following a Sub-Acute Exposure to Manganese.” Journal of Neuroscience Methods 202 (2): 182–91. Doi:10.1016/j.jneumeth.2011.06.019.

Khan N. L., Graham E., Critchley P., et al. 2003. “Parkin Disease: A Phenotypic Study of a Large Case Series.” Brain: A Journal of Neurology 126 (Pt 6): 1279–92.

Kim J. M., Hong S., Kim G. P., et al. 2007. “Importance of Low-Range CAG Expansion and CAA Interruption in SCA2 Parkinsonism.” Archives of Neurology 64 (10): 1510–18. Doi:10.1001/archneur.64.10.1510.

Klein C., and Lohmann-Hedrich K. 2007. “Impact of Recent Genetic Findings in Parkinson’s Disease.” Current Opinion in Neurology 20 (4): 453–64. Doi:10.1097/WCO.0b013e3281e6692b.

Klein C., Lohmann-Hedrich K., Rogaeva E., et al. 2007. “Deciphering the Role of Heterozygous Mutations in Genes Associated with Parkinsonism.” Lancet Neurology 6 (7): 652–62. Doi: 10.1016/S1474-4422(07)70174-6.

Klein C., and Schlossmacher M. G. 2006. “The Genetics of Parkinson Disease: Implications for Neurological Care.” Nature Clinical Practice. Neurology 2 (3): 136–46. Doi: 10.1038/ncpneuro0126.

Klockgether T. 2011. “Update on Degenerative Ataxias.” Current Opinion in Neurology 24 (4): 339–45. Doi:10.1097/WCO.0b013e32834875ba.

Korolchuk V. I., Menzies F. M, and Rubinsztein D. 2010. “Mechanisms of Cross-Talk between the Ubiquitin-Proteasome and Autophagy-Lysosome Systems.” FEBS Letters 584 (7): 1393–98. Doi:10.1016/j.febslet.2009.12.047.

Kruger R., Kuhn W., Leenders K. L., et al. 2001. “Familial Parkinsonism with Synuclein Pathology: Clinical and PET Studies of A30P Mutation Carriers.” Neurology 56 (10): 1355–62. Doi:10.1212/WNL.56.10.1355.

Krzysztof S., and Krzyzosiak W. J. 2004. “Patterns of CAG Repeat Interruptions in SCA1 and SCA2 Genes in Relation to Repeat Instability.” Human Mutation 24 (3): 236–47. Doi:10.1002/humu.20075.

Kubo S.-I., Nemani V. M., Chalkley R. J., et al. 2005. “A Combinatorial Code for the Interaction of -Synuclein with Membranes.” Journal of Biological Chemistry 280 (36): 31664–72. Doi:10.1074/jbc.M504894200.

Kumazawa R., Tomiyama H., Li Y., et al. 2008. “Mutation Analysis of the PINK1 Gene in 391 Patients with Parkinson Disease.” Archives of Neurology 65 (6): 802–8. Doi:10.1001/archneur.65.6.802.

Langston J. W., Ballard P., Tetrud J. W., and Irwin I. 1983. “Chronic Parkinsonism in Humans due to a Product of Meperidine-Analog Synthesis.” Science (New York, N.Y.) 219 (4587): 979–80.

Langston, J. W., and Irwin I. 1986. “MPTP: Current Concepts and Controversies.” Clinical Neuropharmacology 9 (6): 485–507.

Lee R., and Mason A. 2011. Population Aging and the Generational Economy: A Global Perspective. Har/Chrt edition. Edward Elgar Pub.

Lekoubou A., Echouffo-Tcheugui J., and Kengne A. 2014. “Epidemiology of Neurodegenerative Diseases in Sub-Saharan Africa: A Systematic Review.” BMC Public Health 14 (1): 653. Doi: 10.1186/1471-2458-14-653.

Lesage S., Condroyer C., Klebe S., Honoré A., et al. 2012. “Identification of VPS35 Mutations Replicated in French Families with Parkinson Disease.” Neurology 78 (18): 1449–50. Doi:10.1212/WNL.0b013e318253d5f2.

Lesage S., Anheim M., Condroyer C., et al. 2011. “Large-Scale Screening of the Gaucher’s Disease-Related Glucocerebrosidase Gene in Europeans with Parkinson’s Disease.” Human Molecular Genetics 20 (1): 202–10. Doi:10.1093/hmg/ddq454.

Lesage S., and Brice A. 2009. “Parkinson’s Disease: From Monogenic Forms to Genetic Susceptibility Factors.” Human Molecular Genetics 18 (R1): R48–59. Doi:10.1093/hmg/ddp012.

Lesage S., Dürr A., Tazir M., et al. 2006. “LRRK2 G2019S as a Cause of Parkinson’s Disease in North African Arabs.” The New England Journal of Medicine 354 (4): 422–23. Doi: 10.1056/NEJMc055540.

Lesage S., Leutenegger A.-L., Ibanez P., et al. 2005. “LRRK2 Haplotype Analyses in European and North African Families with Parkinson Disease: A Common Founder for the G2019S Mutation Dating from the 13th Century.” American Journal of Human Genetics 77 (2): 330–32.

Li J., Doyle M, Saeed I., et al. 2014. “Bioinformatics Pipelines for Targeted Resequencing and Whole-Exome Sequencing of Human and Mouse Genomes: A Virtual Appliance Approach for Instant Deployment.” PLoS ONE 9 (4): e95217. Doi:10.1371/journal.pone.0095217.

Lin X., Tang W., Ahmad S., Lu J., Colby C., Zhu J., and Yu Q. 2012. “Applications of Targeted Gene Capture and next-Generation Sequencing Technologies in Studies of Human Deafness and Other Genetic Disabilities.” Hearing Research, Annual Reviews 2012, 288 (1–2): 67–76. Doi:10.1016/j.heares.2012.01.004.

Liu L., Li Y., Li S., Hu N., He Y., Pong R., Lin D., Lu L., and Law M. 2012. “Comparison of Next-Generation Sequencing Systems.” BioMed Research International 2012 (July): e251364. Doi:10.1155/2012/251364.

Li W., Matsuoka M., Kai M., Thapa P., Khadge S., Hagge D., Brennan P. J., and Vissa V. 2012. “Real-Time PCR and High-Resolution Melt Analysis for Rapid Detection of Mycobacterium Leprae Drug Resistance Mutations and Strain Types.” Journal of Clinical Microbiology 50 (3): 742–53. Doi:10.1128/JCM.05183-11.

Lorenzetti D., Bohlega S., and Zoghbi H. Y. 1997. “The Expansion of the CAG Repeat in Ataxin-2 Is a Frequent Cause of Autosomal Dominant Spinocerebellar Ataxia.” Neurology 49 (4): 1009–13.

Lu C.-S., Chou Y., Kuo P.-C., Chang H.-C., and Weng Y.-H. 2004. “The Parkinsonian Phenotype of Spinocerebellar Ataxia Type 2.” Archives of Neurology 61 (1): 35–38. Doi:10.1001/archneur.61.1.35.

Lücking C. B., Dürr A., Bonifati V., et al. 2000. “Association between Early-Onset Parkinson’s Disease and Mutations in the Parkin Gene.” The New England Journal of Medicine 342 (21): 1560–67. Doi: 10.1056/NEJM200005253422103.

Lyon E., and Wittwer C. T. 2009. “LightCycler Technology in Molecular Diagnostics.” The Journal of Molecular Diagnostics : JMD 11 (2): 93–101. Doi:10.2353/jmoldx.2009.080094.

Majounie E., Wardle M., Muzaimi M., Cross W. C., Robertson N. P., Williams N., and Morris H. 2007. “Case Control Analysis of Repeat Expansion Size in Ataxia.” Neuroscience Letters 429 (1): 28–32. Doi:10.1016/j.neulet.2007.09.055.

Mammucari C., and Rizzuto R. 2010. “Signalling Pathways in Mitochondrial Dysfunction and Aging.” Mechanisms of Ageing and Development 131 (7-8): 536–43. Doi:10.1016/j.mad.2010.07.003.

Marsden C. D. 1994. “Problems with Long-Term Levodopa Therapy for Parkinson’s Disease.” Clinical Neuropharmacology 17 Suppl 2: S32–44.

Marttila R. J, and Rinne U. K. 1976. “Epidemiology of Parkinson’s Disease in Finland.” Acta Neurologica Scandinavica 53 (2): 81–102.

Matsuda N., Sato S., Shiba K., et al. 2010. “PINK1 Stabilized by Mitochondrial Depolarization Recruits Parkin to Damaged Mitochondria and Activates Latent Parkin for Mitophagy.” The Journal of Cell Biology 189 (2): 211–21. Doi:10.1083/jcb.200910140.

Matsumine H., Saito M., Shimoda-Matsubayashi S., et al. 1997. “Localization of a Gene for an Autosomal Recessive Form of Juvenile Parkinsonism to Chromosome 6q25.2-27.” American Journal of Human Genetics 60 (3): 588–96.

Mayeux R, Marder K., Cote L. J., et al. 1995. “The Frequency of Idiopathic Parkinson’s Disease by Age, Ethnic Group, and Sex in Northern Manhattan, 1988-1993.” American Journal of Epidemiology 142 (8): 820–27.

McCormack A. L., Thiruchelvam M., Manning-Bog A., et al. 2002. “Environmental Risk Factors and Parkinson’s Disease: Selective Degeneration of Nigral Dopaminergic Neurons Caused by the Herbicide Paraquat.” Neurobiology of Disease 10 (2): 119–27.

McInerney-Leo A., Gwinn-Hardy K., and Nussbaum R. L. 2004. “Prevalence of Parkinson’s Disease in Populations of African Ancestry: A Review.” Journal of the National Medical Association 96 (7): 974–79.

McNaught K. St P., Mytilineou C., Jnobaptiste R., et al. 2002. “Impairment of the Ubiquitin-Proteasome System Causes Dopaminergic Cell Death and Inclusion Body Formation in Ventral Mesencephalic Cultures.” Journal of Neurochemistry 81 (2): 301–6.

McNaught K. St P., Perl D. P., Brownell A.-L., and Olanow C.W. 2004. “Systemic Exposure to Proteasome Inhibitors Causes a Progressive Model of Parkinson’s Disease.” Annals of Neurology 56 (1): 149–62. Doi:10.1002/ana.20186.

McQuillan R., Leutenegger A.-L., Abdel-Rahman R., et al. 2008. “Runs of Homozygosity in European Populations.” American Journal of Human Genetics 83 (3): 359–72. Doi:10.1016/j.ajhg.2008.08.007.

Meeus B., Theuns J., and van Broeckhoven C. 2010. “GIGYF2 in Parkinson’s Disease: Innocent until Proven Otherwise.” Neurobiology of Aging 31 (6): 1072–74. Doi:10.1016/j.neurobiolaging.2009.11.005.

Metzker M. L. 2010. “Sequencing Technologies - the next Generation.” Nature Reviews. Genetics 11 (1): 31–46. Doi: 10.1038/nrg2626.

Mizushima K., Watanabe M., Kondo I., Okamoto K., Shizuka M., Abe K., Aoki M., and Shoji M. 1999. “Analysis of Spinocerebellar Ataxia Type 2 Gene and Haplotype Analysis: (CCG) 1-2 Polymorphism and Contribution to Founder Effect.” Journal of Medical Genetics 36 (2): 112–14.

Montgomery J., Wittwer C. T., Kent J. O., and Zhou L. 2007. “Scanning the Cystic Fibrosis Transmembrane Conductance Regulator Gene Using High-Resolution DNA Melting Analysis.” Clinical Chemistry 53 (11): 1891–98. Doi:10.1373/clinchem.2007.092361.

Moore D. J. 2006. “Parkin: A Multifaceted Ubiquitin Ligase.” Biochemical Society Transactions 34 (Pt 5): 749–53. Doi: 10.1042/BST0340749.

Morelli M., and Wardas J. 2001. “Adenosine A (2a) Receptor Antagonists: Potential Therapeutic and Neuroprotective Effects in Parkinson’s Disease.” Neurotoxicity Research 3 (6): 545–56.

Mshana G., Dotchin C., and Walker R. 2011. “‘We Call It the Shaking Illness’: Perceptions and Experiences of Parkinson’s Disease in Rural Northern Tanzania.” BMC Public Health 11: 219. Doi: 10.1186/1471-2458-11-219.

Muangpaisan W., Hori H., and Brayne C. 2009. “Systematic Review of the Prevalence and Incidence of Parkinson’s Disease in Asia.” Journal of Epidemiology / Japan Epidemiological Association 19 (6): 281–93.

Muangpaisan W., Mathews A., Hori H., and Seidel D. 2011. “A Systematic Review of the Worldwide Prevalence and Incidence of Parkinson’s Disease.” Journal of the Medical Association of Thailand = Chotmaihet Thangphaet 94 (6): 749–55.

Mueller J. C., Fuchs J., Hofer A., et al. 2005. “Multiple Regions of Alpha-Synuclein Are Associated with Parkinson’s Disease.” Annals of Neurology 57 (4): 535–41. Doi:10.1002/ana.20438.

Nacimiento W. 2006. “[Parkinson-plus-syndromes].” Fortschritte Der Neurologie-Psychiatrie 74 (3): 165–78; quiz 179–80. Doi: 10.1055/s-2005-915580.

Nakamura K., Jeong S.-Y., Uchihara T., et al. 2001. “SCA17, a Novel Autosomal Dominant Cerebellar Ataxia Caused by an Expanded Polyglutamine in TATA-Binding Protein.” Human Molecular Genetics 10 (14): 1441–48.

Nalls M. A., Duran R., Lopez G., et al. 2013. “A Multicentre Study of Glucocerebrosidase Mutations in Dementia with Lewy Bodies.” JAMA Neurology 70 (6): 727–35. Doi:10.1001/jamaneurol.2013.1925.

Narendra D., Walker J. E., and Youle R. 2012. “Mitochondrial Quality Control Mediated by PINK1 and Parkin: Links to Parkinsonism.” Cold Spring Harbor Perspectives in Biology 4 (11). Doi:10.1101/cshperspect.a011338.

Neuhausen S. L. 2000. “Founder Populations and Their Uses for Breast Cancer Genetics.” Breast Cancer Research: BCR 2 (2): 77–81.

Ng P. C., and Henikoff S. 2001. “Predicting Deleterious Amino Acid Substitutions.” Genome Research 11 (5): 863–74. Doi:10.1101/gr.176601.

Nuscher B., Kamp F., Mehnert T., et al. 2004. “Alpha-Synuclein Has a High Affinity for Packing Defects in a Bilayer Membrane: A Thermodynamics Study.” The Journal of Biological Chemistry 279 (21): 21966–75. Doi:10.1074/jbc.M401076200.

Nuytemans K., Bademci G., Inchausti V., et al. 2013. “Whole Exome Sequencing of Rare Variants in EIF4G1 and VPS35 in Parkinson Disease.” Neurology 80 (11): 982–89. Doi:10.1212/WNL.0b013e31828727d4.

Nuytemans K., Theuns J., Cruts M., and van Broeckhoven C. 2010. “Genetic Etiology of Parkinson Disease Associated with Mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 Genes: A Mutation Update.” Human Mutation 31 (7): 763–80. Doi:10.1002/humu.21277.

Oakley A. E., Collingwood J. F., Dobson J, et al. 2007. “Individual Dopaminergic Neurons Show Raised Iron Levels in Parkinson Disease.” Neurology 68 (21): 1820–25. Doi:10.1212/01.wnl.0000262033.01945.9a.

Obeso J. A., Rodriguez M. C., Guridi J., Alvarez L., et al. 2001. “Lesion of the Basal Ganglia and Surgery for Parkinson Disease.” Archives of Neurology 58 (7): 1165–66.

Okubadejo N., Britton A., Crews C., et al. 2008. “Analysis of Nigerians with Apparently Sporadic Parkinson Disease for Mutations in LRRK2, PRKN and ATXN3.” PLoS ONE 3 (10). Doi:10.1371/journal.pone.0003421.

Osuntokun B. O., Adeuja A., Schoenberg B. S., et al. 1987. “Neurological Disorders in Nigerian Africans: A Community-Based Study.” Acta Neurologica Scandinavica 75 (1): 13–21.

Ozelius L. J., Senthil G., Saunders-Pullman R., et al. 2006. “LRRK2 G2019S as a Cause of Parkinson’s Disease in Ashkenazi Jews.” The New England Journal of Medicine 354 (4): 424–25. Doi: 10.1056/NEJMc055509.

Paganini-Hill A. 2001. “Risk Factors for Parkinson’s Disease: The Leisure World Cohort Study.” Neuroepidemiology 20 (2): 118–24. Doi: 54770.

Paisán-Ruíz C., Jain S., Evans E. W., et al. 2004. “Cloning of the Gene Containing Mutations That Cause PARK8-Linked Parkinson’s Disease.” Neuron 44 (4): 595–600. Doi:10.1016/j.neuron.2004.10.023.

Pankratz N., Beecham G. W., DeStefano A. L., et al. 2012. “Meta-Analysis of Parkinson’s Disease: Identification of a Novel Locus, RIT2.” Annals of Neurology 71 (3): 370–84. Doi:10.1002/ana.22687.

Papadimitriou A., Veletza V, Hadjigeorgiou G. M., et al. 1999. “Mutated Alpha-Synuclein Gene in Two Greek Kindreds with Familial PD: Incomplete Penetrance?” Neurology 52 (3): 651–54.

Papenhausen P., Schwartz S., Risheg H., et al. 2011. “UPD Detection Using Homozygosity Profiling with a SNP Genotyping Microarray.” American Journal of Medical Genetics. Part A 155A (4): 757–68. Doi:10.1002/ajmg.a.33939.

Parker W. D., Boyson S. J., and Parks J. K. 1989. “Abnormalities of the Electron Transport Chain in Idiopathic Parkinson’s Disease.” Annals of Neurology 26 (6): 719–23. Doi:10.1002/ana.410260606.

Parkinson J. 2002. “An Essay on the Shaking Palsy. 1817.” The Journal of Neuropsychiatry and Clinical Neurosciences 14 (2): 223–36; discussion 222.

Park J.-S., Mehta P., Cooper A., et al. 2011. “Pathogenic Effects of Novel Mutations in the P-Type ATPase ATP13A2 (PARK9) Causing Kufor-Rakeb Syndrome, a form of Early-Onset Parkinsonism.” Human Mutation 32 (8): 956–64. Doi:10.1002/humu.21527.

Pearce V., and Wilson I. 2007. “Parkinson’s Disease in Africa.” Age and Ageing 36 (2): 116–17. Doi:10.1093/ageing/afl173.

Pennington S., Snell K., Lee M., and Walker R. 2010. “The Cause of Death in Idiopathic Parkinson’s Disease.” Parkinsonism & Related Disorders 16 (7): 434–37. Doi:10.1016/j.parkreldis.2010.04.010.

Periquet M., Latouche M., Lohmann E., et al. 2003. “Parkin Mutations Are Frequent in Patients with Isolated Early-Onset Parkinsonism.” Brain: A Journal of Neurology 126 (Pt 6): 1271–78.

Podhajska A., Musso A., Trancikova A., et al. 2012. Common pathogenic effects of missense mutations in the P-type ATPase ATP13A2 (PARK9) associated with early-onset parkinsonism. PLos One 7: e39942.

Polymeropoulos M. H, Higgins J. J., Golbe L., Johnson W. G., et al. 1996. “Mapping of a Gene for Parkinson’s Disease to Chromosome 4q21-q23.” Science (New York, N.Y.) 274 (5290): 1197–99.

Polymeropoulos M. H., Lavedan C., Leroy E., et al. 1997. “Mutation in the Alpha-Synuclein Gene Identified in Families with Parkinson’s Disease.” Science (New York, N.Y.) 276 (5321): 2045–47.

Popat R. A., Van Den Eeden S. K., Tanner C. M., et al. 2005. “Effect of Reproductive Factors and Postmenopausal Hormone Use on the Risk of Parkinson Disease.” Neurology 65 (3): 383–90. Doi:10.1212/01.wnl.0000171344.87802.94.

Poulopoulos M., Levy O.A., and Alcalay R. N. 2012. “The Neuropathology of Genetic Parkinson’s Disease.” Movement Disorders: Official Journal of the Movement Disorder Society 27 (7): 831–42. Doi:10.1002/mds.24962.

Pramstaller P., Schlossmacher M. G., Jacques T. S., et al. 2005. “Lewy Body Parkinson’s Disease in a Large Pedigree with 77 Parkin Mutation Carriers.” Annals of Neurology 58 (3): 411–22. Doi:10.1002/ana.20587.

Pringsheim T., Jette N., Frolkis A., and Steeves T. 2014. “The Prevalence of Parkinson’s Disease: A Systematic Review and Meta-Analysis.” Movement Disorders: Official Journal of the Movement Disorder Society, June. Doi:10.1002/mds.25945.

Proukakis C., Dudzik C. G., Brier T., et al. 2013. “A Novel A-Synuclein Missense Mutation in Parkinson Disease.” Neurology 80 (11): 1062–64. Doi:10.1212/WNL.0b013e31828727ba.

Pulst S. M., Nechiporuk A., Nechiporuk T., et al. 1996. “Moderate Expansion of a Normally Biallelic Trinucleotide Repeat in Spinocerebellar Ataxia Type 2.” Nature Genetics 14 (3): 269–76. Doi: 10.1038/ng1196-269.

Qi H., and Li S. 2013. “Dose-Response Meta-Analysis on Coffee, Tea and Caffeine Consumption with Risk of Parkinson’s Disease.” Geriatrics & Gerontology International, July. Doi:10.1111/ggi.12123.

Quail M. A., Smith M., Coupland P., et al. 2012. “A Tale of Three next Generation Sequencing Platforms: Comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq Sequencers.” BMC Genomics 13 (1): 341. Doi: 10.1186/1471-2164-13-341.

Quik M., and Kulak J. M. 2002. “Nicotine and Nicotinic Receptors; Relevance to Parkinson’s Disease.” Neurotoxicology 23 (4-5): 581–94.

Ragothaman M., Sarangmath N., Chaudhary S., et al. 2004. “Complex Phenotypes in an Indian Family with Homozygous SCA2 Mutations.” Annals of Neurology 55 (1): 130–33. Doi:10.1002/ana.10815.

Ramirez A., Heimbach A., Gründemann J., et al. 2006. “Hereditary Parkinsonism with Dementia Is Caused by Mutations in ATP13A2, Encoding a Lysosomal Type 5 P-Type ATPase.” Nature Genetics 38 (10): 1184–91. Doi: 10.1038/ng1884.

Ramsay M., Tiemessen C.T., Choudhury A., and Soodyall H. 2011. “Africa: The next Frontier for Human Disease Gene Discovery?” Human Molecular Genetics 20 (R2): R214–20. Doi:10.1093/hmg/ddr401.

Ravikumar B., Vacher C., Berger Z., et al. 2004. “Inhibition of mTOR Induces Autophagy and Reduces Toxicity of Polyglutamine Expansions in Fly and Mouse Models of Huntington Disease.” Nature Genetics 36 (6): 585–95. Doi: 10.1038/ng1362.

Reed G. H., and Wittwer C.T. 2004. “Sensitivity and Specificity of Single-Nucleotide Polymorphism Scanning by High-Resolution Melting Analysis.” Clinical Chemistry 50 (10): 1748–54. Doi:10.1373/clinchem.2003.029751.

Richards, M., and Chaudhuri K. R. 1996. “Parkinson’s Disease in Populations of African Origin: A Review.” Neuroepidemiology 15 (4): 214–21.

Rocca W. A., McDonnell S. K., Strain K. J., et al. 2004. “Familial Aggregation of Parkinson’s Disease: The Mayo Clinic Family Study.” Annals of Neurology 56 (4): 495–502. Doi:10.1002/ana.20228.

Rodriguez-Oroz M. C., Obeso J. A, Lang A. E., et al. 2005. “Bilateral Deep Brain Stimulation in Parkinson’s Disease: A Multicentre Study with 4 Years Follow-Up.” Brain: A Journal of Neurology 128 (Pt 10): 2240–49. Doi:10.1093/brain/awh571.

Rogaeva E., Johnson J., Lang A. E., et al. 2004. “Analysis of the PINK1 Gene in a Large Cohort of Cases with Parkinson Disease.” Archives of Neurology 61 (12): 1898–1904. Doi:10.1001/archneur.61.12.1898.

Romeo G., and McKusick V. A. 1994. “Phenotypic Diversity, Allelic Series and Modifier Genes.” Nature Genetics 7 (4): 451–53. Doi: 10.1038/ng0894-451.

Ross G. W, Abbott R. D., Petrovitch H., Morens D. M., et al. 2000. “Association of Coffee and Caffeine Intake with the Risk of Parkinson Disease.” JAMA: The Journal of the American Medical Association 283 (20): 2674–79.

Ross O. A., Braithwaite A. T., Skipper L. M., et al. 2008. “Genomic Investigation of A-Synuclein Multiplication and Parkinsonism.” Annals of Neurology 63 (6): 743–50. Doi:10.1002/ana.21380.

Ross O. A., Soto-Ortolaza A. I., Heckman M. G., et al. 2011. “Association of LRRK2 Exonic Variants with Susceptibility to Parkinson’s Disease: A Case-Control Study.” Lancet Neurology 10 (10): 898–908. Doi: 10.1016/S1474-4422(11)70175-2.

Rothfuss O., Fischer H., Hasegawa T., et al. 2009. “Parkin Protects Mitochondrial Genome Integrity and Supports Mitochondrial DNA Repair.” Human Molecular Genetics 18 (20): 3832–50. Doi:10.1093/hmg/ddp327.

Rubinsztein D. C. 2006. “The Roles of Intracellular Protein-Degradation Pathways in Neurodegeneration.” Nature 443 (7113): 780–86. Doi: 10.1038/nature05291.

Rüb U., Schöls L., Paulson H., et al. 2013. “Clinical Features, Neurogenetics and Neuropathology of the Polyglutamine Spinocerebellar Ataxias Type 1, 2, 3, 6 and 7.” Progress in Neurobiology 104 (May): 38–66. Doi:10.1016/j.pneurobio.2013.01.001.

Rudenko I. N., Kaganovich A., Hauser D. N., et al. 2012. “The G2385R Variant of Leucine-Rich Repeat Kinase 2 Associated with Parkinson’s Disease Is a Partial Loss-of-Function Mutation.” The Biochemical Journal 446 (1): 99–111. Doi: 10.1042/BJ20120637.

Salamone J. D., and Correa M. 2012. “The Mysterious Motivational Functions of Mesolimbic Dopamine.” Neuron 76 (3): 470–85. Doi:10.1016/j.neuron.2012.10.021.

Samaranch L., Lorenzo-Betancor O., Arbelo J. M., et al. 2010. “PINK1-Linked Parkinsonism Is Associated with Lewy Body Pathology.” Brain: A Journal of Neurology 133 (Pt 4): 1128–42. Doi:10.1093/brain/awq051.

Sambrook J., and Russell D. W. 2006. “Purification of Nucleic Acids by Extraction with Phenol: Chloroform.” Cold Spring Harbor Protocols 2006 (1): pdb.prot4455. Doi:10.1101/pdb.prot4455.

Sanderson W. C., and Scherbov S. 2014. “Measuring the Speed of Aging across Population Subgroups.” PloS One 9 (5): e96289. Doi:10.1371/journal.pone.0096289.

Scarciolla O., Brancati F., Valente E., et al. 2007. “Multiplex Ligation-Dependent Probe Amplification Assay for Simultaneous Detection of Parkinson’s Disease Gene Rearrangements.” Movement Disorders: Official Journal of the Movement Disorder Society 22 (15): 2274–78. Doi:10.1002/mds.21532.

Schapira A. H. 2007. “Mitochondrial Dysfunction in Parkinson’s Disease.” Cell Death & Differentiation 14 (7): 1261–66. Doi:10.1038/sj.cdd.4402160.

Schoenberg B. S., Osuntokun B. O., Adeuja A. O., et al. 1988. “Comparison of the Prevalence of Parkinson’s Disease in Black Populations in the Rural United States and in Rural Nigeria: Door-to-Door Community Studies.” Neurology 38 (4): 645–46.

Scholefield J., Greenberg J., Weinberg M., et al. 2009. “Design of RNAi Hairpins for Mutation-Specific Silencing of Ataxin-7 and Correction of a SCA7 Phenotype.” PloS One 4 (9): e7232. Doi:10.1371/journal.pone.0007232.

Schöls L., Bauer P., Schmidt T., Schulte T., and Riess O. 2004. “Autosomal Dominant Cerebellar Ataxias: Clinical Features, Genetics, and Pathogenesis.” Lancet Neurology 3 (5): 291–304. Doi: 10.1016/S1474-4422(04)00737-9.

Schouten J. P., McElgunn C., Waaijer R., et al. 2002. “Relative Quantification of 40 Nucleic Acid Sequences by Multiplex Ligation-Dependent Probe Amplification.” Nucleic Acids Research 30 (12): e57.

Schulte E. C, Mollenhauer B., Zimprich A., et al. 2012. “Variants in Eukaryotic Translation Initiation Factor 4G1 in Sporadic Parkinson’s Disease.” Neurogenetics 13 (3): 281–85. Doi: 10.1007/s10048-012-0334-9.

Schultheis P., Hagen T., Toole K., et al. 2004. “Characterization of the P5 Subfamily of P-Type Transport ATPases in Mice.” Biochemical and Biophysical Research Communications 323 (3): 731–38. Doi:10.1016/j.bbrc.2004.08.156.

Sequeiros J., Martindale J., Seneca S., et al. 2010. “EMQN Best Practice Guidelines for Molecular Genetic Testing of SCAs.” European Journal of Human Genetics: EJHG 18 (11): 1173–76. Doi:10.1038/ejhg.2010.8.

Shimura H., Hattori N., Kubo S., Mizuno Y., et al. 2000. “Familial Parkinson Disease Gene Product, Parkin, Is an Ubiquitin-Protein Ligase.” Nature Genetics 25 (3): 302–5. Doi: 10.1038/77060.

Shimura H., Hattori N., Kubo S., Yoshikawa M., et al. 1999. “Immunohistochemical and Subcellular Localization of Parkin Protein: Absence of Protein in Autosomal Recessive Juvenile Parkinsonism Patients.” Annals of Neurology 45 (5): 668–72.

Sidransky E., Nalls M. A., Aasly J. O., Aharon-Peretz J., et al. 2009. “Multicenter Analysis of Glucocerebrosidase Mutations in Parkinson’s Disease.” The New England Journal of Medicine 361 (17): 1651–61. Doi: 10.1056/NEJMoa0901281.

Sierra M., González-Aramburu I., Sánchez-Juan P., et al. 2011. “High Frequency and Reduced Penetrance of lRRK2 g2019S Mutation among Parkinson’s Disease Patients in Cantabria (Spain).” Movement Disorders 26 (13): 2343–46. Doi:10.1002/mds.23965.

Sim N.-L., Kumar P., Hu J., Henikoff S., Schneider G., and Ng P. 2012. “SIFT Web Server: Predicting Effects of Amino Acid Substitutions on Proteins.” Nucleic Acids Research 40 (Web Server issue): W452–57. Doi:10.1093/nar/gks539.

Singleton A. B., Farrer M., Johnson J., Singleton A., Hague S., Kachergus J., Hulihan M., et al. 2003. “Alpha-Synuclein Locus Triplication Causes Parkinson’s Disease.” Science (New York, N.Y.) 302 (5646): 841. Doi:10.1126/science.1090278.

Sironi F., Primignani P., Ricca S., et al. 2013. “DJ-1 Analysis in a Large Cohort of Italian Early Onset Parkinson Disease Patients.” Neuroscience Letters 557 (PB): 165–70. Doi:10.1016/j.neulet.2013.10.048.

Smith D. C., Bryer A., Watson L. M., and Greenberg J. 2012. “Inherited Polyglutamine Spinocerebellar Ataxias in South Africa.” South African Medical Journal = Suid-Afrikaanse Tydskrif Vir Geneeskunde 102 (8): 683–86.

Sorge J., Gross E., West C., and Beutler E. 1990. “High Level Transcription of the Glucocerebrosidase Pseudogene in Normal Subjects and Patients with Gaucher Disease.” The Journal of Clinical Investigation 86 (4): 1137–41. Doi: 10.1172/JCI114818.

Spatola M., and Wider C. 2014. “Genetics of Parkinson’s Disease: The Yield.” Parkinsonism & Related Disorders 20 Suppl 1 (January): S35–38. Doi: 10.1016/S1353-8020(13)70011-7.

Sriram S. R., Li X., Ko H., et al. 2005. “Familial-Associated Mutations Differentially Disrupt the Solubility, Localization, Binding and Ubiquitination Properties of Parkin.” Human Molecular Genetics 14 (17): 2571–86. Doi:10.1093/hmg/ddi292.

Stevanin G., Dürr A., and Brice A. 2000. “Clinical and Molecular Advances in Autosomal Dominant Cerebellar Ataxias: From Genotype to Phenotype and Physiopathology.” European Journal of Human Genetics: EJHG 8 (1): 4–18. Doi:10.1038/sj.ejhg.5200403.

Sun M., Latourelle J., Wooten G. F., et al. 2006. “Influence of Heterozygosity for Parkin Mutation on Onset Age in Familial Parkinson Disease: The GenePD Study.” Archives of Neurology 63 (6): 826–32. Doi:10.1001/archneur.63.6.826.

Sunyaev S., Ramensky V., Koch I., Lathe W., Kondrashov A. S., and Bork P. 2001. “Prediction of Deleterious Human Alleles.” Human Molecular Genetics 10 (6): 591–97.

Sveinbjörnsdottir S., Hicks A. A., Jonsson T., Pétursson H., et al. 2000. “Familial Aggregation of Parkinson’s Disease in Iceland.” The New England Journal of Medicine 343 (24): 1765–70. Doi: 10.1056/NEJM200012143432404.

Takano H., Cancel G., Ikeuchi T., Lorenzetti D., et al. 1998. “Close Associations between prevalence’s of dominantly Inherited Spinocerebellar Ataxias with CAG-Repeat Expansions and Frequencies of Large Normal CAG Alleles in Japanese and Caucasian Populations.” American Journal of Human Genetics 63 (4): 1060–66. Doi: 10.1086/302067.

Tanaka M., Machida Y., Niu S., et al. 2004. “Trehalose Alleviates Polyglutamine-Mediated Pathology in a Mouse Model of Huntington Disease.” Nature Medicine 10 (2): 148–54. Doi: 10.1038/nm985.

Tang S., Zhang Z., Kavitha G., Tan E., and Ng S. 2009. “MDPD: An Integrated Genetic Information Resource for Parkinson’s Disease.” Nucleic Acids Research 37 (Database issue): D858–62. Doi:10.1093/nar/gkn770.

Tanner C. M., Kamel F., Ross G. W., et al. 2011. “Rotenone, Paraquat, and Parkinson’s Disease.” Environmental Health Perspectives 119 (6): 866–72. Doi:10.1289/ehp.1002839.

Tayebi N., Walker J., Stubblefield B., Orvisky E., et al. 2003. “Gaucher Disease with Parkinsonian Manifestations: Does Glucocerebrosidase Deficiency Contribute to a Vulnerability to Parkinsonism?” Molecular Genetics and Metabolism 79 (2): 104–9. Doi: 10.1016/S1096-7192(03)00071-4.

Tekle-Haimanot R., Abebe M., Gebre-Mariam A., et al. 1990. “Community-Based Study of Neurological Disorders in Rural Central Ethiopia.” Neuroepidemiology 9 (5): 263–77.

Thanvi B., and Lo T. 2004. “Long Term Motor Complications of Levodopa: Clinical Features, Mechanisms, and Management Strategies.” Postgraduate Medical Journal 80 (946): 452–58. Doi:10.1136/pgmj.2003.013912.

Thomas K. J., McCoy M. K., Blackinton J., et al. 2011. “DJ-1 Acts in Parallel to the PINK1/parkin Pathway to Control Mitochondrial Function and Autophagy.” Human Molecular Genetics 20 (1): 40–50. Doi:10.1093/hmg/ddq430.

Tishkoff S. A., Reed F., Friedlaender F. R., et al. 2009. “The Genetic Structure and History of Africans and African Americans.” Science (New York, N.Y.) 324 (5930): 1035–44. Doi:10.1126/science.1172257.

Tison F., Dartigues J. F., Dubes L., Zuber M., Alperovitch A., and Henry P. 1994. “Prevalence of Parkinson’s Disease in the Elderly: A Population Study in Gironde, France.” Acta Neurologica Scandinavica 90 (2): 111–15.

Trinh J., Guella I., and Farrer M. 2014. “Disease Penetrance of Late-Onset Parkinsonism: A Meta-Analysis.” JAMA Neurology, October. Doi:10.1001/jamaneurol.2014.1909.

Trinh J., and Farrer M. 2013. “Advances in the Genetics of Parkinson Disease.” Nature Reviews Neurology 9 (8): 445–54. Doi:10.1038/nrneurol.2013.132.

Tsang K. L., Ho S. L., and Lo S. K. 2000. “Estrogen Improves Motor Disability in Parkinsonian Postmenopausal Women with Motor Fluctuations.” Neurology 54 (12): 2292–98.

Tucci A., Charlesworth G., Sheerin U.M., et al. 2012. “Study of the Genetic Variability in a Parkinson’s Disease Gene: EIF4G1.” Neuroscience Letters 518 (1): 19–22. Doi:10.1016/j.neulet.2012.04.033.

Ugolino J., Fang S., Kubisch C., and MonteiroM.J. 2011. “Mutant Atp13a2 Proteins Involved in Parkinsonism Are Degraded by ER-Associated Degradation and Sensitize Cells to ER-Stress Induced Cell Death.” Human Molecular Genetics 20 (18): 3565–77. Doi:10.1093/hmg/ddr274.

UK Parkinson’s Disease Consortium, Welcome Trust Case Control Consortium 2, et al. 2011. “Dissection of the Genetics of Parkinson’s Disease Identifies an Additional Association 5’ of SNCA and Multiple Associated Haplotypes at 17q21.” Human Molecular Genetics 20 (2): 345–53. Doi:10.1093/hmg/ddq469.

Valente E. M., Bentivoglio A. R., Dixon P. H., et al. 2001. “Localization of a Novel Locus for Autosomal Recessive Early-Onset Parkinsonism, PARK6, on Human Chromosome 1p35-p36.” American Journal of Human Genetics 68 (4): 895–900.

Valente E. M., Brancati F., Ferraris A., et al. 2002. “PARK6-Linked Parkinsonism Occurs in Several European Families.” Annals of Neurology 51 (1): 14–18.

Van Den Eeden S., Tanner C. M., et al. 2003. “Incidence of Parkinson’s Disease: Variation by Age, Gender, and Race/ethnicity.” American Journal of Epidemiology 157 (11): 1015–22.

Van de Warrenburg B. P., Lammens M., Lücking C. B., et al. 2001. “Clinical and Pathologic Abnormalities in a Family with Parkinsonism and Parkin Gene Mutations.” Neurology 56 (4): 555–57.

Van Dijk E. L., Auger H., Jaszczyszyn Y., and Thermes C. 2014. “Ten Years of next-Generation Sequencing Technology.” Trends in Genetics: TIG, August. Doi:10.1016/j.tig.2014.07.001.

Vaughan J., Durr A., Tassin J., et al. 1998. “The A-Synuclein Ala53Thr Mutation Is Not a Common Cause of Familial Parkinson’s Disease: A Study of 230 European Cases.” Annals of Neurology 44 (2): 270–73. Doi:10.1002/ana.410440221.

Vilariño-Güell C., Wider C., Ross O. A., et al. 2011. “VPS35 Mutations in Parkinson Disease.” The American Journal of Human Genetics 89 (1): 162–67. Doi:10.1016/j.ajhg.2011.06.001.

Von Campenhausen S., Bornschein B., Wick R., et al. 2005. “Prevalence and Incidence of Parkinson’s Disease in Europe.” European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology 15 (4): 473–90. Doi:10.1016/j.euroneuro.2005.04.007.

Vossen R. H., Aten A. M., Roos A., and den Dunnen J. T. 2009. “High-Resolution Melting Analysis (HRMA): More than Just Sequence Variant Screening.” Human Mutation 30 (6): 860–66. Doi:10.1002/humu.21019.

Walker J. M., Lwin A., Tayebi N., LaMarca M. E., Orvisky E., and Sidransky E. 2003. “Glucocerebrosidase Mutation T369M Appears to Be Another Polymorphism.” Clinical Genetics 63 (3): 237–38.

Wang K., Li M., and Hakonarson H. 2010. “ANNOVAR: Functional Annotation of Genetic Variants from High-Throughput Sequencing Data.” Nucleic Acids Research 38 (16): e164. Doi:10.1093/nar/gkq603.

Warne, J. P., Barron L. H., Goudie D., Kelly K., Dow D., Fitzpatrick D. R., and Brock D. J. 1996. “A General Method for the Detection of Large CAG Repeat Expansions by Fluorescent PCR.” Journal of Medical Genetics 33 (12): 1022–26.

Warren O., Kieburtz K., and Stocchi F. 2014. “Initiating Levodopa Therapy for Parkinson’s Disease.” Movement Disorders: Official Journal of the Movement Disorder Society, January. Doi:10.1002/mds.25805.

Wertheimer J., Gottuso A. Y., et al. 2014. “The Impact of STN Deep Brain Stimulation on Speech in Individuals with Parkinson’s Disease: The Patient’s Perspective.” Parkinsonism & Related Disorders, June. Doi:10.1016/j.parkreldis.2014.06.010.

Wesseling C., van Wendel B., Ruepert C., et al. 2001. “Paraquat in Developing Countries.” International Journal of Occupational and Environmental Health 7 (4): 275–86. Doi:10.1179/oeh.2001.7.4.275.

Wider C., Dickson D. W., and Wszolek Z. K. 2010. “Leucine-Rich Repeat Kinase 2 Gene-Associated Disease: Redefining Genotype-Phenotype Correlation.” Neuro-Degenerative Diseases 7 (1-3): 175–79. Doi: 10.1159/000289232.

Wider C., Skipper L., Solida A., et al. 2008. “Autosomal Dominant Dopa-Responsive Parkinsonism in a Multigenerational Swiss Family.” Parkinsonism & Related Disorders 14 (6): 465–70. Doi:10.1016/j.parkreldis.2007.11.013.

Wider C., Vilariño-Güell C., Jasinska-Myga B., et al. 2010. “Association of the MAPT Locus with Parkinson’s Disease.” European Journal of Neurology: The Official Journal of the European Federation of Neurological Societies 17 (3): 483–86. Doi:10.1111/j.1468-1331.2009.02847.x.

Wilkins A., Brown J. M., and Barker R. A. 2004. “SCA2 Presenting as Levodopa-Responsive Parkinsonism in a Young Patient from the United Kingdom: A Case Report.” Movement Disorders: Official Journal of the Movement Disorder Society 19 (5): 593–95. Doi:10.1002/mds.10715.

Winkler A., Tütüncü E., Trendafilova A., et al. 2010. “Parkinsonism in a Population of Northern Tanzania: A Community-Based Door-to-Door Study in Combination with a Prospective Hospital-Based Evaluation.” Journal of Neurology 257 (5): 799–805. Doi: 10.1007/s00415-009-5420-z.

Wittwer C. T. 2009. “High-Resolution DNA Melting Analysis: Advancements and Limitations.” Human Mutation 30 (6): 857–59. Doi:10.1002/humu.20951.

Wright W., Bradley A., Evanoff A., Lian M., Criswell S. R., and Racette B. 2010. “Geographic and Ethnic Variation in Parkinson Disease: A Population-Based Study of US Medicare Beneficiaries.” Neuroepidemiology 34 (3): 143–51. Doi: 10.1159/000275491.

Wu J., and Jiang R. 2013. “Prediction of Deleterious Nonsynonymous Single-Nucleotide Polymorphism for Human Diseases.” The Scientific World Journal 2013 (January): e675851. Doi:10.1155/2013/675851.

Xu K., Bastia E., and Schwarzschild M. 2005. “Therapeutic Potential of Adenosine A (2A) Receptor Antagonists in Parkinson’s Disease.” Pharmacology & Therapeutics 105 (3): 267–310. Doi:10.1016/j.pharmthera.2004.10.007.

Yonova-Doing E., and Atadzhanov M. 2012. “Analysis of LRRK2, SNCA, Parkin, PINK1, and DJ-1 in Zambian Patients with Parkinson Disease.” Parkinsonism and Related Disorders 18 (5): 567–71. Doi:10.1016/j.parkreldis.2012.02.018.

Zabetian P., Hutter C. M., Yearout D., et al. 2006. “LRRK2 G2019S in Families with Parkinson Disease Who Originated from Europe and the Middle East: Evidence of Two Distinct Founding Events Beginning Two Millennia Ago.” American Journal of Human Genetics 79 (4): 752–58. Doi: 10.1086/508025.

Zeng B.-Y., Bukhatwa S., Hikima A., Rose S., and Jenner P. 2006. “Reproducible Nigral Cell Loss after Systemic Proteasomal Inhibitor Administration to Rats.” Annals of Neurology 60 (2): 248–52. Doi:10.1002/ana.20932.

Zhang Y., Gao J., Chung K., Huang H., Dawson V.L., and Dawson T.M. 2000. “Parkin Functions as an E2-Dependent Ubiquitin- Protein Ligase and Promotes the Degradation of the Synaptic Vesicle-Associated Protein, CDCrel-1.” Proceedings of the National Academy of Sciences of the United States of America 97 (24): 13354–59. Doi:10.1073/pnas.240347797.

Zimprich A., Benet-Pagès A., Struhal W., et al. 2011. "A Mutation in VPS35, Encoding a Subunit of the Retromer Complex, Causes Late-Onset Parkinson Disease." The American Journal of Human Genetics 89 (1): 168–75. Doi:10.1016/j.ajhg.2011.06.008.

Zimprich A., Müller-Myhsok B., Farrer M., et al. 2004. "The PARK8 Locus in Autosomal Dominant Parkinsonism: Confirmation of Linkage and Further Delineation of the Disease-Containing Interval." American Journal of Human Genetics 74 (1): 11–19. Doi: 10.1086/380647.

Zoghbi H. Y. 1996. "The Expanding World of Ataxins." Nature Genetics 14 (3): 237–38. Doi: 10.1038/ng1196-237.

5.2 Online Databases and other Web Resources

- NCBI Reference Sequence: [http://www.ncbi.nlm.nih.gov/RefSeq/]: Date accessed 10/02/2013
- SMART Protein Analyzer (http://smart.embl-heidelberg.de/): Date accessed 25/03/2014
- MutationTaster: (http://www.mutationtaster.org/): Date accessed 25/10/2014
- UCSC Genome browser: http://genome.ucsc.edu: Date accessed 25/06/2013
- Primer3: http://frodo.wi.mit.edu/primer3: Date accessed 25/02/2013
- Mutation Database for Parkinson's disease (MDPD): http://datam.i2r.a-star.edu.sg/mdpd/index.php: Date accessed 25/06/2013
- Parkinson Disease Mutation database (PDmutDB): http://www.molgen.ua.ac.be/PDmutDB: Date accessed: 25/06/2013
- PDGENE: http://www.pdgene.org: Date accessed: 25/06/2013
- The international HapMap project: http://pngu.mgh.harvard.edu: Date accessed 14/10/2013
- 1000 Genomes Project: http://www.1000genomes.org:: Date accessed 14/10/2013
- dbSNP - National Centre for Biotechnology Information Database of single nucleotide polymorphisms: http://www.ncbi.nlm.nih.gov/snp: Date accessed 25/06/2013
- Human Genome Variation Society (HGVS) - Nomenclature for the description of sequence: http://www.hgvs.org/mutnomen: Date accessed 25/06/2013
- Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim: Date accessed: 25/06/2013
- PolyPhen-2: http://genetics.bwh.harvard.edu/pph2: Date accessed: 14/10/2014
- PubMed: http://ncbi.nlm.nih.gov/sites/entrez?db=PubMed: Date accessed: 25/06/2013
- UniProt Protein Knowledgebase: http://www.uniprot.org Date accessed 14/10/2014
- SWISS-MODEL- protein tertiary and quaternary structure modelling: http://swissmodel.expasy.org/interactive/qmGNhg/models/;http://swissmodel.expasy.org/interactive/SLdsLD/models/ Date accessed14/10/2014
- Project Hope : http://www.cmbi.ru.nl/hope/report/2757?2 Date accessed 14/10/2014

Appendix A: Individual protein domain structures

Supplementary Material to Chapter 1

Figures 1.5 to 1.10 have been adapted from Corti et al. (2011).

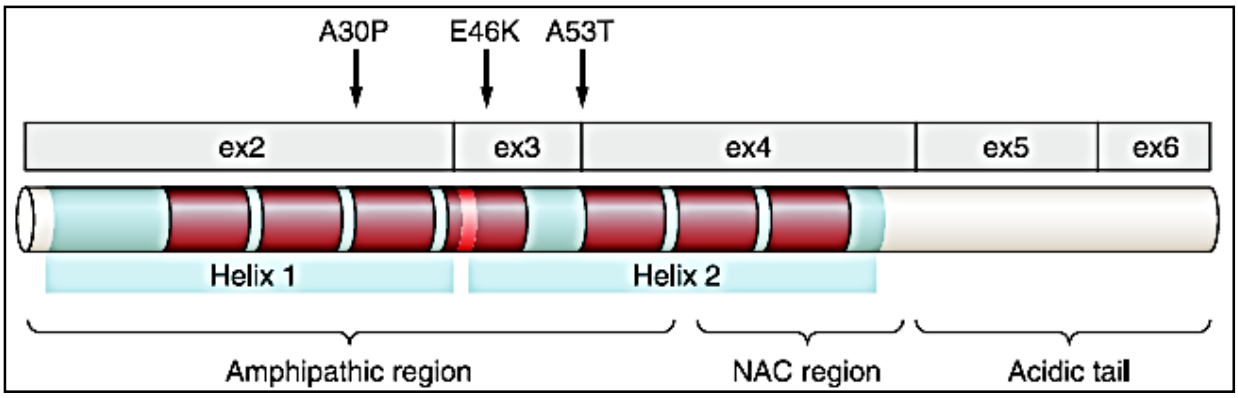


Figure 1.5 Schematic representation of the α-synuclein protein containing 3 pathogenic point mutations. αSYN consists of a NH2 terminus, a central hydrophobic NAC (nonamyloid component) region, and an acidic COOH-terminal region. Exon numbers above the protein structure indicate the boundaries of each domain.

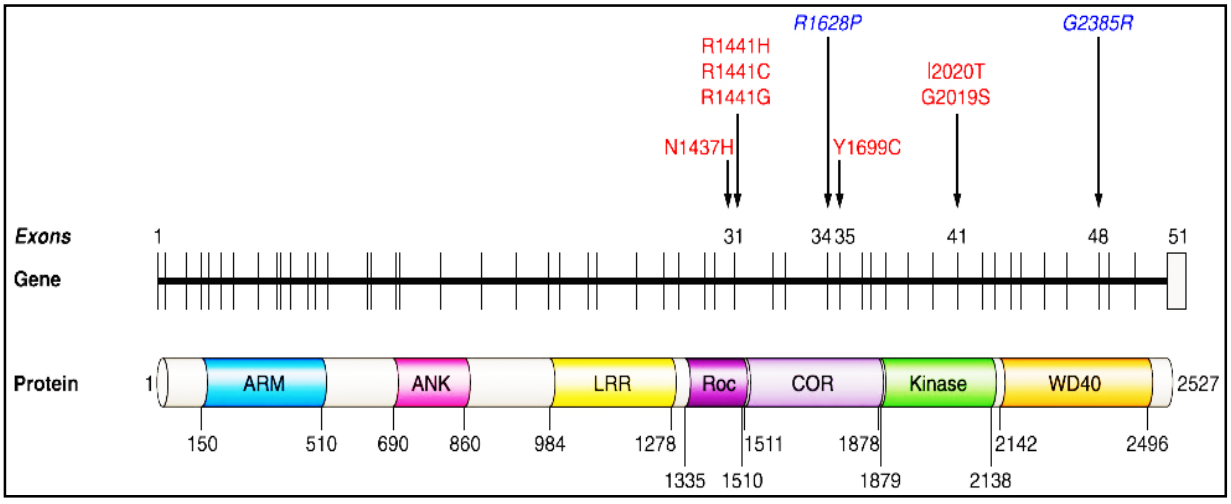


Figure 1.6 Schematic representation of the LRRK2 protein. Pathogenic mutations are shown in red and mutations that are specific for the Asian population are shown in blue. The amino acid residue numbers beneath each domain indicate the estimated domain boundaries.

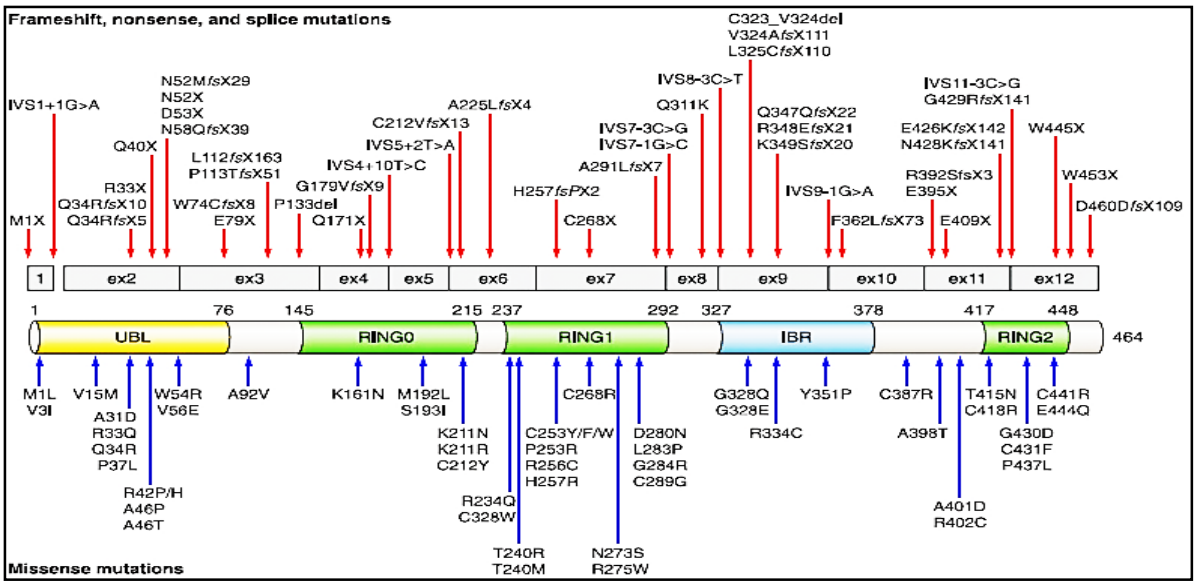


Figure 1.7 Schematic representation of the parkin protein structure. Predicted locations of pathogenic frameshift mutations above the transcript (red lines), and protein organizations and missense mutations below the transcript (blue lines).Parkin contains an NH2-terminal ubiquitin-like (UBL) domain followed by three RING (really interesting new gene) finger domains (RING 0–2) separated by a IBR (in-between-ring) domain in the COOH-terminal part. Numbers under the protein line indicate the boundaries of each domain.

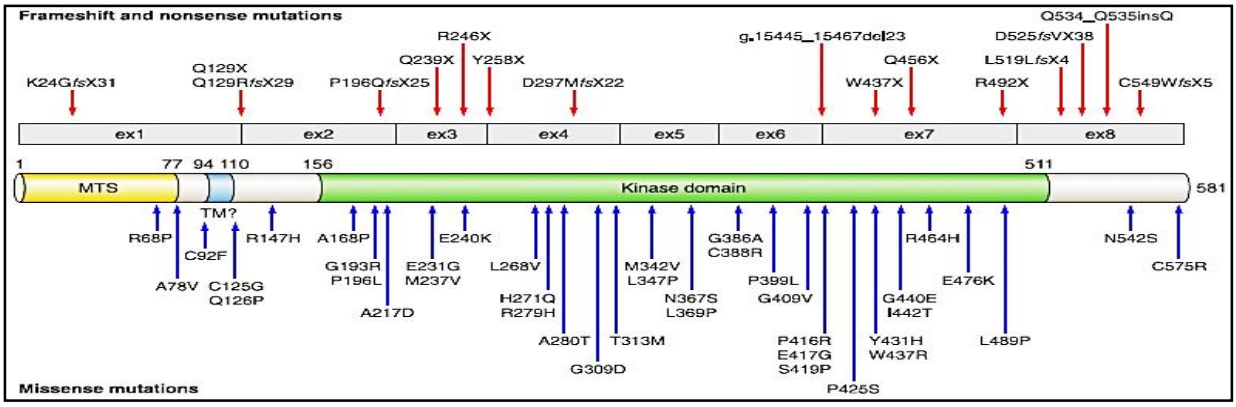


Figure 1.8. Schematic representation of the PINK1 *protein*. Predicted locations of pathogenic frameshift mutations (red lines), and missense mutations (blue lines), and protein organisations. PINK1 consists of a NH2-terminal mitochondrial targeting signal (MTS) motif, a putative transmembrane (TM) region, and a serine-threonine kinase. Numbers under the protein line indicate the boundaries of each domain.

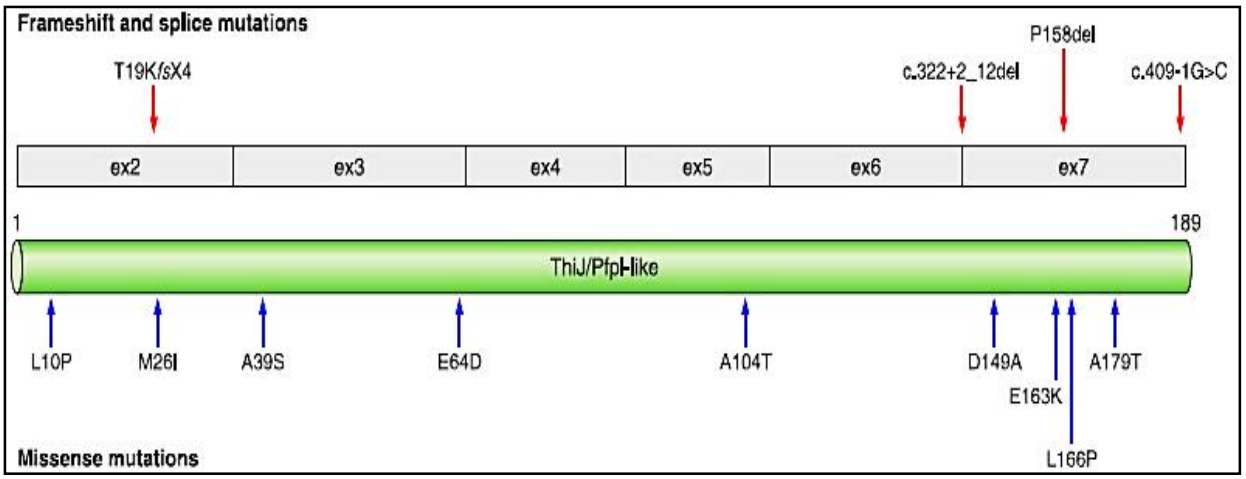


Figure 1.9 Schematic representation the DJ-1 protein. Predicted locations of pathogenic frameshift mutations above the transcript (red lines) and protein organisation and missense mutations below the protein (blue lines). DJ-1 consists of a single domain protein, and a critical cysteine residue (Cys106) which can be modified under oxidative stress conditions.

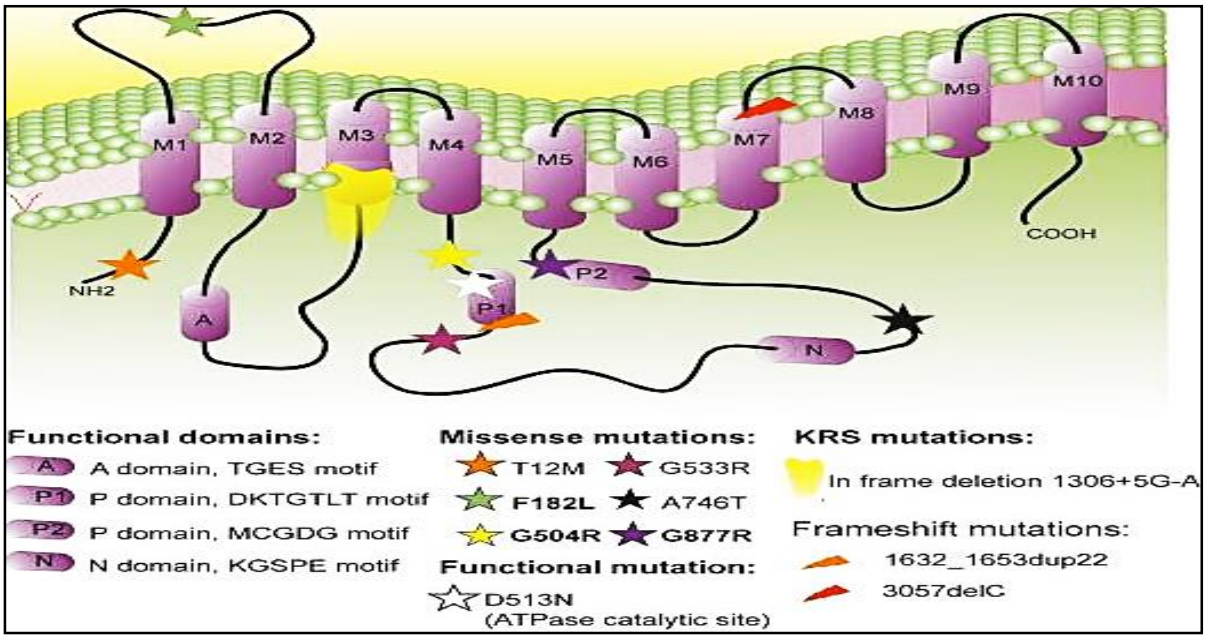
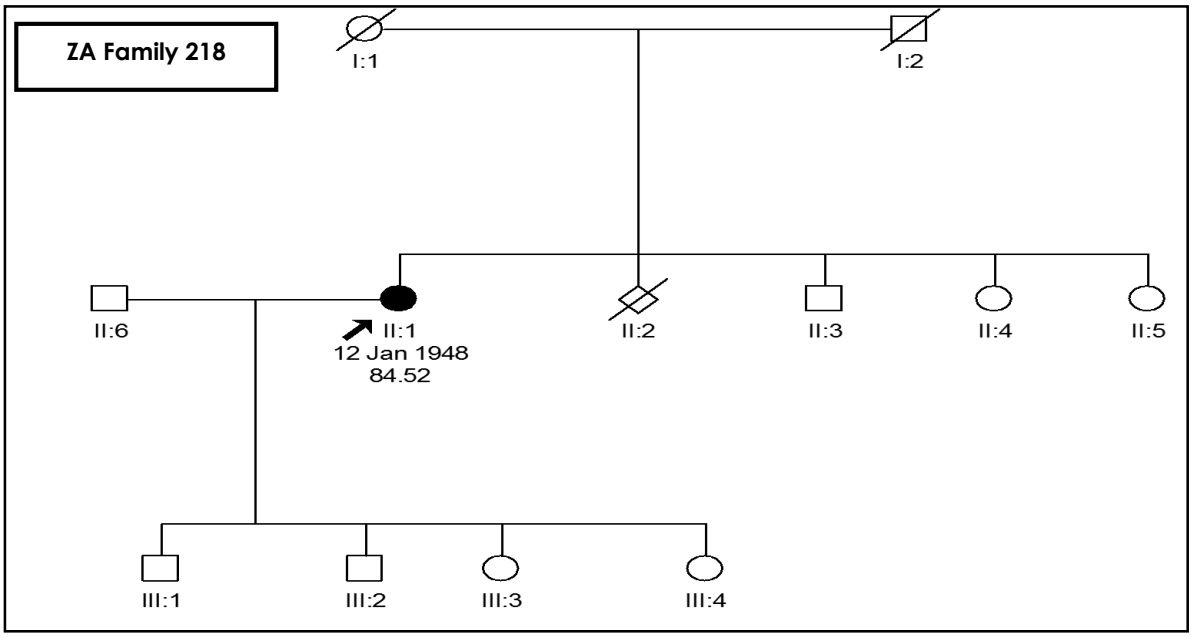
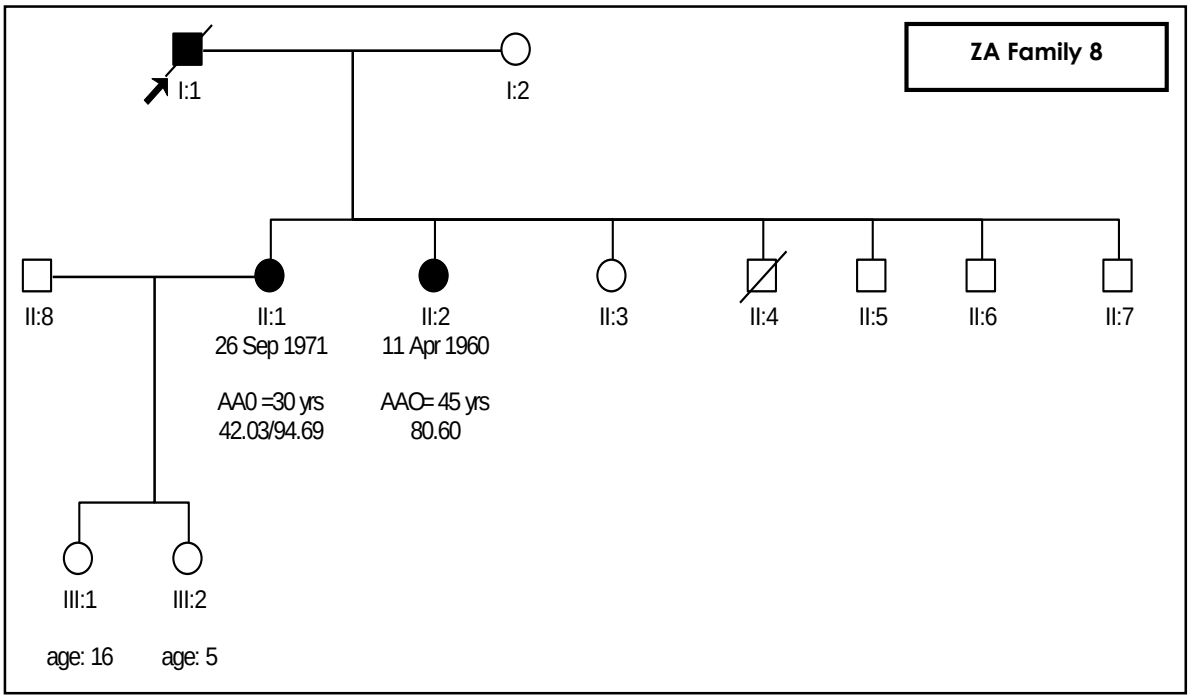
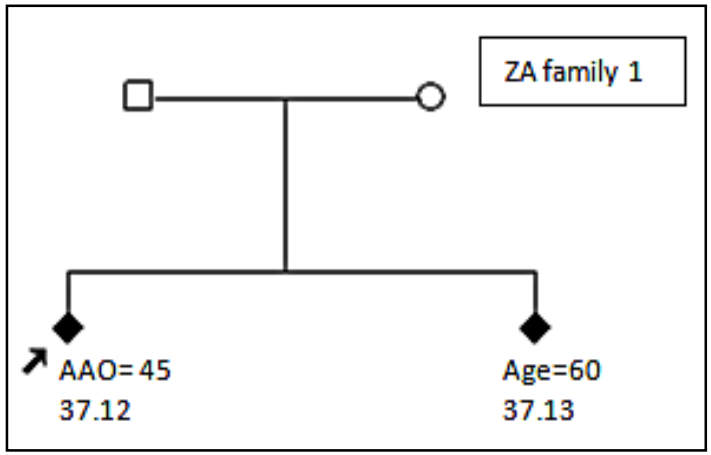


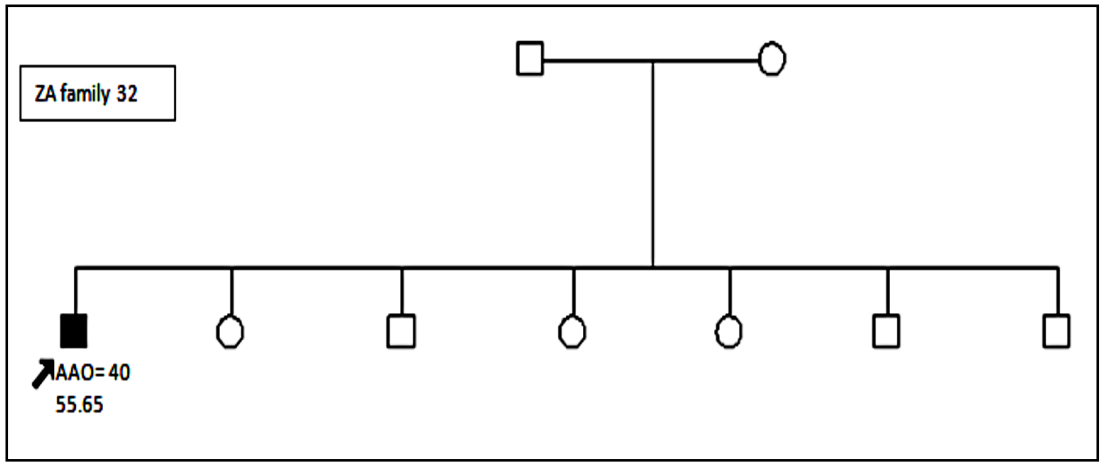
Figure 1.10 Schematic representation of the ATP13A2 transmembrane protein**.** ATP13A2 consists of 10 transmembrane domains (M1-10). Functional domains include the A, P1, P2, and N. The positions of different mutations are indicated by stars. The position of the in-frame deletion is shown by yellow shading [taken from (Podhajska et al., 2012)].

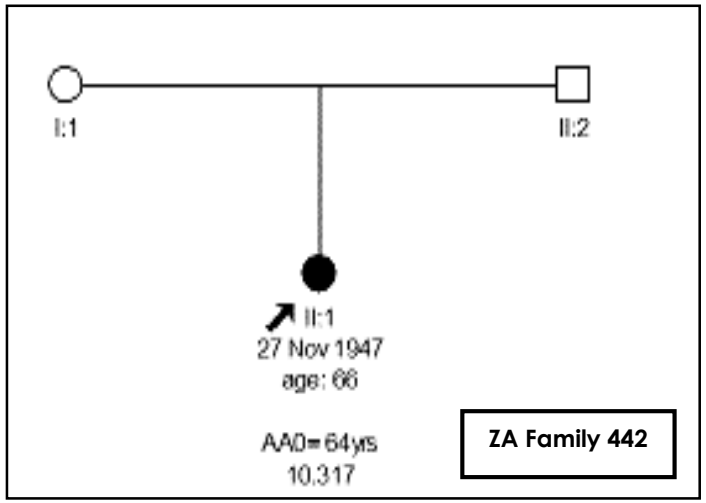
Appendix B: Pedigrees of the Black South African PD patients

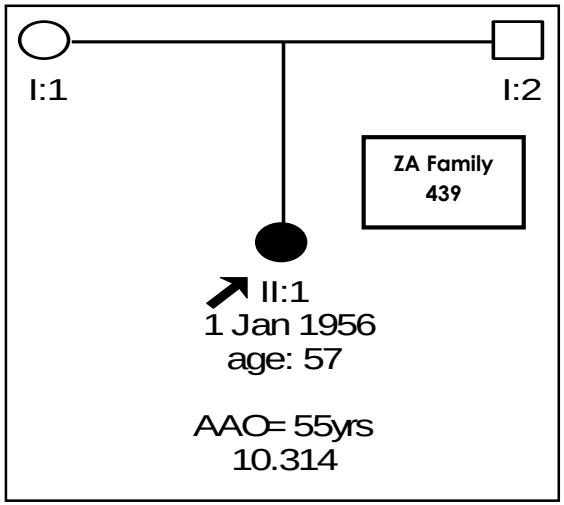


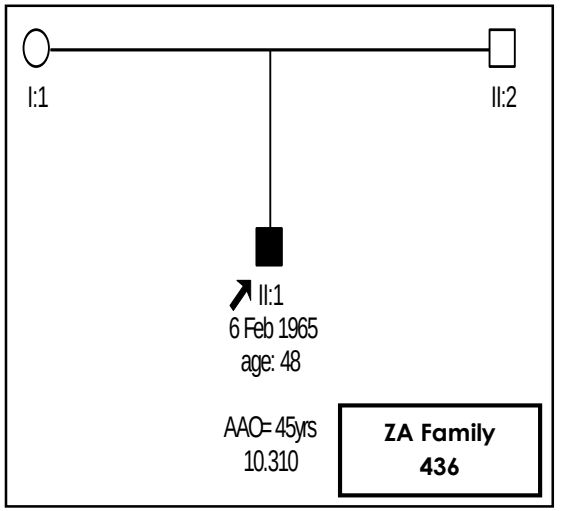


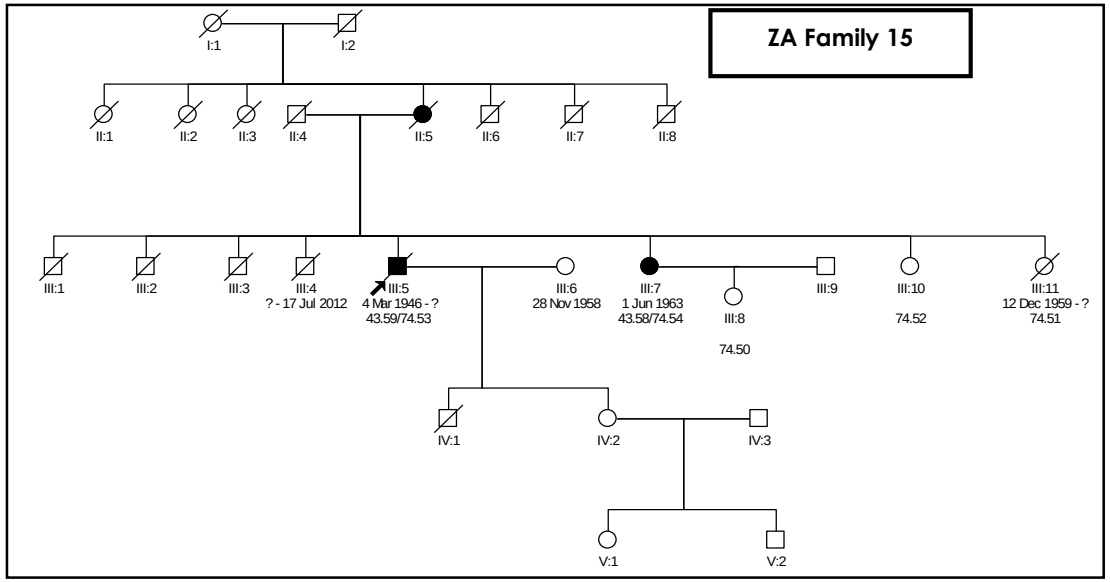


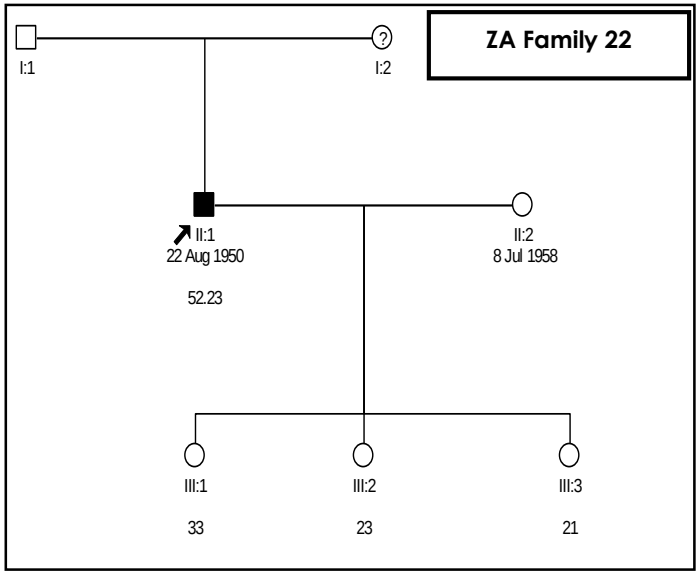


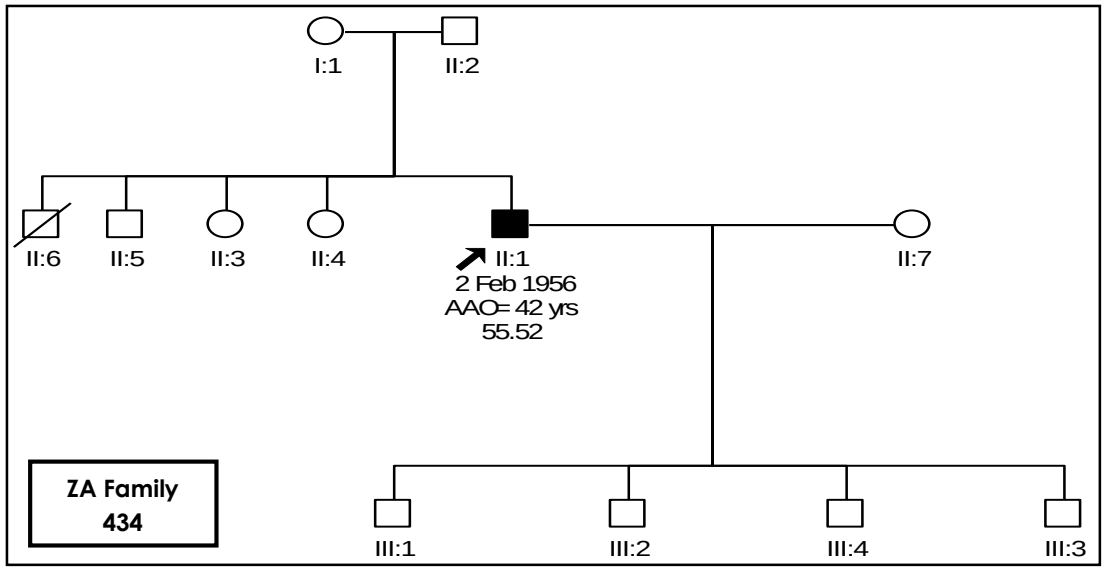


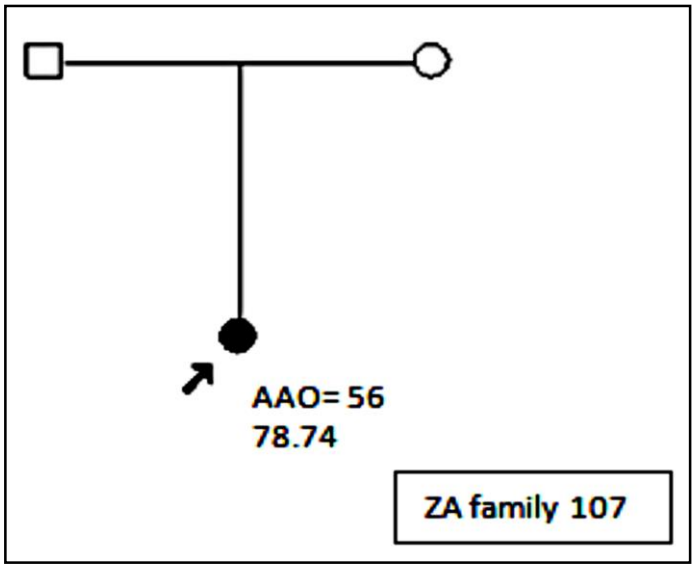


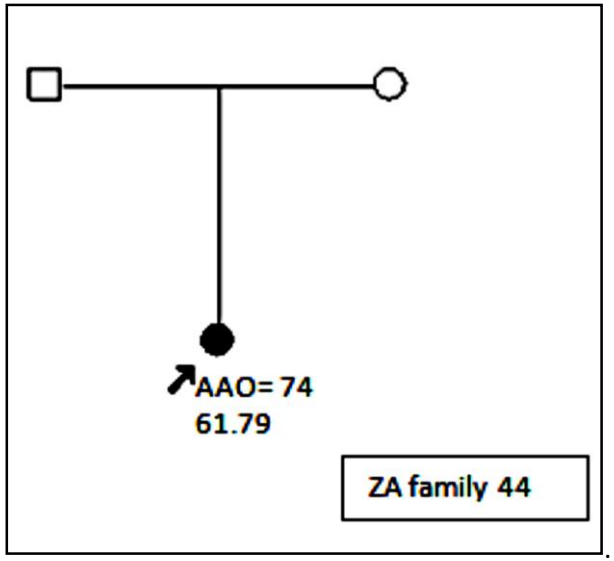


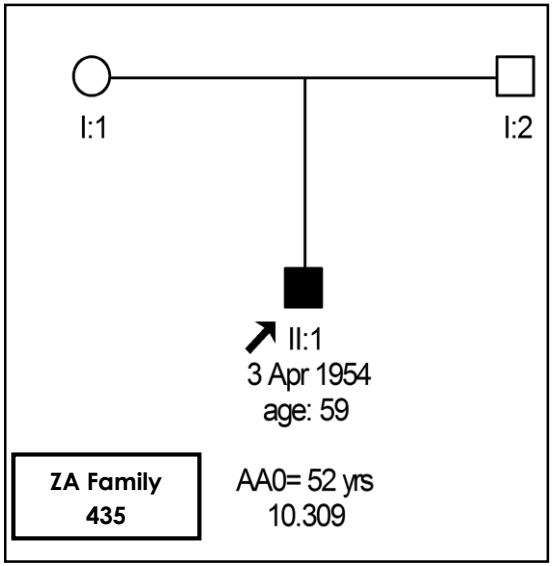


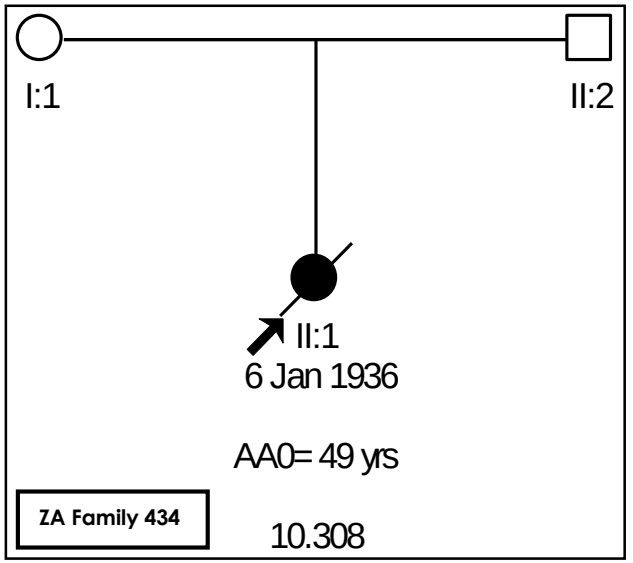


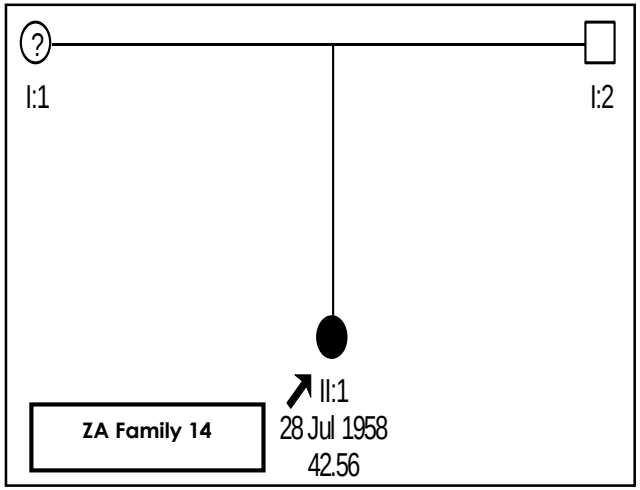


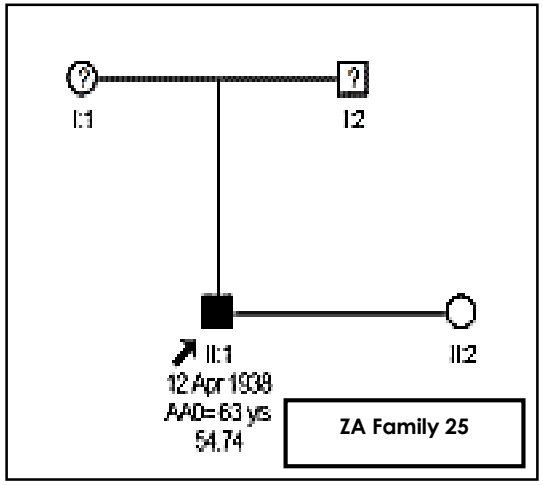


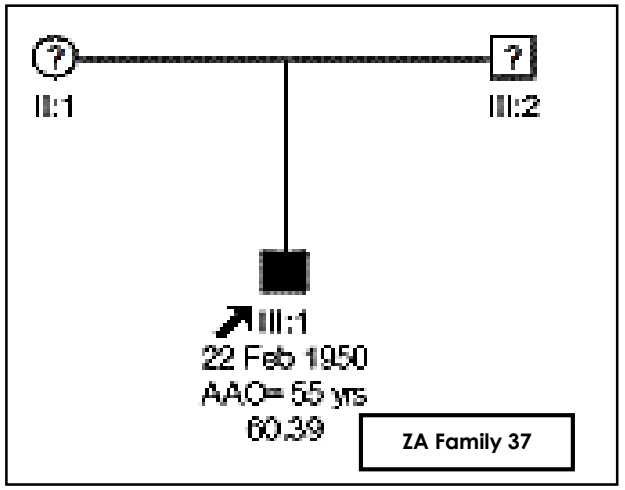


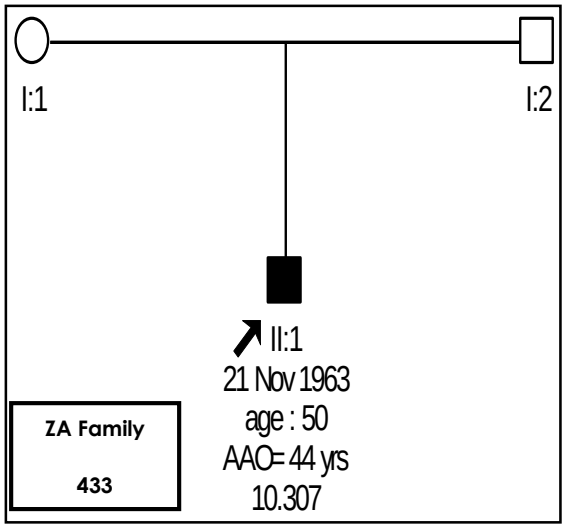


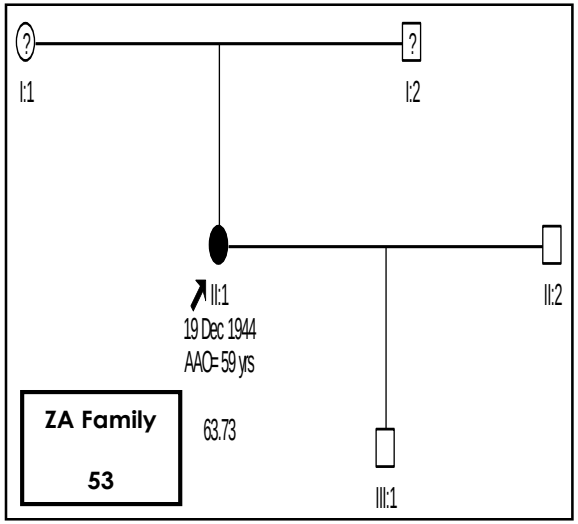


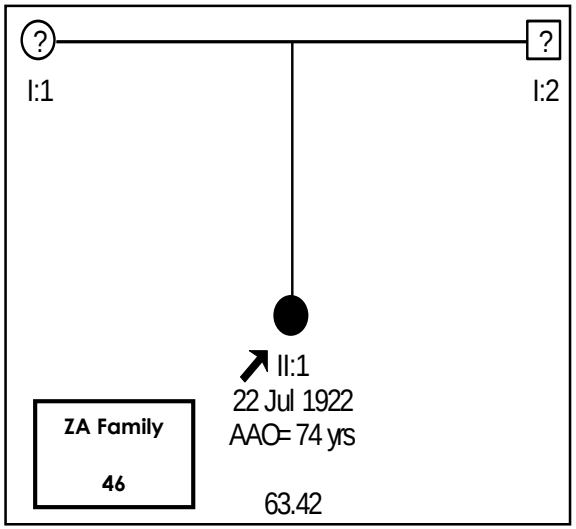


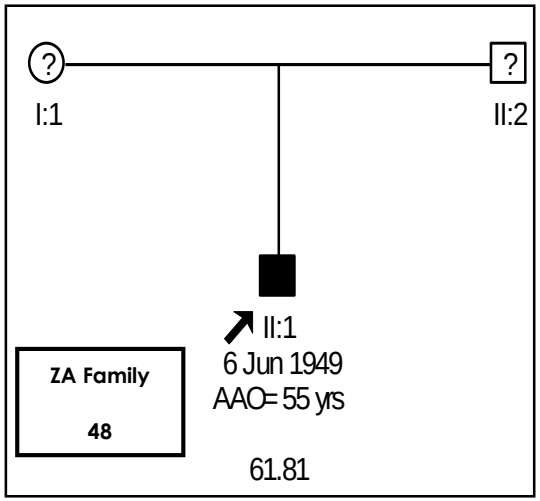


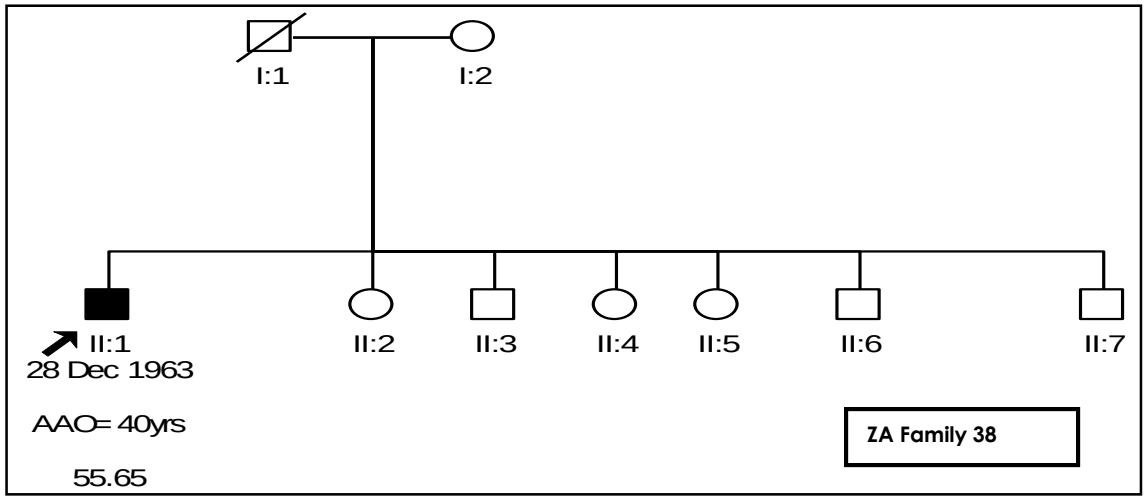












Appendix C: Primers designed for mutation screening

Supplementary Material to Chapter 2

Table 2.2. Primers used and PCR conditions for amplification, and sequencing of *PARK2*, *DJ-1*, *LRRK2*, *VPS35,* and *EIF4G1.*

Gene	Exonic region	Primer Sequence (5´-3´)	Size of PCR fragment (bp)	Annealing temp. (^{0}C)	PCR Conditions
PARK2	Exon 1	For: gaa cta cga ctc cca gca g Rev: ccc gtc att gac agt tgg	300	58	2.5 mM $MgCl_2$ 5.0 M Betaine
PARK2	Exon 2	For: cac cat tta agg gct tcg ag Rev: tca ggc atg aat gtc aga ttg	313	55	1.5 mM $MgCl_2$
PARK2	Exon 3	For: tct cgc att tca tgt ttg aca Rev: gca gac tgc act aaa caa aca	364	55	1.5 mM $MgCl_2$
PARK2	Exon 4	For: gct ttt aaa gag ttt ctt gtc Rev: ttt ctt ttc aaa gac ggg tga	299	55	1.5mM $MgCl_2$
PARK2	Exon 5	For: gga aac atg tct taa gga gt Rev: ttc ctg gca aac agt gaa ga	223	60	2.5mM $MgCl_2$ 5% Formamide
PARK2	Exon 6	For: cca aag aga ttg ttt act gtg Rev: ggg gga gtg atg cta ttt tt	276	55	2.5 mM $MgCl_2$ 5% Formamide
PARK2	Exon 7	For: cct cca gga tta cag aaa ttg Rev: gtt ctt ctg ttc ttc att agc	280	55	1.5 mM $MgCl_2$
VPS35	Exon 15 D620N mutation	For: agg ctg cag aag tct cac agg a Rev: agg gca ggg gga cag tga aga	257	55	1.5 mM $MgCl_2$
EIF4G1	Exon 23 R1205H mutation	For: aca agg ccc aac agt tgc tca g Rev: agg gac ttt tca gtc ttc cca gca	243	56	1.5 mM $MgCl_2$

Table 2.2. Primers used and PCR conditions for amplification, and sequencing of PARK2, DJ-1, LRRK2, VPS35, and EIF4G1. Continued

Gene	Exonic region	Primer Sequence (5´-3´)	Size of PCR fragment (bp)	Annealing temp. (^{0}C)	PCR Conditions
PARK2	Exon 8	For: ggc aac act ggc agt tga ta Rev: ggg gag ccc aaa ctg tct	232	55	1.5 mM $MgCl_2$
PARK2	Exon 9	For: tcc cat gca ctg tag ctc ct Rev: cca gcc cat gtg caa aag	297	55	1.5 mM $MgCl_2$
PARK2	Exon 10	For: caa gcc aga gga atg aat at Rev: gga act ctc cat gac ctc ca	272	55	1.5 mM $MgCl_2$
PARK2	Exon 11	For: ccg acg tac agg gaa cat aaa Rev: ggc acc ttc aga cag cat ct	300	55	1.5 mM $MgCl_2$
PARK2	Exon 12 (set A)	For: tct agg cta gcg tgc tgg tt Rev: gcg tgt gtg tgt gtg ttt ga	296	55	1.5 mM $MgCl_2$
SNCA	Exon 2	For: ccc cca agt tct cat tca a Rev: ccc atc act cat gaa caa gc	235	55	2.5 mM $MgCl_2$
SNCA	Exon 3	For: ttt aag gct agc ttg aga ct Rev: cca cac taa tca cta gat ac	146	40	2.5 mM MgCl2
LRRK2	Exon 31	For: tga atg tca cgg aaa gca aa Rev: ttc tca act gcc tag aaa tgt cag	173	55	1.5 mM $MgCl_2$
LRRK2	Exon 41	For: gca cag aat ttt tga tgc ttg Rev: gga tgg ata acc act gac ctc	313	55	1.5 mM $MgCl_2$
DJ-1	Exon 1	For: cac cca ggg ctg tcc agc ta Rev: caa cgc tga agc gtc cag aa	238	55	1.5 mM $MgCl_2$

Table 2.2. Primers used and PCR conditions for amplification, and sequencing of PARK2, DJ-1, LRRK2, VPS35, and EIF4G1. Continued

Gene	Exonic region	**Primer Sequence (5´-3´)**	**Size of PCR fragment (bp**	**Annealing temp. (^{0}C)**	**PCR Conditions**
DJ-1	Exon 2	For: ctc tgc ttg aaa atg ctc ct Rev: aat gtt ttt gga ttt taa a	220	55	1.5 mM $MgCl_2$
DJ-1	Exon 3	For: cag ggt gag acc cca tct ctc t Rev: gga gtt att cct tca tat ggc ttc tttg tt	256	55	1.5 mM $MgCl_2$
DJ-1	Exon 4	For: tgt caa ttt aat gca cag ttg a Rev: ttc aaa tat ttc ggg agg agg	117	55	1.5 mM $MgCl_2$
DJ-1	Exon 5	For: agg tca gag agc ttg tgg ttt Rev:ggt agc ctt tca ttc gat ggt	152	55	1.5 mM $MgCl_2$
DJ-1	Exon 6	For: cca ggc act att gcg att tt Rev: ttg aga tgg agt ctc gct cc	243	55	1.5 mM $MgCl_2$
DJ-1	Exon 7	For: cgt ctt tct cgt cac ata gcc Rev: aat gtg ttc aga agt cgc tgt cct	177	57	1.5 mM $MgCl_2$

Supplementary Material to Chapter 2 continued.

Table 2.3 Primers used for amplification of GBA.

Exonic regions	**Primer Sequence (5´-3´)**	
	Forward	**Reverse**
Exons 1-5	CCTAAAGTTGTCACCCATAC	AGCAGACCTACCCTACAGTTT
Exons 5-7	GACCTCAAATGATATACCTG	AGTTTGGGAGCCAGTCATTT
Exons 8-11	TGTGTGCAAGGTCCAGGATCAG	ACCACCTAGAGGGGAAAGTG
Individual primer sequences		
Exons and flanking regions	**Forward**	**Reverse**
Exon 1	CCTAGTGCCTATAGCTAAGG	CATTCATTAAATTCCAGTGCC
Exon 2	GTCCTAATGAATGTGGGAGACC	TTGAGCACCTACTAAAAGTC
Exon 3	GCAAGGCAGGTCTCAAACTC	GGAAACTCCATGGTGATCAC
Exon 4	AATCACACGGGCACAGGTAA	GCAGAGTGAGATTCTGCCTC
Exon 5	GCAAGTGATAAGCAGAGTCC	AGCAGACCTACCCTACAGTTT
Exon 6	TGAACCGGATGCACTGGTTG	GGAAGTGGAACTAGGTTGAGG
Exon 7	TTGGCCGGATCATTCATGAC	CTCTAAGTTTGGGAGCCAGTC
Exon 8	GCTTCTGTCAGTCTTTGGTG	CTGGACAGGAAGGGCTTCT
Exon 9	CTCGTGGTGTAGAGTGATGTAAG	GCTCCCTCGTGGTGTAGAGT
Exon 10 &11	GTGGGCTGAAGACAGCGTTGG	GAGGCACATCCTTAGAGGAG

The primer sequences were custom designed to avoid amplifying and sequencing the neighboring pseudogene.

Fragment 1 (Exons 1-5) = 2972 bp, fragment 2 (Exons 5-7) = 2049 bp, and fragment 3 (Exons -11) = 1682 bp

Table 2.4 Customised touch down (TD) PCR conditions used for each GBA fragment.

Fragment 1 (2972pb)			**Fragment 2 (2049pb)**			**Fragment 3 (1682pb)**		
TD 64-54			TD 64-54			Unique program		
96°C	5 min		96°C	5 min		94°C	5 min	
96°C	30 sec		96°C	30 sec		94°C	45 sec	
64°C	30 sec	2 cycles	64°C	30 sec	2 cycles	62-60°C	45 sec	20 cycles
72°C	3 min		72°C	2 min		72°C	3 min	
96°C	30 sec		96°C	30 sec		94°C	45 sec	
63°C	30 sec	2 cycles	63°C	30 sec	2 cycles	60°C	45 sec	18 cycles
72°C	3 min		72°C	2 min		72°C	3 min	
96°C	30 sec		96°C	30 sec		72°C	7 min	
62°C	30 sec	2 cycles	62°C	30 sec	2 cycles			
72°C	3 min		72°C	2 min				
96°C	30 sec		96°C	30 sec				
61°C	30 sec	2 cycles	61°C	30 sec	2 cycles			
72°C	3 min		72°C	2 min				
96°C	30 sec		96°C	30 sec				
60°C	30 sec	2 cycles	60°C	30 sec	2 cycles			
72°C	3 min		72°C	2 min				
96°C	30 sec		96°C	30 sec				
59°C	30 sec	2 cycles	59°C	30 sec	2 cycles			
72°C	3 min		72°C	2 min				

Table 2.4 Customised touch down (TD) PCR conditions used for each GBA fragment. Continued

Fragment 1 (2972pb)		Fragment 2 (2049pb)	
96°C 30 sec 58°C 30 sec 72°C 3 min	2 cycles	96°C 30 sec 58°C 30 sec 72°C 2 min	2 cycles
96°C 30 sec 57°C 30 sec 72°C 3 min	2 cycles	96°C 30 sec 57°C 30 sec 72°C 2 min	2 cycles
96°C 30 sec 56°C 30 sec 72°C 3 min	2 cycles	96°C 30 sec 56°C 30 sec 72°C 2 min	2 cycles
96°C 30 sec 55°C 30 sec 72°C 3 min	2 cycles	96°C 30 sec 55°C 30 sec 72°C 2 min	2 cycles
96°C 30 sec 54°C 30 sec 72°C 3 min	25 cycles	96°C 30 sec 54°C 30 sec 72°C 2 min	25 cycles
72°C 10 min		72°C 10 min	

Appendix D: List of genes included on the customized target-capture panel

Table 2.5 List of genes included in the neurodegeneration screening panel.

Disease/ associated pathway	Gene
ACCPN	SLC12A6
AD	APOE
AD	APP
AD	BACE1
AD	BIN1
AD	CLU
AD	GAB2
AD	PICALM
AD	PSEN1
AD	PSEN2
AD3	SERPINA3
ALS	FUS
ALS	SOD1
ALS	SPG11
ALS	TARDBP
ALS/FTD	C9orf72
ALS12	OPTN
ALS2	ALS2
ALS4	SETX
ALS8	VAPB
ALS9	ANG
bipolar	NR2E1
Cadasil	NOTCH3
CCFDN	CTDP1
CJD	PRNP
CMT1	ARHGEF10
CMT1/2	MPZ
CMT1/2	NEFL
CMT1A	PMP22
CMT1C	LITAF
CMT1D	EGR2
CMT20	DYNC1H1
CMT2A2	MFN2
CMT2B	RAB7A
CMT2B1	LMNA
CMT2D	GARS
CMT2F	HSPB1
CMT2K/4A	GDAP1
CMT2L	HSPB8
CMT2M	DNM2
CMT4B1	MTMR2
CMT4B2	SBF2
CMT4C	SH3TC2
CMT4D	NDRG1
CMT4F	PRX
CMT4H	FGD4

Table 2.5 List of genes included in the neurodegeneration screening panel. Continued.

Disease/ associated pathway	Gene
CMTDIC	YARS
CMTX1	GJB1
CMTX5	PRPS1
dHMN V	BSCL2
dSMA1	IGHMBP2
Dyskinesia	PRRT2
Dystonia	ACTB
Dystonia	ATP1A3
Dystonia	DRD2
Dystonia	GCDH
Dystonia	GCH1
Dystonia	MR1
Dystonia	PRKRA
Dystonia	SGCE
Dystonia	SLC2A1
Dystonia	SLC6A3
Dystonia	TAF1
Dystonia	THAP1
Dystonia	TIMM8A
Dystonia	TOR1A
FTD	GRN
FTD	MAPT
FTLD	CHMP2B
FTLD/FTD	VCP
GAN1	GAN
HNA	SEPT9
HSAN1A	SPTLC1
HSAN2A	WNK1
HSAN3	IKBKAP
HSAN4	NTRK1
HSAN5	NGF
Kufor-Rakeb	ATP13A2
NBIA1	C19orf12
NBIA1	PANK2
NBIA2A	PLA2G6
NBIA3	FTL
PARK16	RAB7L1
CMT4J	FIG4
PD	BST1
PD	EIF4G1
PD	GABP1
PD	GAK
PD	GBA
PD	GPNMB
PD	HLA-DRB5
PD	HSP40
PD	LRRK2
PD	MAOA

Table 2.5 List of genes included in the neurodegeneration screening panel. Continued.

Disease/ associated pathway	Gene
PD	MAOB
PD	MCCC1
PD	PARK2
PD	PARK7
PD	PINK1
PD	PM20D1
PD	RAB25
PD	SETD1A
PD	SNCA
PD	SNCB
PD	SNCG
PD	SPR
PD	STK39
PD	TH
PD	UCHL1
PD	VPS35
PD - ET	LINGO1
PERRY	DCTN1
PKPS	FBXO7
PSP	STX6
Schizophrenia	COMT
Schizophrenia	DTNBP1
SCZD2	FXYD6
SCZD4	PRODH
SCZD9	DISC1
SMA	SMN1
SMA	SMN2
Wilson's	ATP7B
WS4C	SOX10
Dopamine pathway	DRD1
Dopamine pathway	DRD3
Dopamine pathway	DRD4
Dopamine pathway	DRD5
Dopamine pathway	SLC18A2
EIF complex	EIF4G3
EIF complex	PABPC1
EIF complex	PABPC1L
Retromer complex	SNX1
Retromer complex	SNX2
Retromer complex	SNX32
Retromer complex	SNX5
Retromer complex	SNX6
Retromer complex	VPS26A
Retromer complex	VPS26B
Retromer complex	VPS29
Retromer complex	VPS54
Retromer complex	WASH
Vesicle dynamics	DNM1
Vesicle dynamics	DNM3
Vesicle dynamics	EHD1
Vesicle dynamics	EHD2
Vesicle dynamics	EHD3
Vesicle dynamics	FCHO1
Vesicle dynamics	FCHO2
Vesicle dynamics	HGS
Vesicle dynamics	HSPA8
Vesicle dynamics	RAC1

Table 2.5 List of genes included in the neurodegeneration screening panel. Continued.

Disease/ associated pathway	Gene
Vesicle dynamics	SH3GL1
Vesicle dynamics	SH3GL2
Vesicle dynamics	SH3GL3
Vesicle dynamics	SYNJ1
Vesicle dynamics	SYNJ2
Vesicle dynamics	DNAJC5
Vesicle dynamics	SGTA
Vesicle dynamics	SH3BP4
Vesicle dynamics	SNAP25
Vesicle dynamics	STX10
Vesicle dynamics	STX16
Vesicle dynamics	VAMP1
Vesicle dynamics	VAMP2
Vesicle dynamics	VAMP4
Vesicle dynamics	VAMP7
Vesicle dynamics	VAMP8
Vesicle dynamics	VTI1A

Appendix E: Primers Designed for Validation of NGS Results

Table 2.6 Individual primer sequences used for confirmation of NGS results.

Gene	Exonic region	Primer Sequence (5´-3´)	Size of PCR fragment (bp)	Annealing temp. (^{0}C)	PCR Conditions
PARK2	Exon 8	For: CAC TGG CAG ATA GCA GAT GTA G Rev: GCT GTC CCA ACT CCT TGA TTA	277	**55**	1.5 mM $MgCl_2$
GBA	Exon 12	**For:** CTG TTG TGG TCG TGC TAA AC Rev: CAG GTA GGT GTG AAT GGA GTA G	207	**58**	1.5 mM $MgCl_2$
DNAJC13	Exon 44	For: TGT CTG CAT TGA TTA GGT TCC A Rev: GGT GTA GTC ACA TAC CTG GAT TG	209	55	1.5 mM $MgCl_2$
DCTN1	Exon 8	For: CTT TCT TTC TGG TCT GCC TCT AC Rev: TGG GTG TCT TGA TTC TCC TTT AC	269	55	1.5 mM $MgCl_2$
ATP13A2	Exon 22	For: AGC TGT ACC TCA CCC AGT Rev: TGA GGA AGG AGA CAG AGC A	278	56	1.5 mM $MgCl_2$
LRRK2	Exon 7	For: TGC TGC CAT CTA TTT ACA GTC TAT Rev: GAG CAA ACA GCA ACT CAC TTC	223	55	1.5 mM $MgCl_2$
LRRK2	Exon 16	For: GCT GGT GAC TGG ATG TCT TTA Rev: CGC ACA AGT TCC CAA ATT CTT	256	55	1.5 mM $MgCl_2$
LRRK2	Exon 36	For: CAC TTG TGT TGT GTG CAG TAG Rev: CTG TCG CAA AGC TTT AAG GTA A	314	55	1.5 mM $MgCl_2$

Table 2.6 Individual primer sequences used for confirmation of NGS results. Continued

Gene	Exonic region	Primer Sequence (5´-3´)	Size of PCR fragment (bp)	Annealing temp. (^{0}C)	PCR Conditions
LRRK2	Exon 49	For: TGG TTC TAG GGA GGT AAT GGT Rev: CCA TCC ATA TGA CAC TGG AAA GT	334	55	1.5 mM $MgCl_2$
MAPT	Exon 11	For: GTG GTG AGC CTG GGA ATG Rev: ATT CTT CAG GTC TGG CAT GG	337	55	1.5 mM $MgCl_2$
PSEN1	Exon 5	For: GAG GTG GTA ATG TGG TTG GT Rev: CCA TAA GAA GAA CAG GGT GGA A	258	55	1.5 mM $MgCl_2$
PSEN1	Exon 7	For: GTT TGG GAG CCA TCA CAT TAT TC Rev: CCC AGC CGA AAT CTT CAA ATG	268	55	1.5 mM $MgCl_2$
PSEN2	Exon 6	For: GCA GGT CCA GAA TCA CTC AA Rev: AGA GCA CCA CCA AGA AGA TG	208	55	1.5 mM $MgCl_2$

Appendix F: Tolerance scores used to make *in silico* protein functional predictions

Table 2.7 Distribution of prediction scores used by SIFT, PolyPhen and MutationTaster [taken from (Cargill et al., 1999; Xi et al., 2004)].

PolyPhen score	Potential impact	SIFT score	Potential impact	MutationTaster score	Potential impact
>2.00	Damaging	0.00	Deleterious	0.5 -1.00	Disease-causing
1.99– 1.75	Probably damaging	0.01– 0.05	Deleterious	0.00 - 0.4	Polymorphism
1.74– 1.50	Probably damaging	0.051– 0.10	intolerant		
1.49– 1.25	Probably damaging	0.101– 0.20	Borderline		
1.24– 1.00	Borderline	0.201– 0.50	Tolerant		
0.99– 0.50	Benign	0.501– 0.99	Tolerant		
0.49– 0.01	Benign	1.00	Tolerant		
0.00	Benign				

The SIFT, PolyPhen and MutationTaster predictive scores, as outlined in Table 2.7, were used to classify the 13 confirmed sequence variants generated using NGS. Although these algorithms are not 100% accurate, they can be effectively used to further reduce the number of variants for further investigation on a function level.

Appendix G: Phenol-chloroform DNA extraction method

Reagents:

- Cell lysis buffer (store at 4˚C for 6-8 weeks)
    - 109.5g Sucrose,
    - 10ml of 1M Tris-HCl pH 7.4,
    - 5ml of 1M $MgCl_2$,
    - 10ml Triton X,
    - Distilled H2O to a final volume of 1L
- Sodium-EDTA, pH8
- 10% SDS
- Proteinase K (10 mg/mL), -20°C
- Sterile, Distilled H2O
- 3M Sodium Acetate (pH 6 with acetic acid)
- Phenol: Chloroform, (1:1, v/v) pH 8, 4°C
- Chloroform:Octonal (24:1 v/v)
- Ice-cold 96% Ethanol, -20°C
- 70% Ethanol (RT)

Extraction procedure:

1. Pool the blood (at least ~10mL in total) into a 50mL centrifuge tube. Fill to 45mL mark with ice-cold cell lysis buffer – invert several times to mix & incubate on ice for 10 minutes. Invert

the tube several times. Pellet cells at 3000rpm for 10 minutes at 4˚C. Pour off the supernatant.

2. Repeat the wash step with cell lysis buffer once more – pellet should be light pink in colour.
3. Add 900µL of Sodium-EDTA and 100µL 10 % SDS to each pellet. May be stored at this stage at -20˚C.
4. Add 100µL Proteinase K to each 50 mL tube. Mix gently by vortexing. Incubate at 37˚C overnight.
5. Add 2mL of distilled H2O and 500µL of Sodium Acetate to each 50 mL tube. Mix gently by shaking.
6. Add 2.5mL Phenol-Chloroform to each 50 mL and close the lids tightly. In the cold room, place the tubes on a shaker and shake at 140 rpm for 10 minutes.
7. Pour the mixture into a 10mL Corex glass tube and spin at 8000 rpm for 12 minutes at 4˚C. Use the Sorval centrifuge with the SS34 head.
8. Using a plastic Pasteur pipette, gently transfer the upper aqueous phase to a clean Corex glass tube without disturbing the interphase.
9. Add 2.5 mL Chloroform: Octonal to each Corex glass tube containing the aqueous phase. Seal with rubber tops and mix slowly by inversion until the mixture turns milky.
10. Remove the rubber tops and spin at 8000 rpm for 12 minutes at 4˚C in the Sorval centrifuge.
11. Transfer the upper aqueous phase to 15 mL plastic Greiner tubes. To precipitate the DNA, add 5-7 mL of ice-cold 96% ethanol. Seal the tube with plastic cap and mix gently by inversion until the DNA precipitates out of solution.
    a. The protocol may be ‘paused’ at this point, prior to the addition of alcohol.
12. With the aid of a pipette, transfer the DNA precipitate to a clean 1.5 mL Eppendorf tube.

13. Add 1 mL of 70% Ethanol to the Eppendorf tube, invert several times to wash the DNA, and spin at 14000 rpm for 3 minutes. Pour off supernatant carefully so as not to disturb the pellet. Repeat this step.
14. Air-dry at room temperature for ~10 minutes.
15. Resuspend the dried DNA in 300-500µL TE solution and incubate overnight at 37˚C, or for 10 minutes at 65˚C.
16. Ensure the Eppendorf tubes are tightly sealed and place into a 50 mL tube. Place tubes on rotating wheel in cold-room and mix for a minimum of 3 days at 30 rpm.
17. Read optical density at 260 nm and 280 nm.

Store at 4˚C for further manipulation, or at -20˚C for long-term storage.