Implementation of Targeted Resequencing Strategies to Identify Pathogenic Mutations in Nigerian and South African Patients with Parkinson's Disease

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DECLARATION

This dissertation includes one original paper published in a peer-reviewed journal, and one unpublished publication (submitted for publication). The development and writing of the published paper were the principal responsibility of myself and for each of the cases where this is not the case a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Declaration by the candidate:

With regard to Chapter 2, the nature and scope of my contribution were as follows: I was the project lead, carried out literature searches, appraised the articles, summarized results, prepared the tables and figures and drafted and revised the manuscript, which has now been published on-line in Parkinsonism and Related Disorders.
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I have obtained a permission from the publisher (Elsevier) to use the accepted version of the manuscript as part of my thesis (Chapter 2).

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 2,

2. no other authors contributed to Chapter 2 besides those specified below, and

3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 2 of this dissertation.

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With regard to Chapter 4, the nature and scope of my contribution were as follows: I led the project, obtained ethics approvals for the study, did literature searches, carried out Sanger sequencing, high resolution melt experiments, performed data analyses using Ion Torrent software, prepared some of the tables and figures and drafted and revised the manuscript that has been submitted for publication.

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 4,

2. no other authors contributed to Chapter 4 besides those specified below, and

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ABSTRACT

Parkinson's disease (PD) is a debilitating neurodegenerative movement disorder. It is characterized by the progressive loss of specific neurons in the brain, the dopaminergic neurons of the *substantia nigra pars compacta* of the midbrain. Increasing age is considered to be one of the strongest risk factors for PD. The symptoms of PD profoundly affect the quality of life of patients and their family members.

The aetiology of PD is not completely understood. Several genes harbour mutations that contribute to developing PD. Little, however is known about the genetics of PD in the sub-Saharan African patients. African populations have greatly admixed ancestry with the greatest genetic diversity in the world. This diversity has important ramifications for genetic research.

Sporadic or idiopathic cases of PD are the most recurrent in South Africa and Nigeria. Familial PD cases are relatively rare. It is also possible that genetic mutations interact with environmental factors to cause PD onset and its progression.

Several genes including *PRKN, PINK1, PARK7, ATP13A2, SNCA, LRRK2, VPS35, EIF4G1, CHCHD2* and *LRP10* have been associated with PD. The mutations in these genes provide new opportunities to understand the disease by suggesting biological pathways that could be involved in PD pathogenesis. The PD genes have been predominantly identified and studied in European, North American, North African and Asian populations. It is possible that the genetic information obtained from other populations may not be transferable to the sub-Saharan African populations.

The present study focused on identifying pathogenic mutations in Black South African and Nigerian PD patients. This study is the first collaborative genetics study on PD between South Africa and Nigeria. This study hypothesized that novel disease-causing genes will be identified in Black South African and Nigerian PD patients.

A total of 33 Black South African and 42 Nigerian PD patients were recruited and screened for genetic defects using various methods. The mean age at onset of PD in the South Africans and Nigerians was 48±8 and 65±10 years, respectively. Two South African patients had a family history of PD.

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This study evaluated the presence of exonic rearrangements such as multiplications or deletions of exons in the PD patients. A total of 42 Nigerian and 15 South African PD patients were studied for exonic rearrangement using a commercially available assay kit.

The results revealed three Nigerian patients with exonic rearrangements: (1) Heterozygous deletion in *PARK7* exon 1; (2) Heterozygous duplications in *PINK1* exon 5 and *PRKN* exon 1; and (3) Two heterozygous duplications in *PRKN* exon 1 and 2 gene.

The study also implemented targeted next-generation sequencing (NGS) to screen the 751 genes known to harbour pathogenic mutations in patients with neurological diseases using the AmpliSeq™ Neurological Disease panel and the Ion Torrent sequencing platform. NGS is fast, scalable, reliable and could reveal putative pathogenic variants.

Bcftools was used for assessing the data quality and to filter the variants. Sequence variants with mapping quality score > 100 and a minimum read depth > 40 were selected for follow-up. We used *annovar* utility software for the annotation. We removed variants with minor allele frequency > 0.01 in any of the frequency databases. We also assessed how often each variant was found in common among samples. Variants predicted to be deleterious were selected based on the prediction scores by ***MetaLR*** and ***MetaSVM*** as these two showed the best performance on curated data sets. We generated radar plots of these selected variants predicted to be deleterious to illustrate samples overall pathogenicity prediction.

All 47 samples passed the quality control after sequencing. In summary, a total of 14,655 rare variants with a minor allele frequency of ≤0.01 were identified. Of which, 7,934 were intronic and 5,695 exonic. They included, 198 UTR5, 341 UTR3, 32 frameshift, 3,175 synonymous and 2,448 missense variants.

Altogether, 14,057 were single and 598 multiple variants comprising of 261 insertions, 600 deletions and 13,794 substitutions. Altogether 60 variants linked to 44 genes were considered pathogenic based on MetaLR and MetaSVM scores. Altogether 41 of these genes were not previously associated with PD.

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Seven novel mutations were identified in three known PD genes *ATP13A2* (S960R), *PRKN* (P153R and D245E) and *PINK1* (S73L, S228F, S284Y and P305A). Finally, four novel variants were selected for Sanger sequencing, which confirmed the NGS results.

In conclusion, the implementation of tNGS and the advanced bioinformatics tools led to identification of novel pathogenic mutations in the Black South African and Nigerian PD patients.

Mutations were found in known PD genes and in genes not previously known to harbour mutations in PD patients. Further studies are required to ascertain the biological functions of these mutations. The results could also advance the discovery and the development of improved treatment approaches and drug interventions for PD.

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OPSOMMING

Parkinson se siekte (PS) is ‘n aftakelende neurodegeneratiewe bewegingskondisie. Dit word gekenmerk deur die geleidelike afsterwing van spesifieke neurone in die brein, naamlik die dopaminergiese neurone van die *substantia nigra pars compacta* in die midbrein. Toenemende ouderdom word beskou as die belangrikste risikofactor vir PS. Die simptome van PS het ‘n diepgaande effek op die lewenskwaliteit van pasiënte en hul familielede.

Die oorsaak van PS word nie ten volle vestaan nie. Verskeie gene bevat mutasies wat tot die ontwikkeling van PS kan bydra. Nietemin is daar min bekend oor die genetika van PS in Afrika suid van die Sahara. Afrika-n bevolkingsgroepe het n komplekse herkoms en is van die mees genetiese uiteenlopende groepe in die wêreld. Hierdie diversiteit het belangrike implikasies vir genetika navorsing.

Sporadiese oftenwel idiopatiese gevalle van PS word meestal in Suid Afrika en Nigerië gevind. Familiële PS gevalle is relatief skaars. Dit is ook moontlik dat daar interaksies tussen genetiese mutaties en omgewingsfaktore is wat bydra tot die ontstaan en vordering van PS.

Verskeie gene, insluitend *PRKN*, *PINK1*, *PARK7*, *ATP13A2*, *SNCA*, *LRRK2*, *VPS35*, *EIF4G1*, *CHCHD2* en *LRP10* is geassosieer met PS. Die mutasies in hierdie gene bied nuwe geleenthede om die siekte beter te verstaan, deur relevante biologiese roetes uit te lig wat kan bydra tot PS. Die PS gene is hoofsaaklik geïdentifiseer en bestudeer in Europeese, Noord-Amerikaanse, Noord-Afrika en Asiatiese populasies. Dit is moontlik dat die genetiese inligting wat verkry is van hierdie populasies nie van toepassing in bevolkingsgroepe van Afrika suid van die Sahara nie.

Die huidige studie het beoog om patogeniese mutaties in Swart Suid-Afrikaanse en Nigeriese PS-pasiënte te identifiseer. Hierdie studie is die eerste gesamentlike PS-genetika studie tussen Suid-Afrika en Nigerië. Daar word voorgestel dat nuwe siekte-veroorsakende gene in hierdie pasiënte gevind sal word.

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‘n Somtotaal van 33 Swart Suid-Afrikaanse en 42 Nigeriese PS pasiënte is bestudeer vir genetiese defekte deur middel van verskeie metodes. Die gemiddelde aanvangsouderdom van PS was onderskydelik 48±8 en 65±10 jaar in die Suid-Afrikaners en Nigeriërs. Twee Suid-Afrikaanse pasiënte het ‘n familie geskiedenis van PS. Hierdie studie het eers gesoek vir die teenwoordigheid van eksoniese herrangskikkings soos multiplikasies of delesies in PS-gene. A total van 42 Nigeriese en 12 Suid-Afrikaanse PS pasiënte is bestudeer vir eksoniese herrangskikkings met behulp van ‘n kommmersiële toetsstel. Die resultate dui op drie Nigeriese pasiënte met eksoniese herrangskikkings: (1) heterosigotiese delesie in *PARK7* ekson 1; (2) heterosigotiese duplikasies in *PINK1* ekson 5 en *PRKN* ekson 1; en (3) twee heterosigotiese duplikasies in *PRKN* eksone 1 en 2.

Die studie het ook ‘n geteikende volgende-generasie volgordebepaling (VGV) benadering gebruik om te soek vir mutasies in 751 gene wat voorheen geassosieer is met neurologiese siektes, deur middel van die AmpliSeq™ Neurological Disease Panel as ook die Ion Torrent volgordebepalings-platform. VGV is vinnig, opskaalbaar en betroubaar, en kan patogeniese variante ontdek. Vir die VGV deel van die studie is daar DNS monsters van 33 Swart Suid-Afrikaanse en 14 Nigeriëse PS pasiënte gebruik.

Bcftools is gebruik om die gehalte van die data te asseseer en om deur die variante te sif. Volgordevariante wat ‘n karteringsgehalte-telling > 100 en ‘n minimum leesdiepte > 40 het was geselekteer vir verdere studie. Ons het *annovar* sagteware gebruik vir die annotasie. Ons het variante met ‘n allel-frekwensie > 0.01 in enige van die databasisse bewyder. Ons het gekyk na hoe gereeld elke variant in meer as een DNS monster voorgekom het. Patogeniese variante is gekies op grond van ***MetaLR*** en ***MetaSVM*** tellings, aangesien hierdie twee die beste gedoen het op saamgestelde datastelle. Ons het radarplotte genereer van die geselekteerde patogeniese variante om die algehele patogenesiteit-voorspelling uit te beeld.

Al 47 DNS monsters wat gebruik is vir VGV het voldoen aan gehaltebeheer toetse na volgordebepaling. Ter opsomming, 14,655 rare variante is geïdentifiseer met n alleel-frekwensie van ≤0.01. Dit sluit in 32 raamverskuiwing-, 3,175 sinonieme en 2,448 nie-sinonieme variante. Altesaam was daar 14,057 enkelbasis variante en 598 multibasis variante,

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insluitende 261 insersies, 600 delesies en 13,794 substitusies. ‘n Totaal van 60 variante in 44 verskillende gene was as patogenies aanskou na aanleiding van MetaLR en MetaSVM tellings. Sewe nuwe mutasies is geïdenfiseer in drie bekende PS gene, *AP13A2* (S960R), *PRKN* (P153R en D245E) en *PINK1* (S73L, S228F, S284Y en P305A). Vier van die nuwe gene was gekies vir Sanger volgordebepaling, wat die VGV resultate bevestig het.

Ter slotte, the soektog vir genetiese herrangskikkings en die implementering van VGV tesame met gevorderde bioinformatika hulpmiddels het gelei tot die identifisering van nuwe patogeniese mutasies in Swart Suid-Afrikaanse en Nigeriese PS pasiënte.

Mutasies is gevind in beide bekende PS gene en in gene waarin PS mutasies nog nie voorheen beskryf is. Verdere studies word benodig om die biologiese funksies van hierdie mutasies vas te stel. Die resultate kan ook bydra tot die ontdekking en ontwikkeling van beter behandeling-benaderinge en medikasies vir PS.

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RESEARCH OUTPUT

- A review article was published titled: Parkinson's disease in Nigeria: A review of published studies and recommendations for future research.

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- A research article was submitted titled: Targeted Next-Generation Sequencing Identifies Novel Variants in Candidate Genes for Parkinson's Disease in Black South African and Nigerian Patients.

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LIST OF ABBREVIATIONS

'3: Three prime

'5: Five prime

1KGP: 1000 Genomes Project

A: Adenine

AAO: Age at onset

AD: Autosomal dominant

ADP: adenosine diphosphate

AR: Autosomal recessive

ATP: Adenosine triphosphate

ATP13A2: ATPase type 13 A2

ATXN2: Ataxin-2

BAM: Binary alignment mapping

BAM: Binary Alignment/Map

BLAST: Basic Local Alignment Search Tool

C: Cytosine

CAF: Central Analytical Facility

CGH: Comparative genomic hybridization

CHCHD2: coiled-coil-helix-coiled-coil-helix domain 2

CHR: Chromosome

CNS: Central nervous system

CNV: Copy number variation

COMT: catechol-O-methyl transferase

DAT file: Data file

ddNTP: Di-deoxyribonucleotide triphosphate

DJ-1: Daisuke-Junko-1

DLP1: Dynamin like protein 1

dNTP Deoxyribonucleotide triphosphate

DOPAC: dihydroxyphenylacetic acid

DSB: Double-strand break

EIF4G1: Eukaryotic translation initiation factor 4 gamma

ExAC: Exome Aggregation Consortium

ExoI: Exonuclease I

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FADH2: Flavin adenine dinucleotide

FATHMM: Functional Analysis Through Hidden Markov Models

FBOX7: F-box only protein 7

FISH: Fluorescence in situ hybridization

FMRS: Fragment MLPA reaction score

FoSTeS: Fork stalling and template switching

G: Guanine

GBA: Glucocerebrosidase

GIGYF2: GRB10 Interacting GYF Protein 2

GO: Gene Ontology

GTP: Guanosine triphosphate

GWAS: Genome wide association studies

Hg19: Human Genome Reference built 19

HR: Homologous recombination

HRM: High Resolution Melt

HVA: homovanillic acid

IL-1b: Interleukin 1 beta

IL-6: Interleukin 6

ISPs: Ion Spheres™ Particles

KO: Knockout

LB: Lewy body

LRRK2: Leucine rich repeat kinase 2

MAF: Minor Allele Frequency

MAM: Mitochondrial associated membrane

MAO: Monoamine oxidase

MAP3K: Mitogen-activated protein kinase kinase kinase

MAPT: Microtubule-associated protein tau

MetaLR: *M*eta-analytic likelihood ratio

*MetaSVM: M*eta-analytic support vector machine

MLPA: Multiplex Ligation-Dependent Probe Amplification

MMBIR: Microhomology- mediated breakpoint-induced repair

MNVs: Multiple nucleotide variations

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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mPTP: Mitochondrial permeability transition pore

MRI: Magnetic resonance imaging

NADH: Nicotinamide adenine dinucleotide

NAHR: Non-allelic homologous recombination

NCX: Sodium/calcium exchanger

NGS: Next generation sequencing

NHEJ: Nonhomologous end joining

NMDA: N-methyl-D-aspartate

NMS: Non-motor symptoms

NO: Nitric oxide

PCR: Polymerase chain reaction

PD: Parkinson's disease

PET: Positron emission tomography

PGE: Prostaglandins E

PGM: Personal Genomic Machine

PINK1: PTEN-induced kinase 1

PRKN: Parkin

QC: Quality Control

ROS: Reactive oxygen species

RT PCR: Real-time polymerase chain reaction

SAP: Shrimp alkaline phosphatase

SIFT: Sorting Intolerant From Tolerant

SNCA: α-synuclein

SNP: Single nucleotide polymorphism

SNpc: Substantia nigra pars compacta

SNPs: Single nucleotide polymorphisms

SNV: Single nucleotide variant

SPECT: Single photon emission computerized tomography

SSA: sub-Saharan Africa

SYNJ1: Synaptojanin-1

T: Thymine

Ta: Annealing temperature

TAPER: Tool for Automated selection and Prioritisation for Efficient Retrieval of sequence variants

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Tm: Melting temperature

TNF-a: Tumor necrosis factor alpha

tNGS: Targeted Next Generation Sequencing

Ub: Ubiquitin

UBL: Ubiquitin-like domain

UCHL1: Ubiquitin C-terminal hydrolase L1

UPS: Ubiquitin proteasome system

UTR: Untranslated region

VPS13C: vacuolar protein sorting 13C

VPS35: Vacuolar protein sorting-associated protein 35

WT: Wild type

αSYN: α-synuclein protein

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OUTLINE OF THE DISSERTATION

This dissertation provides information on a study that screened for pathogenic mutations in Nigerian and South African Parkinson's disease patients over a three-year period. This dissertation is divided into five chapters:

CHAPTER ONE lays the background to this study. Therein, Parkinson's disease (PD) and its pathobiology are described. Current global prevalences of PD and the future projection of PD prevelance in sub-Saharan Africa (SSA) are elucidated. The genetic mutations identified in PD genes in the literature are listed, as well the mechanisms of pathogenicity of there mutations. The bioinformatics approach used in this study, to characterize mutations is explained. The principles of next-generation sequencing and other experimental procedures used in this study are explained. Chapter one further gives insight for the study rationale and objectives, by linking the facts that PD genetics is understudied in Black South African and Nigerian populations.

CHAPTER TWO was submitted as a review article for publication. It covers the topic of PD in Nigeria. The study provides a comprehensive and critical review on all published studies on PD in Nigeria in a concise manner. It highlights the need for increased awareness among medical professionals in Africa about PD. It also highlights the need for genetic studies on PD in Nigeria.

CHAPTER THREE is a study on copy number variation (CNV) detection in PD genes in 57 Black South African and Nigerian PD patients. Four putative CNVs were identified in three PD genes (*PARK7, PINK1* and *PRKN*) in three patients. Probable mechanisms of these CNVs identified in PD are highlighted. Recommendations are made for further studies as there were several limitations in the current study.

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CHAPTER FOUR was submitted as a research article for publication. It describes the implementation of next-generation sequencing to screen for pathogenic mutations in 47 Black South African and Nigerian PD patients using a commercially available panel of 751 genes associated with various neurological diseases. A total of 60 novel putative pathogenic mutations were identified. Known mutations in PD were not identified, suggesting that, unique mutations could be responsible for PD in SSA patients. Recommendations are made for further studies as there were several limitations in the current study.

CHAPTER FIVE is the general discussion and final conclusions. Therein, the findings in the study are related with the current knowledge of PD genetics. Furthermore, the challenges and benefits of collaborations with South Africa and Nigeria to study PD genetics are emphasized.

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CHAPTER ONE

INTRODUCTION

1.1 History of Parkinson's disease

Parkinson's disease (PD) is one of the neurological diseases that has no cure. It is a neurodegenerative condition that has been categorized under movement disorders. Studies have revealed that there was evidence of PD as far back as 5000 years ago (Manyam, 1990). In the ancient Indian medicinal practices, they referred to it as "Kampavata" (Manyam, 1990). There was, however, no documented report that people were being cured of the ailment. A physician Galen described the ailment based on his observations as "shaking palsy" in AD 175. It was not until 1817 that a detailed medical essay was published on the "shaking palsy" by a British physician James Parkinson, who originally described PD by carefully outlining the major motor signs (bradykinesia, rigidity and tremor) of the disease that are still today considered the hallmarks of PD (Lill and Klein, 2017).

In his original famous essay on the shaking palsy, James Parkinson described the patients with the disease whom he had examined or observed on the street for his report (Parkinson, 1817 and Parkinson, 2002). He suggested that trauma and possibly inflammation as potential causes of PD.

The interest to study the causes of PD continued with Paul Marie (1849-1933), a French anatomist, physiologist, sculptor and anatomical artist who was an assistant to Jean-Martin Charcot, and who in 1880 described the anatomical changes in PD patients (**Figure 1.1**). Dr. Leroux, one of Charcot scholars, also concluded that hereditary factors could be the cause of paralysis agitans (Lill and Klein, 2017).

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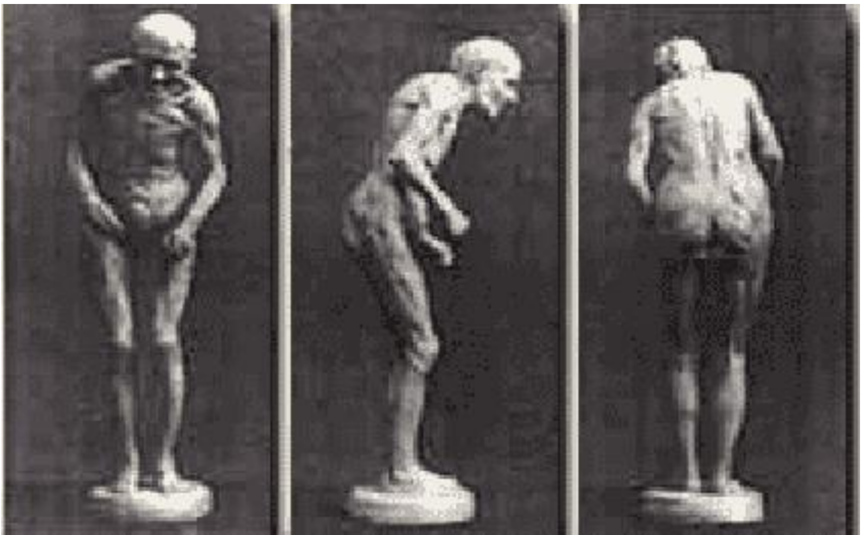

Figure 1.1: Anatomical depiction of PD by Jean-Martin Charcot.
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1.2 Signs and Symptoms of PD

1.2.1 Motor symptoms

Motor symptoms are the most obvious first signs of PD and the symptoms are progressive. Because PD is neurodegenerative, patients usually go from being strong, independent and healthy individuals to being progressively, and persistently unable to move efficiently (**Figure 1.2**). The PD symptoms include:

- Tremors: resting tremor spreads throughout the whole arm, and stiff limbs, sometimes occurring in the lips, feet or tongue**.**
- Bradykinesia: Slow movements that progress to muscle stiffness.
- Akinesia: Muscle stiffness produced by muscle rigidity affecting mostly the legs and neck. When it affects the muscles of the face, it forms like a mask.
- Leg discomfort: Burning sensations and cramp in the legs.
- Balance: A progressive loss of coordination and posture balance, leading to risk of falls.

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1.2.2 Non-motors symptoms

In addition to the predominant motor symptoms, Non-motor symptoms in PD also affect the brain and the peripheral nervous system. In about two decades before onset of PD motor symptoms, non-motor symptoms like constipation, sleep disorder and hyposmia (loss of smell sense) precede the onset of the main motor symptoms. The condition is described as prodromal PD. Some patients experience anxiety, anger, apathy, depression, hallucination and delusion as early signs of PD, or these symptoms occur throughout the course of the disease. These symptoms may be related to Braak's theory of PD progression, which suggests that neurodegeneration starts in the enteric nervous system of the gut and in the medulla and olfactory bulb before reaching through dopaminergic (DA) neurons of the substantia nigra (Braak et al., 2003). This theory is supported by post-mortem studies that showed classical neurodegenerations in PD, related to non-motor symptoms and were thought to precede classical motor symptoms by several years (Hayes et al., 2010; Lees, 2010; Tibar et al., 2018).

There are other non-motor symptoms evident in PD but they may present differently in patients. Low blood pressure, which can result in light-headedness and fainting. Temperature sensitivity increases in PD, perception of temperature can be affected, and may result in hot flashes and excessive sweating. Other signs described among PD patients are idiopathic rapid eye movement and sleep behaviour disorder (Chaudhuri et al., 2006, 2011, Tibar et al., 2018).

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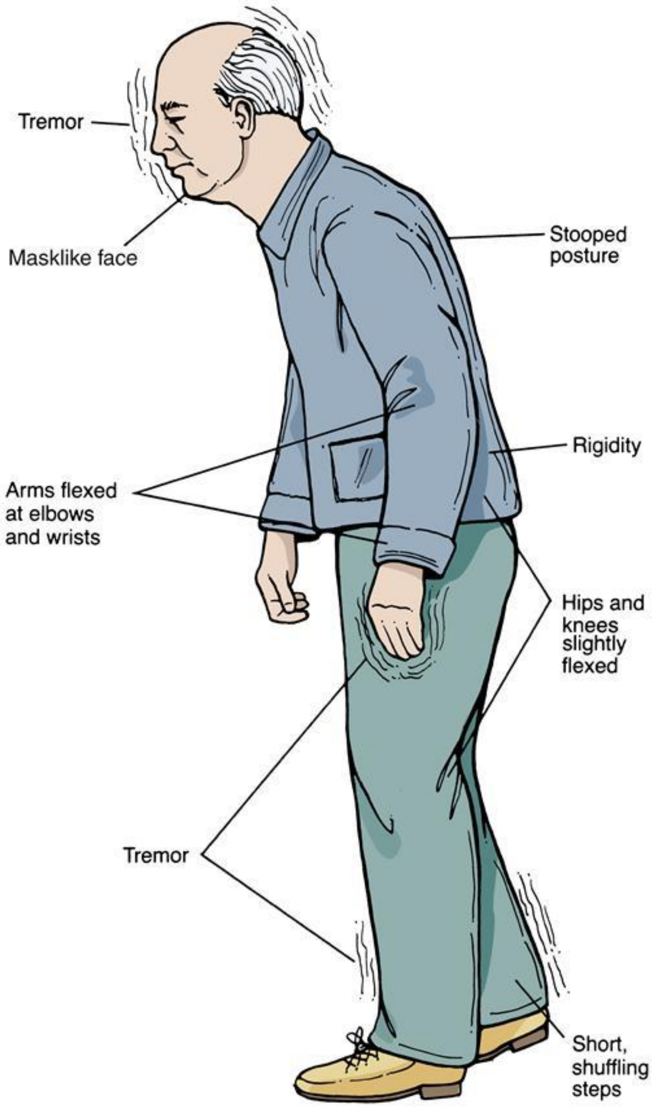

Figure 1.2: Typical motor signs of PD.
From Monahan FD, Neighbors M: Phipps' medical-surgical nursing: health and illness perspectives, edition 8, Philadelphia, 2007, Saunders, p 1446.) Permission from Saunders Publishing.

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1.3 Diagnosis of PD

1.3.1 Clinical diagnosis

The diagnosis of PD is mainly clinical by using criteria of the UK PD society Brain Bank (UKPDSBBC) (Hughes et al., 1992). The patient must display bradykinesia plus any two of the three other cardinal symptoms (tremor, rigidity, postural instatbility). Often, PD patients do not have full set of symptoms described on the UKPDSBBC criteria, hence, taken into account these clinical manifestations will make the diagnostic process straightforward. The UKPDSBBC are reported to be accurate in over 90% of diagnosed cases clinically suspected to have PD (Dotchin et al., 2007). The Hoehn and Yahr scale can be used to classify the severity of PD (Goetz et al., 2004). It has been shown to improve diagnotic accuracy in patients (Goetz et al., 2004).

1.3.2 Neuroimaging examination

The emergence of neuroimaging techniques has made it possible to use a single-photon emission computed tomography (SPECT) along with a radio-labelled compound to study dopamine levels in the brain. The compound will bind to dopamine receptors and can be viewed using SPECT. This method allows the measurement of the amount of dopamine releasing neurons and can be compared with healthy individuals (**Figure 1.6**). Neuroimaging is a supportive technique that helps the clinicians to accurately diagnose PD.

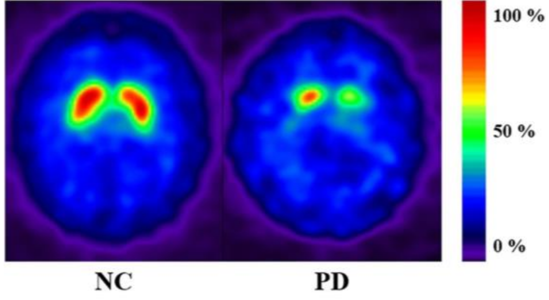

Figure 1.3: SPECT image showing dopamine levels in a healthy brain verus in a PD brain. Taken from *(Son et al., 2016)*, licenced by creative commons https://www.nature.com/articles/srep38070#rightslink

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1.4 Aetiology of PD

The neuropathological hallmarks of PD can be explained by the progressive loss of specific neurons in the brain, especially the dopaminergic neurons of the *substantia nigra pars compacta* of the midbrain (Beal, 1992; Biskup et al., 2006; Chu and Kordower, 2007a; Levy et al., 2005 and (Li et al., 2014; Maiti et al., 2017).

Dopaminergic neurons are the main source of dopamine (DA) neurotransmitter in the central nervous system (CNS), with a prominent role in voluntary movement and behavioral processes (Shen and Cookson, 2004).

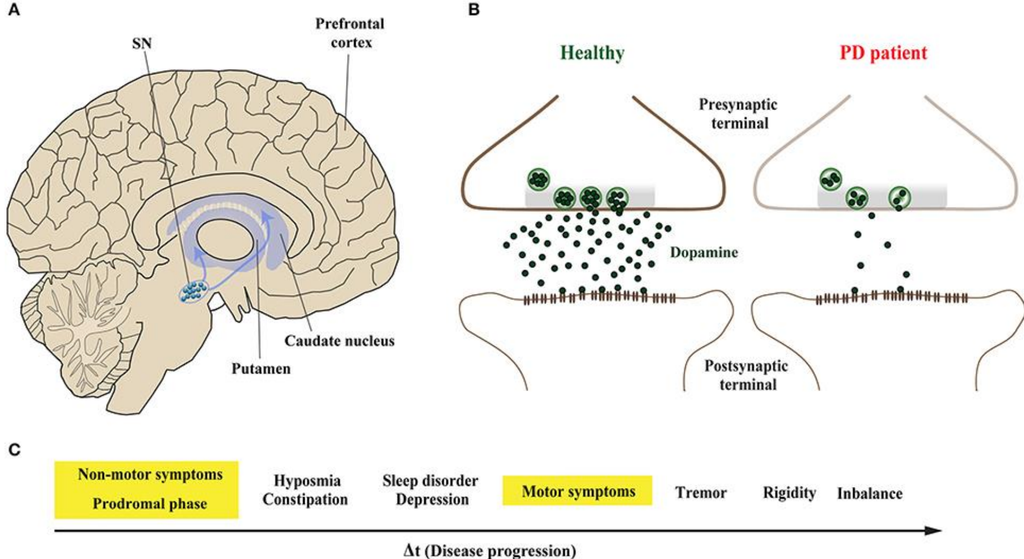

Figure 1.4: Location of the substantia nigra and comparison between PD affected dopaminergic neurons and normal neurons during exocytosis.

The figure shows the difference between the dopamine molecules released at the synaptic cleft during exocytosis in PD patients and healthy individuals. ***(A)*** *The dopamine outflow is low at the synaptic cleft in the PD patients which is the hallmark of the disease.* ***(B)*** *Compared to healthy controls (left), nigrostriatal degeneration results in the depletion and ultimate loss of the neurotransmitter dopamine on synaptic terminals of striatal neurons (right).* ***(C)*** *The resulting motor symptoms, However, PD patients can experience non-motors symptoms these include olfactory dysfunction, sleep disturbances and depression. Taken from Bridi and Hirth 2018* licenced by Creative Commons.

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Studies on DA are still relatively new to the field of monoamine transmitters in mammalian brain. Until the mid-1950s DA was considered as merely a precursor in the biosynthesis of the catecholamines. Later, physiologic and pharmacologic studies explored DA's roles in the brain, and found that they regulate movements via two main pathways. Namely, the direct pathway that promotes voluntary movement and the indirect pathway that inhibits movement. DA signalling from the *substantia nigra* is important for both pathways (Luo and Huang, 2016; Purves et al., 2001).

The discovery of profound depletion of DA in the striatum of PD patients was accompanied by poor motor control. A normal movement occurs in non-PD individuals while a movement disorder is seen in PD patients due to the low levels of DA at the synapses (**Figure 1.4**). Dopaminergic cell loss in excess of 50% usually leads to the onset of PD symptoms (Cheng et al., 2010).

Another important discovery in the understanding of PD pathophysiology was the discovery of the central role of cellular α-synuclein accumulation in genetic and sporadic forms of PD. Depositions of α-synuclein in the cytoplasm of the neurons in several brain regions, as well as the Lewy bodies were observed in the brain. The aggregated α-synuclein are found in dopaminergic brainstem neurons and in neurons in the olfactory system, but α-synuclein is also found in the limbic and neocortical brain regions as PD progresses (Poewe et al., 2017). This α-synuclein pathology is also shared by other PD-related conditions (synucleinopathies) with overlapping symptoms. The combined classical motor disturbances of PD are referred to as parkinsonism or parkinsonian syndrome which are also observable in other PD-related conditions like dementia with lewy body or multiple system atrophy. These conditions make it difficult to diagnose PD correctly in some patients due to sharing the common signs and symptoms of PD which confine their diagnoses.

Furthermore, recent advances support the idea that a prion-like mechanism operates in PD. The propagation of α-synuclein pathology from one neuron to another plays an important role in the progressive worsening of symptoms and

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the gradual involvement of additional brain and autonomic functions as the disease advances. The diversity in the molecular structure of α-synuclein aggregates, and consequently their capacity to interact with different ensembles of partner proteins and to propagate between different brain regions might explain why all PD patients do not follow the same disease course. These findings could also explain why other synucleinopathies (e.g., dementia with Lewy bodies and multiple system atrophy) take a different course to PD (Brundin and Melki, 2017).

1.5 PD risk factors

The aetiology of PD is not fully understood, but different studies have provided evidence that environmental, occupational and genetic factors are potential risk factors for developing PD (Ben-Shlomo, 1997). A number of risk factors are clearly evident in PD development. Lifestyle, including exposure to persistent organic pollutants, or shared genetic factors could contribute to PD in an individual.

Environmental factors

Environmental factors have been thoroughly studied as one of the leading causes of PD. Differences in PD incidence from one area to another have been recognized. The discovery of the effects of the toxin, 1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine (MPTP) in parkinsonism in humans (Langston, 1985; Langston et al., 1983) supported the environmental hypothesis, that humans exposed to numerous pesticides and toxins could develop PD. MPTP is a prodrug to the neurotoxin MPP+, which causes permanent symptoms of PD by destroying DA neurons in the substantia nigra. It has been used to generate disease models in various animal studies (Langston, 2017). However, not everyone exposed to environmental toxins develops PD, although, the incidence of PD could be significantly greater in individuals exposed to certain environmental chemicals such as pesticides and fossil fuels (Brown et al., 2006).

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Smoking cigarettes or drinking caffeine have been suggested to play protective roles against developing PD (Martyn and Gale, 2003), suggesting that environmental factors including personal lifestyles have complex roles in PD pathophysiology.

Similarly, heavy metals toxicities such as lead poisoning were thought to be environmental toxins that can contribute to the development of PD (Chin-Chan et al., 2015). Other herbicides such as rotenone and paraquat have been extensively studied in PD animal models. These chemicals have similar mechanisms of actions as MPTP, by destroying the DA neurons in the brain (Langston, 2017).

Gene-environment interactions in PD

Interactions between genetic factors and environmental exposures could be major contributors to the aetiology of PD (Cannon and Greenamyre, 2013). Although, it is not completely understood how most of these interactions contribute to PD pathobiology in humans, such interactions have the potential to have a major impact on PD. Animal models have also highlighted the importance of genetic susceptibility to toxin exposure in PD. While many pesticides have limited evidence to link them to PD, paraquat and rotenone have been strongly linked to PD (Cannon and Greenamyre, 2013). Similarly, exposure to heavy metals such as iron, lead and manganese and their combinations are associated with an increased risk for developing PD, since they accumulate in the substantia nigra and contribute to oxidative stress (Chin-Chan et al., 2015).

Absorption, distribution, metabolism, and excretion are key factors in how the human body deals with xenobiotic exposures (Cannon and Greenamyre, 2013). Genetic predisposition may affect toxin disposition in a number of ways relevant to PD, including increased toxin accumulation in sensitive brain regions and alterations in metabolism that produce more toxic species. There are indeed some data to support these predispositions (Cannon and Greenamyre, 2013). Polymorphisms in cytochrome P450s (*CYP2D6*) have received the most attention and have been reported as a risk factor for PD, with increased risk of ~2–3-fold (Deng et al., 2004; Elbaz et al., 2004 Cannon and Greenamyre, 2013).

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Ageing

Ageing comes with various changes in the physical and biochemical components of the body. PD generally affects the middle to late age of the population (45-80 years), a small proportion of cases (~10%) present an onset earlier than 45 years of age. Its risks however, continue to increase in the elderly (80 and above) (de Lau *et al* 2006). Notably, increasing age is the strongest risk factor for the development of PD.

The number of DA neurons consistently reduces as part of normal aging (Beal, 1992; Biskup et al., 2006; Chu and Kordower, 2007a; Levy et al., 2005). The progression of PD increases exponentially with age (Collier et al., 2017; de Lau and Breteler, 2006a; Ross et al., 2000).

Gender

Studies have shown that males are more likely to have PD than females. Recent meta-analysis data indicated that PD is twice as common in men as in women (Miller and Cronin-Golomb, 2010). The studies suggested that this may be because males have greater exposure to other risk factors such as toxins or head trauma and other occupational hazards (Poewe *et al* 2017).

Similarly, it has been speculated that oestrogen may have neuro-protective effects, of which it might reduce the risk of developing PD in females. Menopausal women therefore, might be at risk for developing PD.

Genetic factors

Generally, PD occurs as a sporadic disorder. Prior to 1997, it was not known that the aetiology of PD could be linked to genetic factors. However, in the last two decades, it has been postulated that mutations in genes can lead to PD. In most people, it is not a single genetic mutation that causes PD, but there are several genetic changes that increase the risk for PD. This is likely true for sporadic cases of PD.

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However, first-degree family members of affected patients have a 2-3 fold increased risk to develop the disease compared to subjects in the general population or controls (Savica et al., 2016; Sveinbjörnsdottir et al., 2000). In familial forms of PD, mutations in several genes have been reported (**Table 1.1**). Cell-based models are proving to be extremely valuable tools for figuring out what these genes and their proteins do and how PD-linked mutations have altered their functions (Moore et al., 2005).

Presently, about 34 genes have been directly implicated in the aetiology of PD (**Table 1.1**), of which five genes [Alpha-synuclein *(SNCA)*, Leucine-rich repeat kinase 2, (*LRRK2)*, Parkin RBR E3 Ubiquitin Protein *ligase (PRKN)*, PTEN-induced kinase 1 (*PINK1)* and Protein deglycase DJ-1 (*PARK7)*], have been strongly linked to and have been replicated in a Mendelian form of PD. *SNCA* and *LRRK2* have been classified as autosomal dominant (AD) genes whereas *PRKN, PINK1 and PARK7* have been classified as autosomal recessive (AR) genes.

Physiologically, some of these genes regulate normal functioning of the DA pathways. SNCA promotes exocytosis and it can play a part in endocytosis at the synaptic transmissions (Logan et al 2017). LRRK2 regulates phosphorylation of *endophilin A* postsynaptically and the release of *clathrin* coated endocytic vesicles (Lin and Farrer, 2014). Parkin is involved in ubiquitination and proteasomal function (Arkinson and Walden 2018). PINK1 and PARK7 regulate maintenance of mitochondria (Jain et al., 2005; Lin and Farrer, 2014).

PINK1 and Parkin cooperate in several processes that are associated with the mitochondrial functions *(Scott* et al 2017). Parkin is recruited to the mitochondrial surface and activated by PINK1, to exert its E3 ubiquitin protein ligase function (Mouton-Liger et al 2017). This binding is required to mediate autophagosome formation on the damaged mitochondrion, leading to mitophagy. Parkin is also involved in the maintenance of mitochondrial levels of PD-linked mitochondrial enzyme HSD17B10, possibly by promoting its import through the TOM complex (Mouton-Liger et al 2017).

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PINK1 and Parkin can also control localized translation of several mRNAs for nuclear-encoded subunits of respiratory chain complexes. PINK1 and Parkin also control the formation of mitochondria-derived vesicles, leading to the specific degradation of damaged mitochondrial content (Mouton-Liger et al 2017). Parkin also regulates the process of mitochondrial biogenesis by promoting the proteasomal degradation of the PGC-1α transcriptional repressor PARIS, in a PINK1-dependent manner (Mouton-Liger et al 2017).

The functions of these proteins share similar pathways that make genetic studies of PD interesting

The G2019S mutation in *LRRK2* appears to be the most common known mutation underlying PD (including the apparently sporadic form), with frequencies as high as 6% in the European patients diagnosed of PD and 41% in North African Arabs patients (Lesage et al., 2006).

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Table 1.1: A list of genes that are associated with PD and parkinsonism

Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
ARSB	AR	PD	Contains mutation recently replicated in a large cohort PD study	Lof mutation (Q887X stopgain) identified	(Jansen et al., 2017)
ATP7B	AD	Parkinsonism	Contains mutations associated with parkinsonism	Identification of mutation H1069Q (as either homo-or heterozygous) have strong association with a variety of parkinsonism including PD in European populations	(Montes et al., 2014; Oczkowska et al., 2014; Sechi et al., 2007)
ATP13A2	AR	PD and Parkinsonism	Contains mutations considered as non-PD but robustly associated with parkinsonism.	Pathogenic mutations were associated with complex phenotype with parkinsonism, spasticity, and dementia.	(Ramirez et al., 2006)
CHCHD2	AD	PD	Contains mutations robustly associated with PD	Missense mutations (T61I, R145Q) and exon skipping were identified in familiar PD	(Funayama et al., 2015)
DCTN1	AD	Parkinsonism	Contains mutations considered as non-PD but robustly associated with parkinsonism.	Heterozygous mutation, (F52L), segregates with parkinsonism in a family.	(Araki et al., 2014)

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Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
DNAJC13	AD	PD and Parkinsonism	Contains mutations considered as non-PD but robustly associated with parkinsonism.	Mutation in receptor-mediated endocytosis 8/RME-8 (N855S) was found to segregate with disease. Screening of cases and controls identified four additional patients with the mutation, of which two had familial parkinsonism.	(Vilariño-Güell et al., 2011)
DNAJC6	AR	PD	Contains mutations robustly associated with PD	Different homozygous mutations segregating with PD in a family were identified. One of the mutation was flanked by long runs of homozygosity within highest linkage peaks.	(Olgiati et al., 2016)

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Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
EIF4G1	AD	PD	Contains mutations robustly associated with PD	A missense mutation (R1205H) found by genome -wide linkage analysis and disease cosegregation analysis in a large French family with ADPD.	(Chartier-Harlin et al., 2011; Dhungel et al., 2015; Lesage et al., 2012; Tucci et al., 2012a)
FBXO7	AR	PD	Contains variants reported as PD risk factors	A homozygous truncating mutation (R498X) was identified in familial PD	(Conedera et al., 2016; Di Fonzo et al., 2009)
GBA	AR	PD	Contains variants reported as PD risk factors	Multiple rare variants of intermediate-effect strength identified associated with PD and a gene expression study in GBA mutant fibroblast.	(Sanchez-Martinez et al., 2016; Sidransky et al., 2009)
GIGYF2	AD	PD	Contains mutations reported as PD risk factors	A total of seven different missense mutations resulting in single amino acid substitutions were present in 12 unrelated PD index patients (4.8%) and not in controls.	(Lautier et al., 2008)

Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
GPATCH 2L	AR	PD	Contains mutation recently replicated in a large cohort PD study	Lof mutation (R362X stopgain) identified	(Jansen et al., 2017)
GRN	AR	PD	Contains variants reported as PD risk factors	A single nucleotide polymorphism (rs5848) was studied in PD and was considered to be a risk factor in PD patients.	(Chang et al., 2013)
HTRA2	AD	PD	Contains mutations robustly associated with PD	G399S mutation was identified and homozygotes for this allele developed PD.	(Gulsuner et al., 2014)
LRP10	AD	PD	Contains mutations robustly associated with familial autosomal dominant PD cases	Five mutations including (N517del, R533L P699S) that were replicated and studied for mechanisms in cell-based assays	(Quadri et al., 2018)
LRRK2	AD	PD	Contains mutations robustly associated with PD	Six confirmed point mutations: R1441G, R1441C, N1437H, Y1699C, G2019S, I2020T were earlier identified in familiar PD.	(Paisán-Ruíz et al., 2004; Zimprich et al., 2004)
MAPT	AR	PD-dementia	Contains variants reported as PD risk factors	Five SNPs were significantly associated with PD	(Tobin et al., 2008; Wang et al., 2016)

Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
PARK7	AR	PD	Contains mutations robustly associated with PD	Mutations (deletion of 14.082bp and L166P) were identified in PD patients.	(Bonifati et al., 2003)
PINK1	AR	PD	Contains mutations robustly associated with PD	More than 20 mutations, point mutations, INDELs, and exon rearrangements were identified in PD.	(Valente et al., 2004)
PLA2G6	AR	PD	Contains mutations reported as PD risk factors	PLA2G6 mutations identified in levodopa-responsive dystonia-parkinsonism and segregation of R632W with disease.	(Lu et al., 2012; Paisan-Ruiz et al., 2009; Sina et al., 2009)
POLG1	AR	Parkinsonism	Contains mutations robustly associated with parkinsonism	Heterozygous mutation (Y955C) was first identified in progressive external ophthalmoplegias. Subsequently,27 mutations in 14 probands with a variety	(Stewart et al., 2009; Van Goethem et al., 2001; Wong et al., 2008a)

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Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
				of parkinsonism have been reported	
PRKN	AR	PD	Contains mutations robustly associated with PD	Pathogenic mutations, point mutations, INDELs, and exon rearrangements have been implicated in PD.	(Brüggemann and Klein, 1993; Kitada et al., 1998)
PTPRH	AR	PD	Contains mutation recently replicated in a large cohort PD study	Lof mutation (90-2insT stopgain) identified	(Jansen et al., 2017)
SLC30A10	AR	Parkinsonism	Contains mutations confirmed with association and functional studies	Identification of homozygous c.507delG and F167S mutations in parkinsonism in Dutch and italian families.	(Quadri et al., 2012)
SNCA	AD	PD	Contains mutations robustly associated with PD	Three rare mutations: A53E, A30P, E46K and duplications and triplications of the *SNCA* gene were first identified in PD.	(Polymeropoulos et al., 1997; Singleton et al., 2003)

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Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
SPG11			Contains mutations considered as non-PD but robustly associated with parkinsonism.	Two mutations, c.3664insT and c.6331insG, found in a patient with spastic paraplegia and early onset parkinsonism.	(Guidubaldi et al., 2011)
SYNJ1	AR	Parkinsonism	Contains mutations reported as parkinsonism risk factors	Patients with early onset parkinsonism had mutations: R258Q and S1422R	(Quadri et al., 2013)
TMEM230	AD	PD	Contains mutations reported as PD risk factors	Pathogenic mutations; (R141L, Y92C, R171C, I125M, and M64T) cosegregated with PD in a family. Mutations influenced gene expression.	(Deng et al., 2016)
UHRF1BP1L	AR	PD	Contains mutation recently replicated in a large cohort PD study	Lof mutation (K1376X stopgain) identified	(Jansen et al., 2017)

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Gene	Inheritance pattern	Associated with PD/Parkinsonism	Comment	Findings	Study reference
VPS13C	AR	PD	Contains variants reported as parkinsonism risk factors	Mutations (G1389R, Q1593K) associated with a distinct form of early-onset parkinsonism characterized by rapid and severe disease progression and early cognitive decline; resembling diffuse Lewy body disease.	(Lesage et al., 2016)
VPS35	AD	PD	Contains mutations robustly associated with PD	Pathogenic mutations (D620N,R524W,L774M,I241M,M57I) identified in family members with PD.	(Dhungel et al., 2015; Mohan and Mellick, 2017; Vilariño-Güell et al., 2011; Zimprich et al., 2011)

Single neuclotides Polymorphism (SNP), Insertion and Deletion (INDELs), Lof (loss of function), Autosomal recessive (AR), Autosomal dominant (AD)

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Understanding the genetic causes of PD is reshaping the knowledge about PD pathology and how it progresses. However, some genes still lack strong evidences to associate them to PD pathology. These include *PARK3, UCHL1 (PARK5), GIGYF2 (PARK11), HTRA2 (PARK13) and PLA2G6 (PARK14)*. The genetics of PD is complex because there are overlapping mutations in these genes in PD and other diseases such as Alzheimer's disease, dementia and Lewy body disease. To date, there are only five genes that have been robustly linked to PD (**Table 1.2** and **Appendix I** for the list of mutations catalogued in these genes). These will be elaborated on in the section below.

Table 1.2: Loci and genes strongly associated with, and replicated in inherited PD

Locus	Gene symbol	Chromosome	Mode of inheritance	Age at onset
PARK1	*SNCA*	4q	Autosomal dominant	~45
PARK2	*PRKN*	6p	Autosomal recessive	30-60
PARK6	*PINK1*	1p36	Autosomal recessive	36-60
PARK7	*DJ-1*	1p36	Autosomal recessive	27-40
PARK8	*LRRK2*	12p	Autosomal dominant	45-57

SNCA

The study by Polymeropoulos *et al* 1997 was the first study that showed a mutation in the α-synuclein and it was discovered before the identification of α-synuclein in the Lewy Bodies (Spillantini et al., 1997) in PD. A missense mutation (A53T) in α-synuclein (encoded by *SNCA* gene) was identified in a classical positional cloning (Polymeropoulos *et al* 1997). Thus, the discovery of α-synuclein accumulation in Lewy bodies in PD in 1997 (Spillantini et al., 1997).

The two landmark studies were the first to show the effects of genetic component in SNCA gene in association with PD. However, more missense mutations in *SNCA* (A30P, E46K, H50Q, and A53E) were later identified in other studies that confirmed the causative role of α-synuclein in PD (Wang et al., 2016, 2017).

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Mutations identified in the gene in the patients have high penetrance and were associated with early-onset PD within the age 40s or 50s with typical motor symptoms and a good response to L-dopa treatment.

A recent report described another missense mutation, G51D that was associated with parkinsonism pyramidal syndrome in a French family (Lesage et al., 2013).

Three missense mutations (A53E, A30P, E46K), as well as duplications and triplications in the *SNCA* gene have been associated with autosomal dominant PD (Krüger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003). α -synuclein is mostly expressed at the presynaptic space in the CNS. *SNCA* aggregates to form fibrils deposits due to its hydrophobic non-amyloid beta component domain and progresses to form the Lewy bodies that are involved in the pathology of PD. The oligomeric forms of α-synuclein fibrils deposits are cytotoxic. Overexpression of α-synuclein in *in vitro* studies increased the formation of α-synuclein protofibrils (Conway et al., 2000; Fredenburg et al., 2007). In a study that used toxin that specifically targeted and damaged mitochondria in rodents and in cell culture experiments showed the involvement of α-synuclein aggregates in PD (Betarbet et al., 2000; Fornai et al., 2005).

The accumulation of unfolded proteins produced by *SNCA* was linked to the ubiquitin proteasome pathway leading to mitochondrial dysfunction and neuronal cell death (Tanaka et al., 2001). Majorly, protein aggregation and mitochondrial dysfunction were identified to be interwoven and were considered as the causes of PD in human brain studies. Many studies found α-synuclein accumulation in the substantia nigra and striatum of PD patients (Devi et al., 2008).

PRKN

Parkin (encoded by *PRKN*) plays a role in the ubiquitin pathways involving the E1-activating enzyme, an E2-conjugating enzyme, and an E3 ligase. It which has E3 ubiquitin ligase function and is involved in the proteasome-mediated degradation, and during autophagy.

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Ubiquitin signalling plays a central role in protein quality control. Parkin can trigger proteasome-independent ubiquitination, it also has roles in mediating transcriptional regulation, protein trafficking and neuroprotective signalling (Fallon et al., 2006).

Parkin facilitates the polyubiquitination reaction so that the process of proteasomal degradation will enable the clearing of proteins that normally aggregate in the brain and in the mitochondria.

The loss of E3 ligase activity causes accumulation of toxic protein aggregates leading to autosomal-recessive juvenile parkinsonism (Dawson, 2006). The role of parkin is, therefore, essential by protecting cells against further damage. Mutations in the *PRKN* gene predispose cell to damage by compromising the processes of protein degradation.

PINK1

PINK1 mutations cause autosomal recessive early onset PD (Valente et al., 2004). PINK1 gene encodes a mitochondrial serine/threonine-protein kinase. It protects cells from stress-induced mitochondrial dysfunction in different regions of the mitochondrial membrane (Gandhi et al., 2006; Pridgeon et al., 2007).

This protein is found in cells throughout the body. PINK1 also plays crucial roles in mitophagy, a selective degradation of mitochondria by autophagy. Studies have shown in *in vitro* cell-based assays that mutations in this gene can lead to mitochondrial dysfunction, leading to abnormalities in mitochondrial morphology, resulting to a decreased membrane potential, and increased reactive oxygen species (ROS) production, sensitive to apoptosis (Wood-Kaczmar et al., 2008). Wang and colleagues found that PINK1 overexpression restored normal mitochondrial morphology that reduced ROS production indicating that PINK1 is important in the maintainance of mitochondrial morphology and to protect the neurons damages cause by ROS in mice brain (Wang et al., 2011). A study also showed that embryonic fibroblasts derived from PINK1 knockout (KO) mice had low mitochondrial membrane potential, decreased cellular ATP levels and declines mitochondrial respiratory activity (Amo et al., 2011).

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PARK7

Mutations in *PARK7* gene can cause autosomal recessive early onset parkinsonism (Bonifati et al., 2003). *PARK7* is crucial in many tissues and organs, including the brain. *PARK7* has antioxidant and transcription modulation properties. One of the protein's functions may be to help protect cells, particularly brain cells, from oxidative stress. *PARK7* is localized in the cytosol and can also be found in the nucleus and mitochondria (Zhang et al., 2005).
When there is oxidative stress, *PARK7* will translocate to the mitochondria where it will gain a neuroprotective abilities. The translocation of *PARK7* is facilitated by the oxidation of cysteine 106 to cysteine-sulfinic acid which is vital for PARK7 in its function in the mitochondria (Blackinton et al., 2009; Canet-Avilés et al., 2004). When *PARK7* was silenced in neuronal cells, there was increased cell death that was linked to stress and inhibition of the proteasome (Taira et al., 2004; Yokota et al., 2003). Mitochondrial complex I dysfunction is the leading mechanism implicated in PD associated with in *PARK7* mutations.

LRRK2

LRRK2 mutations have been associated with both familial and sporadic forms of PD (Klein and Schlossmacher, 2006; Paisan-Ruiz et al., 2009; Zimprich et al., 2004). The *LRRK2* gene encodes serine/threonine kinase containing mitogen-activated protein kinase kinase kinase (MAP3K), a Roc domain with Ras/GTPase, a WD40-repeat domain and leucine-rich repeats (Biskup and West, 2009; Gandhi et al., 2009; Mata et al., 2006). *LRRK2* mutations is responsible for the neurotoxicity caused by an increase in *LRRK2* kinase activity suggesting a toxic gain of function (Gloeckner et al., 2006; Greggio et al., 2006). Overexpression of *LRRK2* leads to apoptotic neuronal cell death and mitochondrial dysfunction (Iaccarino et al., 2007).

The most common mutation in *LRRK2* is G2019S, it causes defects in mitochondrial morphology and functions, resulting to an increased production of ROS in the neurons. *LRRK2* interacts with Dynamin like protein 1 (DLP1) in neurons leads this interaction leads to translocation of DLP1 from the cytosol into the mitochondria (Niu et al., 2012).

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Similarly, ATP13A2 gene can be classified as a known PD gene, although the gene is not largely implicated in PD pathology, but pathogenic mutations in ATP13A2 show that this gene is associated with parkinsonism and including PD. ATP13A2 gene is involved in autophagy. Mutations in *ATP13A2* can decrease the ability of lysosomes to degrade proteins and mediate clearance of autophagosomes hence leading to aggregation of α-synuclein in neurons (Dehay et al., 2012; Yang and Xu, 2014).

ATP13A2 encodes a lysosomal transmembrane protein that belongs to the 5P-type ATPase subfamily. Its activities are localized in the lysosome, but when mutations are involved in PD, the activities of this gene are localize to the endoplasmic reticulum. *ATP13A2* is highly expressed in the brain especially in the cytosol of the dopaminergic neurons suggesting a brain-specific function. Studies showed that *ATP13A2* expression levels in the substantia nigra were reduced in post-mortem tissue samples of patients with sporadic PD compared to controls without PD.

ATP13A2 can also facilitate the transmembrane transport of manganese, zinc, iron and cadmium that cause neurodegeneration. Elevated levels of manganese and zinc have been found in serum and cerebrospinal fluid of PD patients. Exposure to these metals is a significant environmental risk. *ATP13A2* protects cells from toxicity by regulating the homeostasis of manganese and zinc (Rentschler et al., 2012; Yang and Xu, 2014). In summary, mitochondria are involved in autophagy in the cells (Rentschler et al., 2012). The differentiated cells like the neurons depend on active membrane transport systems that also require mitochondria to function. Hence, the survival of these neurons when they are stressed or damaged depends on the autophagy (Tooze and Schiavo, 2008). Autophagy impairments caused by mutations have been implicated in PD.

In 2011, Zimprich and colleagues identified a mutation in *vacuolar protein sorting 35 homolog gene (VPS3*5, encoding vacuolar protein sorting 35) in a family with inherited PD. This mutation was found in every affected individual in the family and segregates with late-onset PD in a Mendelian autosomal dominant manner (Hernandez et al 2016). VPS35 protein alters the trafficking

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of cathepsin D, a protein that is implicated in the degradation of α-synuclein. *VPS35* is linked to the other autosomal dominant PD genes *SNCA* and *LRRK2* through endosomes and vesicular trafficking and underscores the importance of studying these pathways in health and disease (Hernandez et al 2016).

Beta glucocerebrosidase *(GBA)* is a gene long associated with the autosomal recessive lysosomal storage disorder, Gaucher's disease. Aharon-Peretz and colleagues were able to show that a single mutation in *GBA* gene increased the risk for PD. The function of GBA is well established as an integral enzyme required for the breakdown of glucocerebroside to ceramide. Multiple studies have shown that glucocerebrosidase activity is lower in patients carrying *GBA* mutations and in sporadic PD cases, suggesting a broader role for *GBA* in pathogenesis (Hernandez et al., 2016; Schapira, 2015).

1.6 Biological processes associated with PD

There are various biological processes contribute to the pathophysiology of PD. The roles of environmental toxins and brain trauma in PD pathophysiology have been described earlier, PD-linked genetic mutations have specific pathways by which they interact and lead to PD onset. Mitochondrial dysfunction, calcium imbalance and oxidative stress are the leading biological processes implicated in PD pathology.

1.6.1 Oxidative stress

Oxidative stress is associated with PD as a consequence of mitochondrial dysfunction in the brain tissue. This theory was supported by the identification of mutations in the *PARK7* gene that resulted in cellular oxidative stress. *PARK7* has antioxidant effects but the mutated form had reduced gene expression (Guzman et al., 2010; Poewe et al., 2017).

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It is the process of oxidative phosphorylation in the mitochondria that generates ROS and intracellular oxidative stress. The mitochondrial complex I and complex III are the main sites of ROS production in mitochondria. Superoxide radical is produced when there is a transfer of single electron to oxygen in the respiratory chain. Constant stress perturbation in the dopaminergic neurons causes neurodegeneration.

The mitochondrial complex I activity in the post-mortem substantia nigra of patients with idiopathic PD has been studied (Parker et al., 2008; Schapira et al., 1989). Oxidative damage to the catalytic subunits of complex I had a correlation between increased protein oxidation and reduction in electron transfer ability in PD patients.

1.6.2 Mitochondrial dysfunction

Both sporadic and inherited forms of PD share biochemical, pathological and clinical features. Mitochondrial dysfunction is a major point of emphasis in the study of both forms of PD (Grünewald et al., 2018; Keane et al., 2011). Mitochondrial complex I deficiency was described in a study in 1989 when Schapira and colleagues studied the brains of PD patients (Schapira et al., 1989).

The primary function of the mitochondria in a cell is to produce adenosine triphosphate (ATP) through the process of respiration and oxidative phosphorylation. This process serves as a source of energy in a cell. When there is joining of redox and phosphorylation reactions in the inner membrane of mitochondria, ATP will be synthesised. ATP can be formed when the electron transport chain transfers electrons via the complexes I-IV and nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH2). This will lead to the formation of a proton gradient in the inner mitochondrial membrane. Proton influx from the mitochondrial accumulates in the intermembrane space which then creates an electrochemical gradient. This electrochemical gradient is the driver for the synthesis of ATP from adenosine diphosphate (ADP) by ATP synthase.

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1.6.3 Calcium imbalance

Calcium regulates the functions of mitochondria and endoplasmic reticulum. Calcium influxes in the neurons come through the L-type calcium channels or by the activation of N-methyl-D-aspartate (NMDA) receptors. Calcium binding proteins and the metabotropic glutamate receptors govern the regulations of calcium presence in the intracellular space (**Figure 1.5**). Increased levels of calcium in the neuron increase dopamine metabolism in the substantia nigra pars compacta DA neurons. Also by shifting the levels of calcium influx through the L-type calcium channels in the substantia nigra dopaminergic neurons, a basal mitochondrial oxidant stress was induced and implicated in aging and cell death (Mosharov et al., 2009).

Calcium is transported by a calcium uniporter inside the cell across the plasma membrane to the mitochondria. The mitochondrial membrane pores then allow the communication with the endoplasmic reticulum and mitochondria directly (Rizzuto et al., 2000). Mitochondrial sodium/calcium exchanger and high conductance ion channels like the mitochondrial permeability transition pore regulates the efflux of calcium from mitochondria (Rizzuto et al., 2000). Mitochondrial permeability transition pore has two conductance states, when it is low, it is reversible and involved in calcium handling, when it is high, it is irreversible and leads to mitochondrial swelling and leakage of molecules like cytochrome C that triggers apoptosis in cell (Gogvadze et al., 2006).

1.6.4 Neuroinflammation

The term neuroinflammation is gaining overwhelming support in the understanding of PD pathophysiology. It is explained as a self-propelling cycle of inflammatory process involving the brain immune cells (microglia and astrocytes) that drives the progressive neurodegeneration. Moreover, increasing evidence suggests that early-life occurrence of inflammation in the brain, as a result of either brain injury or exposure to infectious agents, may play a role in the pathogenesis of PD (Hirsch and Hunot, 2009).

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Neuroinflammation usually leads to a cascade of events that assault the neurons (**Figure 1.5**). During neuroinflammation there is microglial activation, astrogliosis, and lymphocytic infiltrations (Hirsch and Hunot, 2009).

These inflammatory cells drive the processes. The release of inflammatory cytokines during neuroinflammation, will amplify the inflammatory response by activating and recruiting other cells to the brain lesion, by activating the microglia, the neurons will be damaged.

This process is being mediated by releasing various toxic substances including the inflammatory cytokines such as *IL-1b, TNF-a, IL-6, NO, PGE*, and superoxide.

1.6.5 Other

In addition, PD symptoms could be amplified in the presence of stress also. Risk factors such as cholesterol intake, dairy products, alcohol, body mass index, caffeine and cigarettes have been investigated in PD but there is little evidence to support their roles in PD pathobiology (Abbas et al., 2018). Since the prevalence of PD is different from one country to another, environmental factors and diet might be responsible (Chaudhuri et al., 1997). Low levels of vitamin B folate have also been linked to severe PD symptoms in mouse studies, suggesting that people with low levels of vitamin B folate could be predisposed to the disease.

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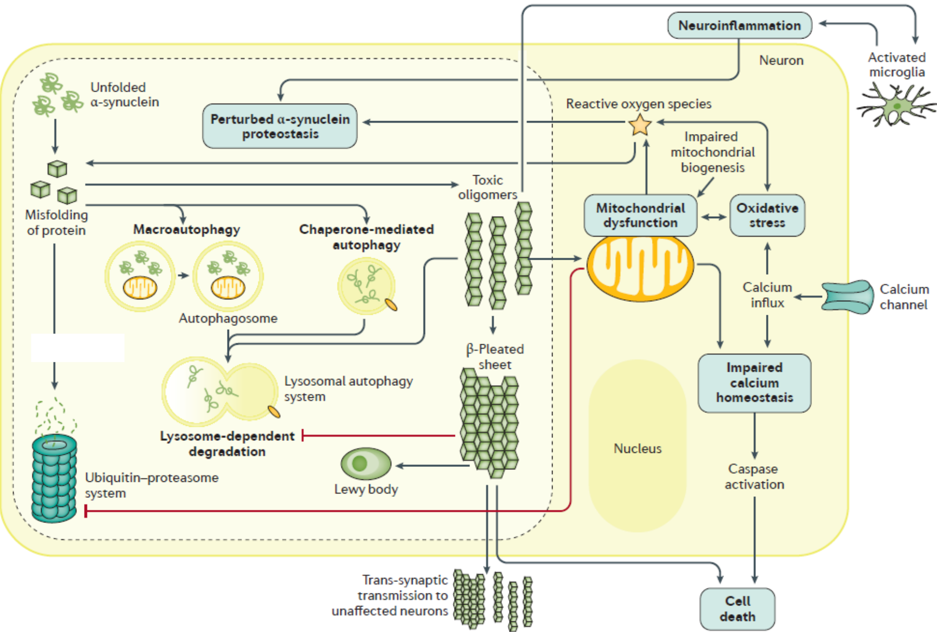

Figure 2.5 Molecular mechanisms involved in PD, the figure shows major interactions between molecular pathways that are implicated in the pathogenesis of PD. Taken from (Poewe *et al*., 2017) Permission from Springer Nature

1.7 Treatment of PD

The discovery that DA loss in the SNPc will cause extrapyramidal PD symptoms, provided the basis for development of treatment. In the 1960s the drug L-dopa was first administered to treat the symptoms and has since become the first-line of drug to treat PD. Similarly, excellent response to L-dopa is a supportive criteria for diagnosing PD with the UKPDSBBC (Hughes et al., 1992), and can also be used to correctly diagnose PD as only PD patients will respond quickly to L-dopa intake. L-dopa structure is closely similar to DA structure (**Figure 1.6**). It has a carboxylic acid group that makes it polar and can cross the blood-brain barrier where DOPA decarboxylase enzymes can convert L-dopa to DA. This occurs both in the peripheral circulation and in the CNS.

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DA synthesis occurs from a rate limiting step from the conversion of the amino acid tyrosine, which when tyrosine gets into the neurons get converted to L-dopa by the enzyme tyrosine hydroxylase. Subsequently, the conversion continues by the L-aromatic amino acid decarboxylase. This enzyme is most active so that even in a healthy individual the level of DA is negligible in the brain. Following the release of DA, it can be degraded to dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by the monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), respectively. DOPAC and HVA are the major metabolites of DA. The roles of MAO and COMT in degrading DA to DOPAC and HVA, respectively, make them major sites for drug actions in the treatment of PD.

A) **B)**

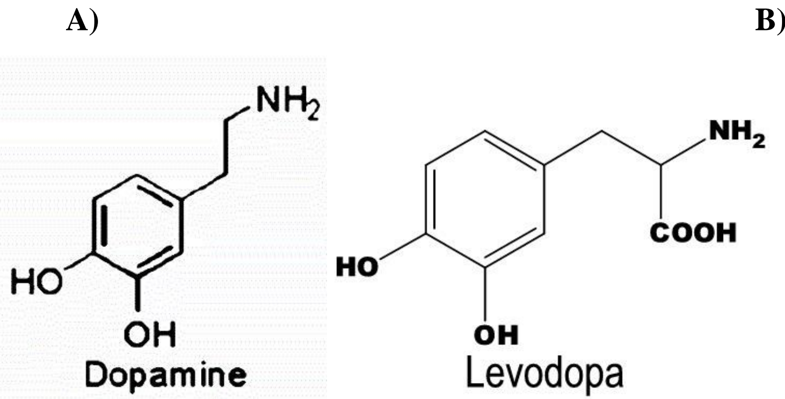

Figure 1.6: Structures of (A) DA neurotransmitter and (B) L-dopa, a DA receptor agonist.

There is currently no drug that can prevent, reverse, or halt the progression of the disease. PD, like other neurodegenerative diseases has complex pathogenesis so patients show a wide range of symptoms which makes its treatment difficult. Currently, drug treatments of PD are based on symptomatic management. When PD symptoms progress, the other non-dopaminergic neurons will be involved, so the drugs that target the dopamine pathways might not be appropriate to treat other non-motor symptoms that arise at later stages of the disease (Burn, 2013). The medications to treat the motor symptoms of PD act on a complex neurologic interaction in the striatum that coordinates movement.

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A combination of L-dopa and carbidopa can be administered as the first line treatment to aid movement deficit in PD (**Figure 1.7** and **Appendix II** for classes of drugs approved by the FDA and used for treating PD). Levodopa can cross the blood brain barrier where it is converted into dopamine, whereas carbidopa inhibits dopamine decarboxylase in the central nervous system to prevent dopamine degradation. Tolcapone can also be administered along with levodopa and carbidopa, it is a selective inhibitor of COMT at the peripheral tissues and central nervous system, so that it prevents the degradation of levodopa. A dopamine agonist, bromocriptine is given to patients to directly stimulate dopamine receptors. In addition, MAO type B inhibitors selegiline administered once daily, can inhibit MAO-B to ensure that dopamine has a longer lasting effect in the brain (Connolly and Lang, 2014). Also, amantadine (100 mg) has been useful in treating gait dysfunction and dyskinesia in PD (Connolly and Lang, 2014)

Certain treatments are recommended to treat the non-motor symptoms in PD. Clozapine is effective for hallucinations and psychiatric disturbances, and recently a new drug nuplazid was approved by the FDA to treat psychosis in PD patients. Some other classes of drugs have been tried in PD patients such as the anti-cholinesterase which is effective for treating dementia **(Figure 1.7** and **Appendix II**). Antidepressants and pramipexole are typically administered to treat depression (Connolly and Lang, 2014). Anticholinergic medications also help to regulate muscle movement.

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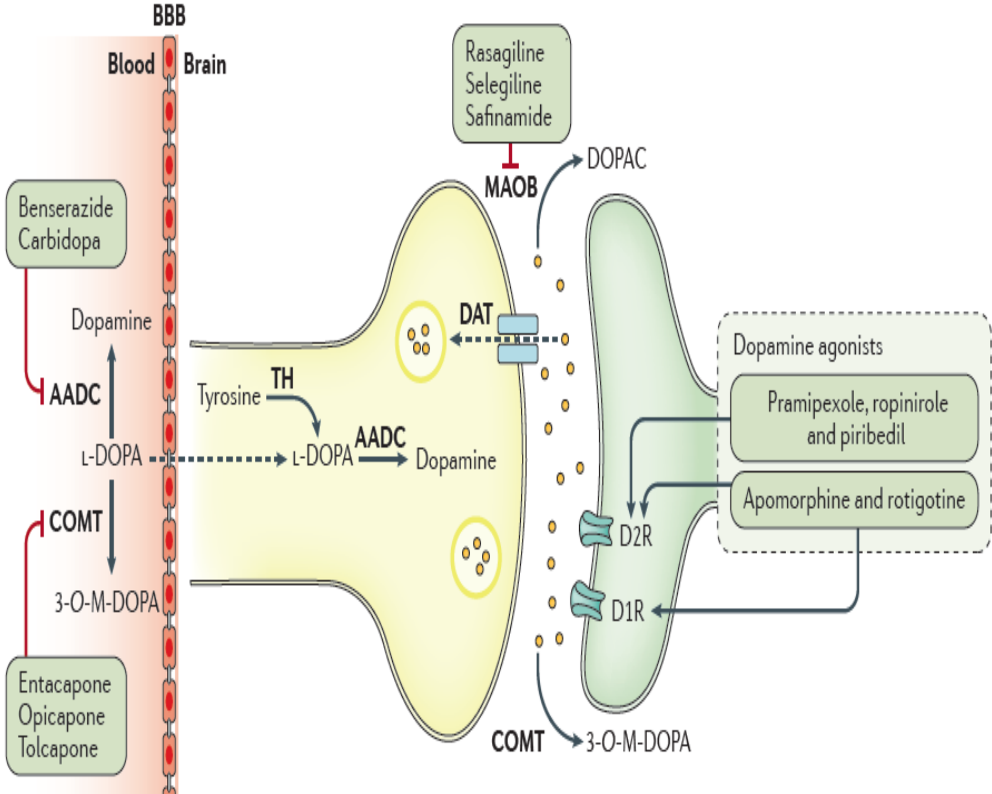

Figure 1.7: Classes of drugs that can activate or inhibit receptors at neuron sites to alleviate PD symptoms. BBB: Blood Brain Barrier; DAT: Dopamine transporter: COMT: catechol-O-methyl transferase; DOPAC: dihydroxyphenylacetic acid, L-DOPA: levodopa; TH: Tyrosine; D2R and D1R: Dopamine receptors 1 and 2; AADC: Aromatic L-amino acid decarboxylase and MAO: monoamine oxidase. Taken from (Poewe et al., 2017), Permission from Springer Nature.

However, these drugs do not cure PD nor can they reverse neuronal loss. Besides, each drug has serious adverse effects that might be unbearable for the patients, hence the more reason to investigate better drug treatments for PD. The symptomatic approach to treat PD has been limited. Still, the drugs and other existing therapies have been used to improve a patient's quality of life. Surgical therapy including deep brain stimulation has also been considered to improve dopamine levels in the brain. In some instances, making lifestyle and dietary changes, and employing physical and speech therapy has been recommended to PD patients and has proven to be beneficial.

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1.8 Incidence and Prevalence of PD

PD occurs globally across all ethnic groups and geographical regions. PD incidence increases with age (**Figure 1.8**). It is generally accepted that the prevalence of the disease ranges from 1-2% among individuals over the age of 60 years (Olanow et al., 2009; von Campenhausen et al., 2005) and affects 4% of the people above 80 years (de Lau and Breteler, 2006a).

The exact global figures for PD prevalence are not available, though it is estimated that 9-10 million people worldwide have PD, and the incidence is predominant in Europe and North America. The incidence of the disease varies in different reports. The global incidence of PD ranges from 5 to >35 new cases per 100,000 yearly (Poewe et al., 2017).

Similarly, an incidence rate of 15.4–27.6/100,000 and 6.1–17.4/100,000 in the door to door and record-based studies was reported in Europe (Abbas et al., 2018). The incidence in Asian studies was lower at 1.5–17/100,000 compared to 9–22/100,000 and 11–13/100,000 in studies from Europe and North American studies respectively (Abbas et al., 2018). A meta-analysis of European studies reported PD incidence of 11–19/100,000. In addition, the median incidence was 14/100,000 cases under the age of 65 years, while at the age group of 70 years or older the median incidence was higher at 160/100,000 (Abbas et al., 2018).

Incidence studies may be affected by misdiagnosis of PD, especially among the most elderly. Likewise, cases of no or poor diagnosis of PD in patients are likely to occur especially in an area where expert neurologists are scarce, and if the patients fail to visit the hospital at the onset of the disease. It is plausible to expect higher incidence rates of PD in the urban areas than in the rural areas due to the lack of neurologists in the rural areas. Also patients in rural areas have limited access to quality health services. High levels of pollutants and environmental toxins in urban cities due to the industrialization could contribute to higher prevalence in these areas.

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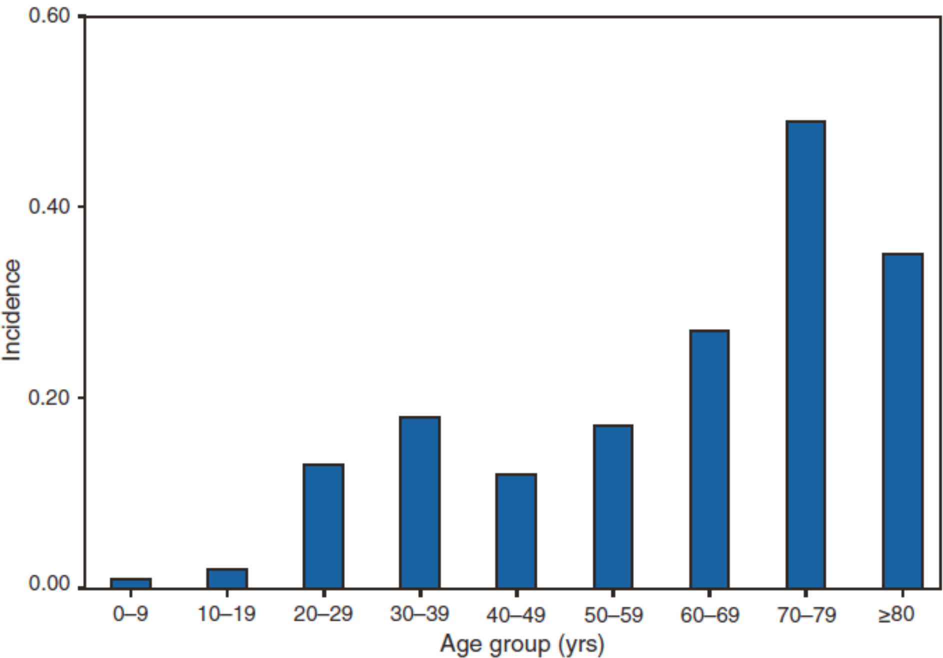

Figure 1.8: PD incidence increases with age the incidence rate is higher in age 60 years and above. Permission from centre for disease control and prevention https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5853a1.htm

A number of epidemiological studies have been conducted across the globe to ascertain the disease burden and the possible risk factors. A study that carried out a meta-analysis of data of PD from 1985-2010 observed an increase in PD prevalence globally. The study reported prevalence data as a number of PD cases per 100,000 individuals. **Table 1.3** provides summaries of prevalence studies of PD from (1985-2010) by age, sex and geographic location.

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Table 2.3: A summary of incidence studies of PD by age, sex and geographic location

Age group	Crude incidence of PD Number of cases/100,000	Incidence of PD by geographical location (N/100,000)			Incidence of PD by sex (N/100,000)	
		Asia	SA	N.A/ Euro /AUS	Female	Male
40-49	41	-	-	-	45	36
50-59	107	88	228	113	41	134
55-64	173	-	-	-	150	233
60-69	428	376	637	540	392	389
65-74	425	-	-	-	610	706
70-79	1087	646	2180	1602	813	932
>80	1903	148	6095	2953	1517	2,101

Table 2.3 was modified from (Pringsheim et al., 2014). (SA)- South America, (N.A)-North America, (Euro)-Europe, (AUS)-Australia.

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Crude prevalence studies by geographic location indicated that PD occurrence was relatively high in South America, North America, Europe, Australia and Asia (Pringsheim et al., 2014). Also, males between the age of 50–59 years had a significantly higher in prevalence of PD of 134/100,000 as compared to females, who had a prevalence of PD of 41/ 100,000 (Pringsheim et al., 2014), suggesting that males are more often affected by PD than females.

1.8.1 PD prevalence studies in sub-Saharan African countries

Prevalence studies on PD in sub-Saharan Africa (SSA) are sparse. Studies from 1982-2006, found only 7 reported cases of PD/100,000 in Ethiopia (Tekle-Haimanot et al., 1990), 10/100,000 in Nigeria (Osuntokun et al., 1987), 20/100000 in Togo (Balogou et al., 2001) and 20/1000 patients in Tanzania (Dotchin et al., 2008a). Still, it is difficult to compare data on prevalence of PD in Africa with other continents.

A study in Tanzania demonstrated that the prevalence of PD in that country is predicted to increase by an alarming 184% by the year 2025 (Dotchin et al., 2012), surpassing the corresponding 92% increase projected for Canada and the USA (Bach et al., 2011).

In Nigeria, age-adjusted PD prevalence rates range from 10 to 67 per 100,000 for community-based studies (Osuntokun et al., 1987; Schoenberg et al., 1988), whereas hospital-based studies report rates of 4.1 to 59.4/ 1000 of all neurological consultations (Ekenze et al., 2010a; Okubadejo et al., 2010a). It was reported in 2014 that roughly 90,000 Nigerians suffer from PD. Epidemiological studies in South Africa are also limited. The prevalence rate for PD from one hospital-based study was ~5/1000 in South African population (Cosnett and Bill, 1988) and 5.3/1000 among South Africans (White, Mixed ancestry, Black and Indian) in a cross sectional study (Cosnett and Bill, 1988). Although, broad prevalence studies on PD in SSA are sparse, the aforementioned evidence is contrary to the view that PD is a disease associated with ethnicity or that it occurs predominantly in the Western countries.

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The population in SSA is growing more rapidly and also ageing more rapidly. The prevalence of PD in SSA is set to increase significantly. A recent study projected that SSA populations will increase significantly in age **(Figure 1.9)**.

This has important implications for PD studies in SSA, especially in the rapidly increasing population countries like South Africa and Nigeria. Similarly, because PD could indirectly affect the socio-economic growth of a particular country by increasing disabilities among the middle-aged and old-aged people and will ultimately reduce their workforce ability. In Africa, based on the theory that increasing industrialization and environmental toxins could predispose people to PD, and since South Africa and Nigeria probably are the two most industrialized countries in the SSA with a large workforce, it is likely that PD prevalence will increase drastically.

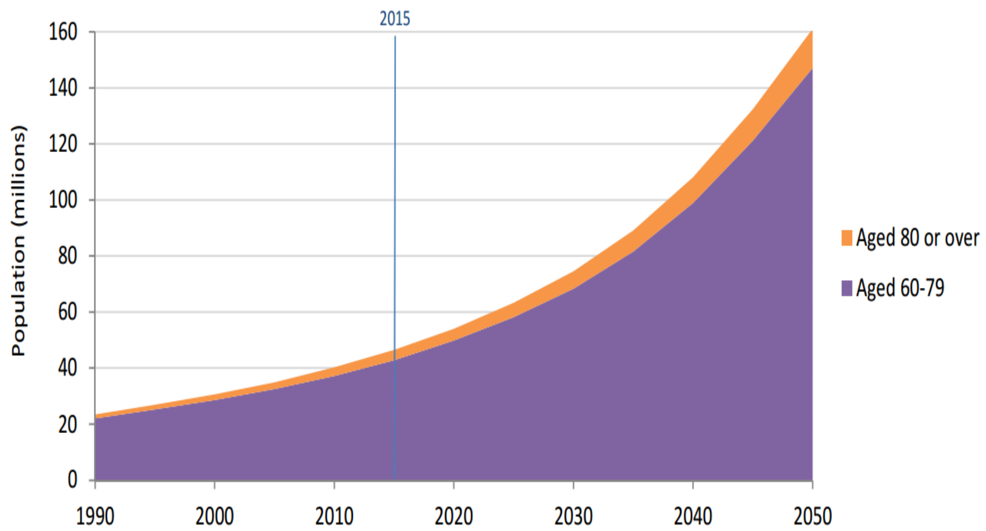

Figure 1.9: Population aged 60-79 years and 80 years or over in SSA, 1990-2050. United Nations, Department of Economic and Social Affairs. Population Division (2015). World Population Prospects: The 2015 Revision. http://www.un.org/en/development/desa/population/publications/pdf/popfacts/PopFacts_2016-1.pdf Reprinted with the permission of the United Nations."

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1.9 Analysis tools for studying genetic diseases

1.9.1 Next-generation sequencing platforms

Next-Generation Sequencing (NGS) technique is an emerging tool that provides a way to explore the genetic basis of various diseases including movement disorders such as PD and has resulted in the discovery of a number of disease-causing genetic variants. NGS is a rapid, robust, and cost-effective high throughput parallel sequencing. Utilization of NGS to identify specific changes in DNA by rapidly and simultaneously sequencing multiple gene targets within multiple samples is making sequencing studies much better. Conventional methods of sequencing like the Sanger sequencing are not efficient for a large-scale rapid sequencing study. The cost of buying the reagents required to sequence a hundred samples using Sanger method might be too expensive for this type of study. Hence NGS was chosen for this study. In contrast to the whole-genome sequencing, a targeted DNA panel is designed to capture and amplify a specific area of interest in the genome.

The key to a targeted sequencing is to amplify genomic regions of interest using PCR and specific sets of pooled primers. Before now, the tasks of designing such primers and optimizing PCR conditions were labour-intensive and time-consuming. Targeted NGS (tNGS) gene-panel tests have also given the field of genomics an edge in identifying causative genes and variants, providing a faster pipeline with a high diagnostic yield in clinical settings. For heterogeneous disorders like PD, tNGS provides higher coverage for known disease-associated genes and can be flexible in including suspected genes in the targeted library based on patient phenotype.

Each sequencing platform however has advantages and disadvantages **(Table 1.4)**. When considering a large-scale sequencing study with accuracy, an NGS approach will be most suitable. Generally, Illumina instruments are used more widely than any other NGS technology, but such broad use of a single technology may introduce systemic bias, especially in variant identification (Chakravorty *et al* 2017).

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Table 1.4: Comparison between the different sequencing platforms.

Platform	Ion Torrent sequencing	Pacific Bio	Pyro-sequencing	Illumina	Sequencing by ligation (SOLiD)	Sanger sequencing
Insert size	250 bp	2900 bp	700 bp	700 bp	50 bp	400 bp
Accuracy (%)	99.4	99	99.9	99.2	99.9	99.9
Error rate (%)	1.17	12.86	N/A	0.80	N/A	0.5
Average reads per run	>5 million	<1million	~1 million	>100 million	>100 million	N/A
Duration	2 hours	2 hours	24 hours	~2 days	~1 week	3 hours
Advantages	Fast & less expensive	Longest read length	Long read size, fast	Potential for high sequence yield	Low cost per base	Most acurate. Useful for many applications
Disadvantages	Homo-polymer errors	Low yield at high accuracy. Expensive equipment	Expensive Homo-polymer errors	N/A	Longer duration than other platforms	Expensive and impractical for larger-scale sequencing projects

Table 1.4 was modified from the (Quail *et al* 2012) study. N/A-Not applicable.

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An Ion Torrent sequencing with a customized Ion AmpliSeq™ designed panel has been used to effectively study sporadic cases of PD and was shown to be a powerful method to screen for mutations in PD patients (Gorostidi et al 2016). In this study a customized panel of genes was available to screen for mutations in genes that are mostly linked to neurological diseases in people.

In the current study, the Ion AmpliSeq™ Neurological Research Panel (Thermo Fisher Scientific) was used for targeted next-generation sequencing (tNGS), it is a commercially available panel for the screening of 751 genes, including 17 of the 34 known PD genes. The panel produces an average base coverage of 165X, 97% reads on target and 99.4% average gene coverage (www.ampliseq.com). Only 10 ng of DNA is needed to generate mutational profiles.

1.9.1.1 Next-Generation Sequencing on Ion Torrent Personal Genomic Machine

Ion Torrent library construction includes DNA fragmentation, hybridization of primer sequences, ligation of adapters, and library purification. Sequence template preparation can be performed manually or by use of an OneTouch™ Machine (Life Technologies). Template preparation is carried out using an emulsion PCR and enrichment system on Ion Spheres™ Particles (ISPs). The ISPs have covalently linked complementary adapter sequences on their surfaces to facilitate amplification on the particles.

Enriched particles are primed for sequencing by annealing a sequencing primer and are then loaded into the wells of an Ion Chip **(Figure 1.10).** The Ion sphere particles with the amplified library can be loaded on the Ion torrent Chip 540 for bidirectional sequencing of amplicons on the Ion S5 Personal Genomic Machine (PGM). The sequencer combines semiconductor-sequencing technology with basic biochemistry to directly translate chemical information into digital data. This eliminates the need for expensive optics, lasers, and complex sequencing chemistries with fluorescently labelled nucleotides.

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Normally, there is a release of a proton when a nucleotide is incorporated by the polymerase in the DNA molecule, and the process will lead to a change in pH. The semiconductor sequencing chip contains million copies of the amplified barcoded beads. The Ion PGM depending on the chip used, can generate to 5.5 million reads, with an output reaching 2 Gb, a reading length up to 400 bases, and a run time between 2 to 7 hour.

The Ion PGM sequencer successively flow-in one nucleotide after another in the chip. To show that one nucleotide complements the sequence of the DNA molecule in a micro-well and has been incorporated, hydrogen ions will be released. The release of hydrogen ions makes a change in pH in the solution that can be detected by the ion sensor (**Figure 1.10**). But if there are two identical bases on the DNA strand, the voltage will be doubled, and the chip will record two identical bases being called. If a nucleotide does not match in a well, no voltage change is recorded and no base will be called. After, standard file formats for the analysis are produced.

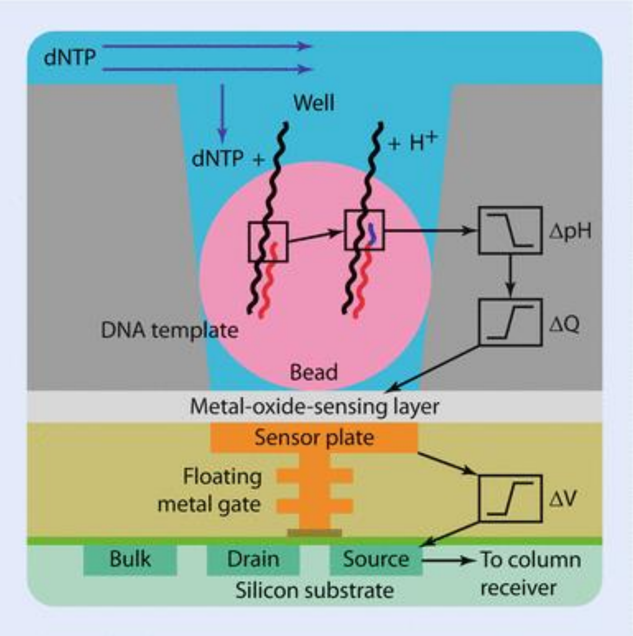

Figure 1.10: Ion Torrent library preparation and sequencing principle in a single well in a sequencing chip. The dNTP is incorporated complementary to the nucleotide to start a reaction that leads to the base calling. Permission from Springer Nature.

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1.9.1.2 Base Calling and Map reading

The binary alignment mapping (BAM) containing raw reads can be generated after sequencing. Base calling and mapping of the raw reads with the hg19 reference sequence can be carried out on Ion Torrent Suite™ Software 5.2. The beginning steps of the Torrent Suite™ Software 5.2 analysis pipeline are described in **Figure 1:11.**

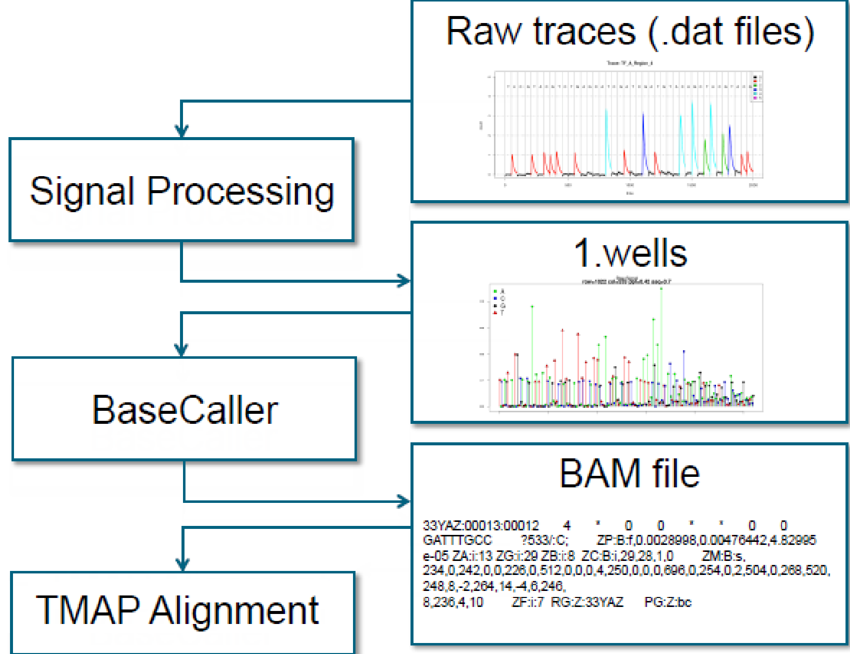

Figure 1.11: The Torrent Mapping Alignment Program (TMAP). It is a sequence alignment software program optimized specifically for Ion Torrent™ data. TMAP contains several mapping algorithms and is recommended for the Ion Torrent suite software 5.10. Taken from http://129.130.90.13/ion-docs/GUID-D13D3B43-FB55-43FD-ADE0-5EC4170FB49C.html Permission from Thermo Fisher Scientific

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1.9.1.3 Steps involved in the Ion Torrent sequencing

Ion Torrent sequencing requires the following steps (**Figure 1.11**)

- The sequencing instrument generates Data (DAT) files of electrical signals' raw traces.
- The signal-processing step converts the raw traces into a single number per flow per well.
- The BaseCaller converts the file information of each well into a sequence of bases and writes the sequence into an unaligned BAM file.
- The BAM file is passed to TMAP for alignment.

The signal-processing steps also mark several types of low-quality reads. These include polyclonal reads which are reads with two template beads instead of one, reads with high signal processing residual indicating an ambiguous signal value and reads that do not contain a valid library. The BaseCaller module also performs read filtering and read trimming.

1.9.2 Variant Calling and Annotation

Ion Reporter™ Software 5.4 is used to annotate variants. The software includes a set of bioinformatics tools for the data analysis, annotation, and translating of sequencing data. Specialized custom analysis and workflow plug-ins are applied for complex sequencing results. To achieve confidence in the NGS sequencing data, certain criteria are looked for in the data **(Table 1.5).** The American College of Medical. Genetics and Genomics and the US Centers for Disease Control and Prevention proposed guidelines for NGS studies and bioinformatics pipelines to produce quality genomic studies. These guidelines address test validation, quality control, proficiency testing, and reference materials. Quality metrics such as QUAL scores, depth, coverage uniformity, mapping quality, and GC bias should be considered in an NGS study (Chakravorty *et al* 2017).

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A comprehensive annotation of coding and noncoding regions and accurate variant classifications are crucial in NGS studies. When genome annotation pipelines have classified variants that are discovered in sequencing, it is necessary to understand each variant identified based on their functions. In addition, variants in other structural domains of genes can also be important for gene function and have regulatory roles in expression.

Table 1.5: Benchmarks used to evaluate sequencing quality in tNGS.

Indicator	Description
Number of mapped reads	Total number of reads mapped to the reference.
Number of reads on target	Total number of reads mapped to any targeted region of the reference.
Target base coverage	A base that is covered by multiple target regions counted once per sequencing read.
Bases in target regions	The total number of bases in all specified target regions of the reference.
Percent of reads on target	The percentage of reads mapped to any targeted region relative to all reads mapped to the reference.
Total aligned base reads	The total number of bases covered by reads aligned to the reference.
Total base reads on target	The total number of target bases covered by any number of aligned reads.

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Indicator	Description
Average base coverage depth	The average number of reads of all targeted reference bases.
Uniformity of base coverage	The percentage of bases in all targeted regions covered by at least 0.2x the average base coverage depth.
Average base read depth	The average number of reads of all targeted reference bases that were read at least one time.
Number of amplicons	The number of amplicons specified in the target regions file.
Uniformity of amplicon coverage	The percentage of bases in all targeted regions covered by at least 0.2x the average base read depth.
Amplicons with no strand bias	The percentage of all amplicons that did not show a bias towards forward or reverse strand read alignments.
Amplicons reading end-to-end	The percentage of all amplicons that were considered to have a sufficient proportion of assigned reads (70%) that covered the whole amplicon target from end-to-end.

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Table 1.6: Types of variants according to the functional annotation.

Annotation	Description
Frameshift insertion	A insertion of one or more nucleotides that causes a frameshift in protein coding sequence.
Frameshift deletion	A deletion of one or more nucleotides that causes a frameshift in protein coding sequence.
Frameshift block substitution	A block substitution of one or more nucleotides that causes a frameshift in protein coding sequence
Stopgain	A nonsynonymous SNV, frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that leads to the immediate creation of a stop codon at the variant site. For frameshift mutations, the creation of stop codon downstream of the variant will not be counted as a stop gain.
Stoploss	A nonsynonymous SNV, frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that leads to the immediate elimination of a stop codon at the variant site

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Nonframeshift insertion	A insertion of 3 or multiples of 3 nucleotides that do not cause frameshift in protein coding sequence
Nonframeshift deletion	A deletion of 3 or multiples of 3 nucleotides that do not cause frameshift in protein coding sequence
Nonframeshift block substitution	A block substitution of one or more nucleotides that do not cause frameshift in protein coding sequence
Nonsynonymous SNV	A single nucleotide change that cause an amino acid change
Synonymous SNV	A single nucleotide change that does not cause an amino acid change
Unknown	Unknown function (due to various errors in the gene structure definition in the database file)

Source: http://annovar.openbioinformatics.org/en/latest/user-guide/gene/

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1.9.3 *In silico* pathogenicity tools

The impact of variants identified in a genetic study can be determined by computational approaches, or *in silico* testing. The purpose is to predict and possibly interpret functionally, the roles of a variant in a disease pathology. It is essential to understand how a gene that harbours a particular variant is relevant to a particular disease. Various *in silico* tools using different implementations and combinations of features have now been developed (**Appendix III**). These *in silico* tools are generally based on: The analyses of sequence conservation at the position of a missense substitution, which is measured from a protein multiple sequence alignment. The severity of a missense substitution with respect to the observed range of variation at its position in an alignment, and structural features of the wild-type and variant proteins are determined (Bryony et al 2013).

Widely used *in silico* tools for annotations of variants.

- SIFT (Sorting Intolerant From Tolerant): is an algorithm which predicts whether an amino acid substitution will affect protein function based on sequence homology and the physical properties of amino acids. A SIFT score of less than 0.05 is predicted to be deleterious. A substitution with a score greater than or equal to 0.05 is predicted to be tolerated (www.sift.jcvi.org/).
- PolyPhen2: is a tool that predicts possible impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations. Structural features such as amino acid atomic contacts and solvent accessibility are also assessed and empirically determined cut-offs used to predict if the substitution is probably damaging, possibly damaging or 'benign' (www.genetics.bwh.harvard.edu/pph2/).
- Grantham score: is a prediction of the effect of substitutions between amino acids based on chemical properties, including polarity and molecular volume, characterised into classes of increasing chemical dissimilarity: conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151).

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- Combined Annotation Dependent Depletion (CADD): is a tool for scoring the deleteriousness of single nucleotide variants as well as insertion/deletion variants in the human genome. CADD can quantitatively prioritize functional, deleterious, and disease causal variants across a wide range of functional categories, effect sizes and genetic architectures and can be used to prioritize causal variation in both research and clinical settings. C-scores strongly correlate with allelic diversity, pathogenicity of both coding and non-coding variants, and experimentally measured regulatory effects, and also highly rank causal variants within individual genome sequence (www.cadd.gs.washington.edu/).

- LRT score: The likelihood ratio test (LRT) is a statistical test of the goodness-of-fit between two models. A relatively more complex model is compared to a simpler model to see if it fits a particular dataset significantly better. If so, the additional parameters of the more complex model are often used in subsequent analyses. The LRT is only valid if used to compare hierarchically nested models (www.evomics.org/resources/likelihood-ratio-test/).

- MutationTaster score: is a web-based application to evaluate DNA sequence variants for their disease-causing potential. The software performs a battery of *in silico* tests including amino acid substitutions, conservation of amino acid, loss of function, effects on slicing and other regulatory region, to estimate the impact of the variant on the gene product/protein. Tests are performed on both, protein and DNA level (www.mutationtaster.org/).

MutationAssessor: predicts the functional impact of amino-acid substitutions in proteins. The functional impact is assessed based on evolutionary conservation of the affected amino acid in protein homologs. The method has been validated on a large set (60k) of disease associated Online Mendelian Inheritance in Man (OMIM) and polymorphic variants (www.mutationassessor.org).

- FATHMM score: is capable of predicting the functional effects of protein missense mutations by combining sequence conservation within hidden Markov models representing the alignment of homologous sequences and conserved protein domains, with "pathogenicity weights" representing the overall tolerance of the protein/domain to mutations (www.fathmm.biocompute.org.uk/).

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1.9.4 High Resolution Melt (HRM) Test

High Resolution Melting (HRM) can be used to screen for genetic variants. HRM is a cost effective, simple and fast method for DNA analysis. HRM analysis is based on DNA melting (dissociation) that is observed at the denaturing stage of a PCR. HRM can detect small sequence variations just by direct melting of the DNA double strands. HRM is a post-PCR method. The region of interest within the DNA sequence is first amplified using PCR and specific oligonucleotides. An intercalating dye (SYTO9) is added to the reaction and as the amplicons were being produced, the dye is incorporated. The dye fluoresces only in the presence of double-stranded DNA.

The amplicons can be heated from 50°C to 95°C applying holds at a time-interval. When the temperature is increasing, at a certain melting temperature the sample DNA will start to denature, leading to a separation of the DNA strands to a double stranded DNA. The melting temperature depends on the sequence context of the DNA fragments. If a DNA fragment is rich in high GC content, it will melt at higher temperature than a DNA fragment rich in high AT content. At a point where the DNA fragment has been separated into a double strand, fluorescence is released, and it is plotted showing the level of fluorescence vs the temperature, generating a melting curve. This HRM melting curve can show the differences, even if a single base is changed in the DNA sample sequence. The melting curves generated after HRM analysis are plotted with fluorescence on the Y axis and temperature on the X axis **(Figure 1.12 and Figure 1.13)**.

The limitation of HRM analysis is that it will not determine the sequence change present in the DNA fragment. It merely indicates that the DNA sequence is likely to be different with different melting profiles. Sanger sequencing is required to confirm that and to identify the sequence change responsible for the difference in melting profiles.

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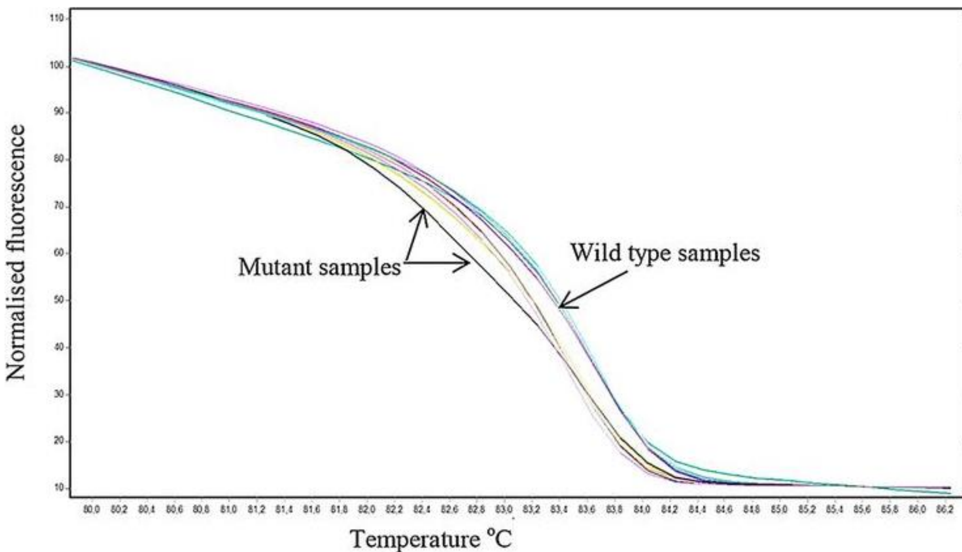

Figure 1.12: Example of a HRM standardized graph. The temperature usually is set at a range of 55–95ºC. There is a correlation between the fluorescence and temperature, allowing identification of variation between different wild type samples and mutant samples respectively. Taken from (Serban et al 2014). Permission by BMJ Publishing Group Ltd.

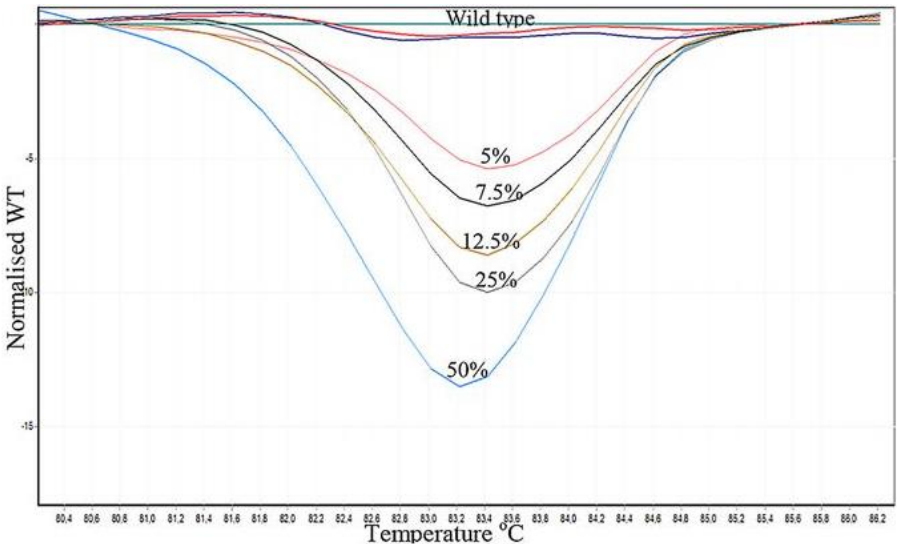

Figure 1.13: Example of a HRM difference graph. This graph is used when a particular genotype is identified and used as a reference/baseline for the other DNA samples. The position of each sample relative to the reference is plotted against the temperature thereby showing differences between various samples. Taken from (Serban et al 2014). Permission by BMJ Publishing Group Ltd.

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1.9.5 Detection of Copy number Variations (CNVs)

1.9.51 Mechanisms CNVs

Changes in the structure of chromosomes occur by two general mechanisms, homologous recombination (HR) and nonhomologous recombination. HR is used in repair of DNA breaks and gaps. There will be no change in structure if a damaged sequence is repaired using homologous sequence in the same chromosomal position in the sister chromosome or homologue, but repair might utilize homologous sequences in different chromosomal positions. This is called non-allelic and could change the structure of the chromosome. The correct choice of recombination partner prevents chromosomal structural change.

Non-allelic homologous recombination (NAHR) occurs if a recombination repair event utilizies a direct repeat as homology, resulting to products that are reciprocally duplicated and deleted for sequence between the repeats. These might segregate from each other at the next cell division, thus changing the copy number in both daughter cells (Hastings et al., 2009), **(Figure 1.14).**

The best studied mechanism of HR is double-strand break (DSB)-induced recombination. The HR-double-strand break repair mechanisms occurs at the break, where 5′ ends are resected to leave 3′ overhanging tails. These catalyze the invasion by one or both ends into homologous sequence forming a D-loop. The 3′ end then primes DNA synthesis that extends and past the position of the original break (Hastings et al., 2009). The second end is incorporated into the D-loop by annealing, and is also extended. Following ligation, which forms a double Holliday junction, the junctions are resolved by endonuclease. The overall effect will be either a non-crossover or a crossover, depending upon whether the two junctions are resolved in the same or different orientations (Hastings et al., 2009), **(Figure 1.14).**

Similarly, the presence of microhomology at a site of nonhomologous recombination has been regarded as the signature of nonhomologous end joining (NHEJ). The NHEJ is like DSB, NHEJ rejoins DSB ends accurately or leads to small 1-4 bp deletions, and also in some cases to insertion of free DNA (Hastings et al 2009; La Cognata et al 2017)**.** It is likely that NHEJ contributes to some chromosomal rearrangements by joining nonhomologous sequences.

The Fork stalling and template switching (FoSTeS) mechanism is proposed to occur in cells under stress. A free single-stranded lagging template might acquire secondary structures, which could block the progress of the replicating fork. The 3′ ends then become free from their templates and might then align to other free single-stranded-template sequence on another replicating fork that shares microhomology, resulting to rearrangement such as duplication, deletion, inversion or translocation depending on the position of the other replicating fork (Hastings et al 2009; La Cognata et al 2017). Fork stalling can be caused by other situations, such as lesions in the template strand or shortage of deoxynucleotide triphosphates (Hastings et al 2009; La Cognata et al 2017), **(Figure 1.14).**

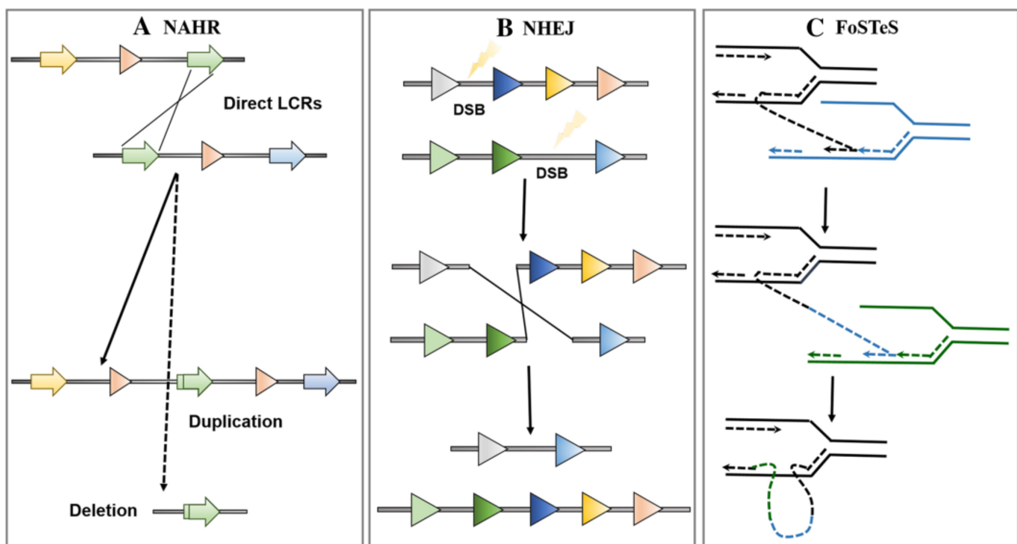

Figure 1.14: Schematic illustration of the three most common events causing genomic rearrangements. A. NAHR generates CNVs when genomic segments with high sequence similarity (direct low-copy repeats sequences, green arrows) recombine. This recombination can generate a duplication of the similar locus (red arrow) on one chromosome, while removing the copy from the other.

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B. Double stranded breaks (DBS) in DNA sequence recruit NHEJ-associated proteins to repair and ligate DNA strands together. First, end-repair protein replaces lost nucleotides on the double-strand break and DNA ligase associates broken DNA fragments together. If fragments from different chromosomes ligate together, duplications or deletions of sequence can occur. **C.** After the original stalling of the replication fork (black line), the lagging strand disengages and anneals to a second fork (blue line), followed by extension of the now 'primed' second fork and DNA synthesis. After the fork disengages, the tethered original fork with its lagging strand (black and blue lines) could invade a third fork (green line). Serial replication fork disengaging and lagging strand invasion could occur several times (e.g., FoSTeS × 2, FoSTeS × 3, etc.) before resumption of replication on the original template. It should be noted that the CNVs created through FoSTeS are difficult to be distinguished from those generated by microhomology- mediated breakpoint-induced repair (MMBIR), a mechanism of end-joining that relies on small-scale homology of DNA sequence at the ends of DSBs. Taken from (La Cognata et al 2017) licenced under creative common (http://creativecommons.org/licenses/by/4.0/)

Mainly, these mechanisms contribute to the deletions and duplications of chromosomal segments and could affect about 12% of human genome leading to changes in the levels of expression of genes, a redundancy in sequence, so that some copies are free to evolve new or modified functions or patterns of expression, while other copies maintain the original function.

This likelihood allow reassorting of exons between different genes by translocation, insertion or deletion, so that proteins might acquire new domains, and hence new or modified activities (Frith and Khan, 2018; La Cognata et al., 2017).

1.9.5.2 Multiplex Ligation-Dependent Probe Amplification (MLPA)

MLPA is a multiplex PCR method, it is simple and robust and can be used for identifying chromosomal DNA copy number changes in many targets. MLPA requires a thermocycler and capillary electrophoresis apparatus. MLPA experiment can simultaneously be carried out in about 96 samples and provide the results within 24 hours.

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Amplification of a probe is dependent on a ligation process, which can only occur if target sequence is present in the DNA sample. Two adjacent probes are designed that contain forward and reverse primer sequence respectively for a short sequence of target DNA (**Figure 1:15**). In addition, one of the two probes contain a stuffer sequence the length of which can be varied. The probes are hybridized to the target DNA and subsequently ligated. Only if ligation occurred, a functional PCR product is produced, so that amplification only happens if target sequence is present in the DNA sample. The amount of PCR product is proportional to the amount of target sequence present in the sample, making the technique suitable for quantitative measurements.

The importation and pre-processing of MLPA raw data utilizes an automated workflow suitable for genetic profile analysis, that begin with raw sequencer chromatogram files or pre-processed peak tables. The multiple probe mixes can then be combined for each probe mix, the MLPA setup can be entered initially in the software including the dye setup, this strategy for automated name and information parsing, and selection of the size standard used. Peaks on the samples are assigned automatically to known probes based upon the size information provided in the probe mix definitions (Applied Biosystems and Beckman MLPA manual).

The MLPA (PO51 and PO52) kit detects CNVs in PD genes (https://www.mlpa.com). It consists of two different kits, each with a separate probe mix. These probe mixes contain oligonucleotides for ligation to the exons of genes known to cause PD, as well as reference probes. The SALSA MLPA P051 (MRC Holland, Netherlands) is designed to amplify exons of *SNCA*, *LRRK2, PRKN, PINK1, PARK7* and *ATP13A2*. SALSA MLPA P052 (MRC Holland, Netherlands) amplifies exons of *PRKN, UCHL1, GCH1, CAV1* and *LRRK2*. The genes included on the SALSA MLPA P051 kit are genes with previously reported CNVs in PD patients.

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The raw data are analysed using the Coffalyser.Net software, version 131211 (http://coffalyser.software.informer.com/download/). The fragment analysis and comparative analysis can be done using the Coffalyser software. The software displays peak signals and size markers for the electropherogram analyses to be differentiated (**Figure 1:15**). Raw data files (ABI files, .fsa format) are exported in Coffalyser where analyses can be done, the process requires some standardization methods as described in the Coffalyser manual.

The analysis results have different ranges of values that can be interpreted as heterozygous or homozygous deletions or duplications. MLPA peak detection can vary in height. The following guidelines are used for the interpretation of results:

- Peak height values between 0.7 and 1.3 are considered to represent results of samples without a deletion or exonic rearrangements
- Values between 0.3 and 0.6 indicate a heterozygous deletion
- Values between 1.4 and 1.6 indicate a heterozygous duplication
- Values higher than or equal to 1.7 indicate a triplication
- Absence of a peak indicates either a possible point mutation at that probe recognition site or a homozygous deletion (ratio 0.0).

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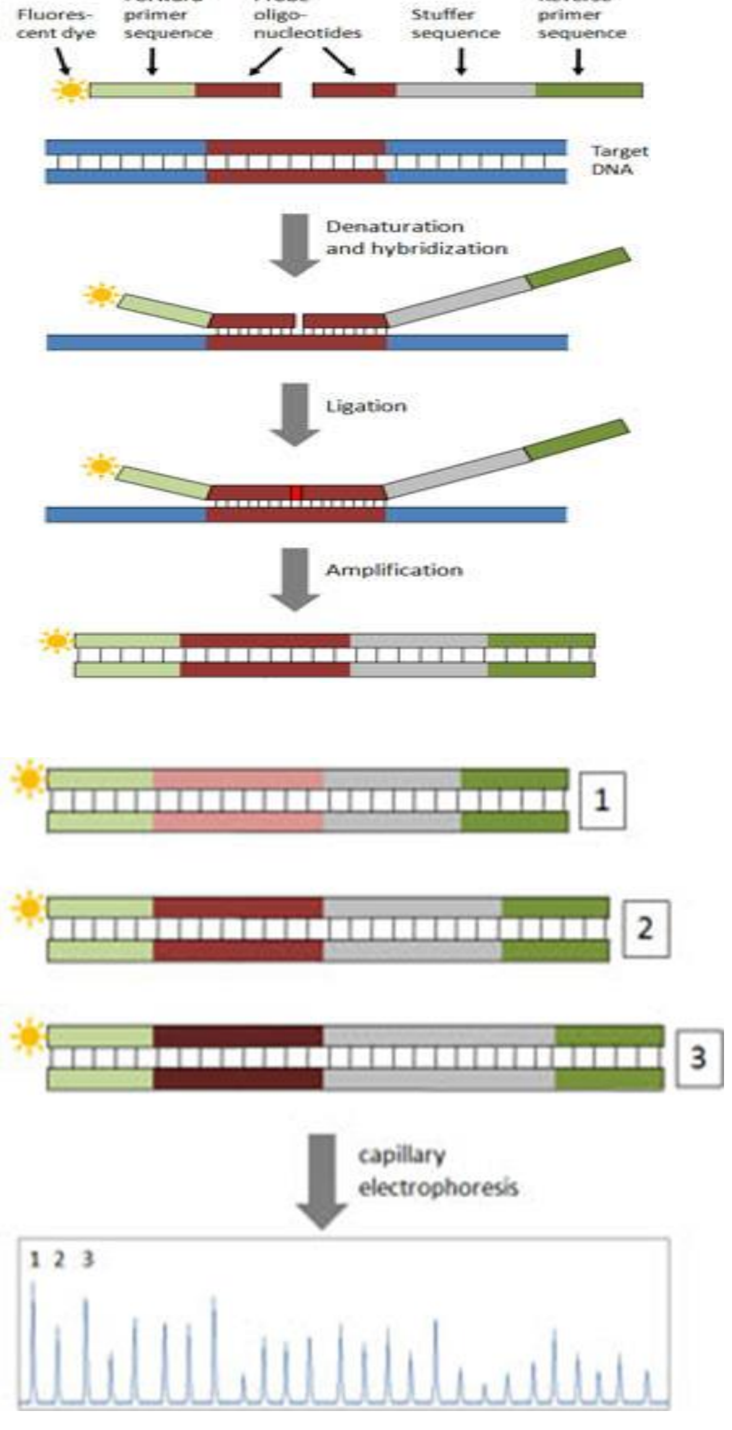

Figure: 1:15: Description of the MLPA processes showing hybridization of probes and capillary electrophoresis signal peaks. Permission from Applied Maths NV, a bioMérieux company Sint-Martens-Latem, Belgium. https://download.appliedmaths.com/sites/default/files/img/lpa1.jpg

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1.10 Study Rationale

The challenges of a genetic PD study in SSA are enormous. Many SSA PD patients do not have access to quality health services. The majority are thought to be undiagnosed and untreated, leading to poor health outcomes. Increasing interests to study the genetics and epidemiology of PD in SSA are advancing and increasing the rates of correct diagnosis. The number of people with PD are set to increase in the SSA. The contributions of genetics to the study of PD in SSA could enhance our understanding of the disease mechanisms. Hence, in this study, a number of different techniques were used, including NGS, MLPA and HRM. The principles of these techniques used in the present study were discussed in section 1.9.

1.10.1 Diversity of SSA population

The DNA in the human genome are constantly changing and this process allows humans to evolve and adapt. The little changes that occur in the genome of an individual contribute largely to the observable phenotypes and may have an impact on disease pathogenesis or responses to treatments. It has been established that mankind evolved from Africa thousands of years ago, and as the earth changed, people began to migrate to all directions. Studies have shown that African ancestry populations are greatly admixed **(Figure 1.16)**. Many factors contributed to this genetic diversity in Africa, which includes migrations, intermarriages, and a genetic bottleneck that has occurred in some environments in the past. African populations have the greatest genomic diversity in the world, and this diversity has important ramifications for genomic research (Rotimi et al 2007). Even among the African populations, the haplotype blocks differ. The functional effects of a genetic variant could have major consequences relating to the pharmacogenomics studies of treatments. The causes of drug treatment idiosyncrasy in people have been the central focus of the pharmacogenomics studies that depend mostly on the understanding of genetics of individuals.

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The observed genetic diversities in Africans warrant further investigations. This is necessary and could potentially explain PD mechanisms in them. Furthermore, the implications of the genetic diversity might lead to the development of specific medications that will be suitable for a particular group of people.

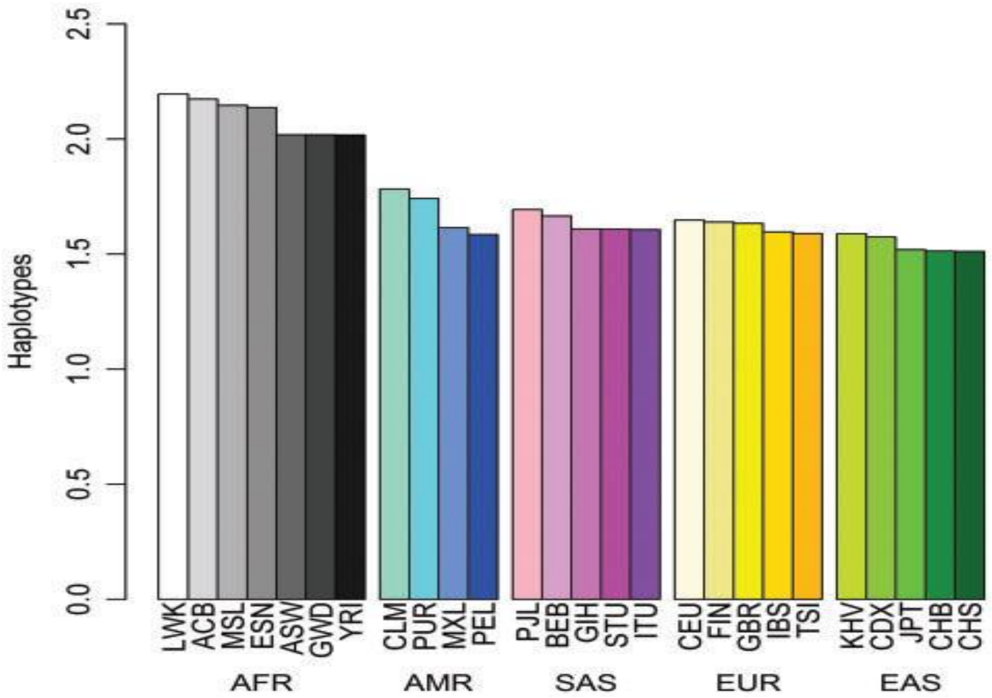

Figure 1.16: Haplotype differences across different ethnicities in a 1000 Genomes Project. African populations have a highest level of haplotype blocks compared to other populations. Taken from Rotimi et al 2018. Permission from Oxford University Press.

Nearly all diseases have ethnic variations in occurrence. It can be said that racial groups are constantly undergoing evolutionary change. Polymorphic genetic markers may be linked to certain groups of people, depending also on geographic locations. Genetic variations such as single nucleotide polymorphisms (SNPs), CNVs, multiple nucleotide variations (MNVs), INDELs, RNA splice site mutations and large genomic rearrangements are often studied as genetic causes of a disease.

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These genetic factors are inheritable and could affect the functionality of a gene. To understand the genetics of PD in SSA, especially in admixed populations of Black origin, the aforementioned genetic markers should be considered. Common variants discovered in PD so far are mostly single nucleotide variations (SNVs), small deletions (less than 20 bp), CNVs and MNVs. Rarely had PD been associated with any form of chromosome defects in patients.

The mutations in PD-causing genes have been predominantly identified and studied in European, North American, North African and Asian populations. Most of these mutations identified in PD and were replicated are considered for genetic testing in a clinical setting. It is possible that the information from other populations may not be transferable to the SSA populations.

1.10.2 Limited genetic studies on Black South African and Nigerian patients with PD

Little is known about the genetics of Black South African and Nigerian PD patients. There are several challenges with genetic studies of PD in SSA. These include relatively small sample sizes, partly because PD patients are reluctant to go to the neurological clinic to aid their diagnoses and treatments. They try to avoid stigmatization because people still believe that PD is a mysterious disease created by some divination. The lack of genetic studies is also due to lack of funding and resources for genetic studies in SSA countries.

In SSA, little is known about the genetics of PD. One study screened for pathogenic mutations in *LRRK2* in Ghanaian PD patients (Cilia et al 2012) and found no mutations, similarly, another study looked at *PRKN* mutations in Zambian PD patients (Yonova-Doing et al 2012), and found exon 2 and 4 deletions; and another study that screened for *PINK1* mutations in a Sudanese PD family (Cazeneuve et al 2009) found a homozygous variant in *PINK1* gene that segregated with PD in the family.

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The previous genetic studies in South African and Nigerian PD patients (**Table 1.7**) suggested that the genetic causes of PD in SSA might be unique. Although, sporadic and familial cases of PD exist in both in South Africa and Nigeria, more studies are needed to investigate the interplay of genetics and the environmental factors in PD in both countries. The projection that there will be an increase in the prevalence of this disorder worldwide, including SSA countries, means that comprehensive genetic studies are urgently needed.

. The first study described the results of 57 Nigerian PD patients screened for pathogenic mutations in the known PD genes (Okubadejo et al., 2008).

The second study utilized Human Genome Diversity Cell Line Panel for screening coding variants in the *EIF4G1* gene in Nigerian controls (Cann et al., 2002; Tucci et al., 2012).

Pathogenic mutations were found in three Black South African PD patients, all with mutations in the *PRKN* gene (Blanckenberg et al., 2013). However, mutations in the *SNCA*, *LRRK2, VPS35, EIF4G*1 or *CHCHD2,* leading to PD with an autosomal dominant inheritance or in *PINK1, PARK7*, or *ATP13A2,* associated with PD with autosomal recessive inheritance were not identified.

Similarly, some other genes like those that control the functions of the brain are yet to be associated with causative PD mutations in the Black South African or Nigerian patients. It is likely that some of the genes that have not been studied have important roles in PD.

Consequently, this project was intended to identify novel disease-causing genes and novel disease-causing mutations in the PD genes in the South African and Nigerian PD patients. By collaborating with expert neurologists in Nigeria and South Africa, well characterized PD patients were identified and recruited for a comprehensive genetic study. High-throughput NGS techniques were considered as the best tools for sequencing.

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Table 1.7: A summary of genetic studies in Black South Africans and Nigerians PD patients.

Autosomal dominant genes	Country	No.of Patients	Screened (Methods)	Result	Reference
LRRK2	Nigeria	57	Exons 31 and 41	No mutation	(Okubadejo et al., 2008a)
SNCA	South Africa	12	All 6 exons +A30P mutation (Exon dosage-MLPA)	No mutation	(Keyser et al., 2010a)
VPS35	South Africa	18	D620N mutation (CASP assay)	No mutation	(Blanckenberg et al., 2014a)
EIF4G1	South Africa Nigeria	18 26	R1205H mutation (CASP assay) Exons 8 and 22 qRT-PCR	No mutation No mutation	(Blanckenberg et al., 2014a) (Tucci et al., 2012b)
Autosomal recessive genes	**Country**	**No.of Patients**	**Screened (Methods)**	**Result**	**Reference**
DJ-1	South Africa	12	Exons 1,3,5&7 (Exon dosage-MLPA)	No mutation	(Keyser et al., 2010a)
PRKN	Nigeria	57	All 12 exons	No mutation	(Okubadejo et al., 2008a)
PRKN	South Africa	17	All 12 exons (SSCP and HRM)	Duplication and deletion of exon 2 and exon 9, respectively in two affected siblings.	(Bardien et al., 2009) (Haylett et al., 2012a)

				Heterozygous G430D mutaton and deleten of exon 4 in another probad	
PRKN	South Africa	21	All 12 exons MLPA assay and qRT-PCR	A single heterozygous exon 4 deletion in *PRKN* found in one proband	(van der Merwe et al., 2016)
PINK 1	South Afica	12	All 8 exons (Exon dosage-MLPA)	No mutation	(Keyser et al., 2010a)
PINK 1	South Afica	18	All 8 exons (Exon dosage-MLPA)	No mutation	(Keyser et al., 2010b)

HRM, high resolution melt; MLPA, multiplex ligation-dependent probe amplification. SSCP, single-strand conformation polymorphism, KASP assay, *Competitive* Allele Specific PCR. qRT-PCR, quantitative reverse transcriptase PCR. **Table 1.7** was modified from Blanckenberg et al., 2013 with permission.

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1.11 Goal of the present study

This study hypothesized that by using targeted NGS sequencing strategies to screen Black South African and Nigerian PD patients, novel pathogenic mutations in genes could be identified.

The significance of the study is that identifying novel pathogenic mutations in genes in these patients will improve our understanding of PD pathobiology specifically on how the mutations in genes affect the SSA PD patients.

1.12 Aims and Objectives

The present study aimed to identify new mutations in known genes and putative deleterious variants in other non-PD genes in the Black South African and Nigerian PD patients

Hence, this study had the following objectives:

- Perform a literature search and compile a review of all studies on PD in Nigeria
- Recruit Black South African and Nigerian PD patients and controls over a three year period for genetic studies
- Record relevant demographic, clinical and genetic data of PD patients in a REDCap database
- Screen for exonic rearrangements in candidate genes using MLPA
- Set up and optimise Ion Ampliseq targeted resequencing panel for mutation screening of the patients
- Screen for sequence changes in Black South African and Nigerian PD patients using a panel of 751 genes
- Validate selected novel variants predicted to be deleterious using Sanger sequencing techniques
- Screen for selected novel variants predicted to be deleterious frequency using High resolution melt assay
- Protein structures and modelling for functional prediction of selected novel variants predicted to be deleterious

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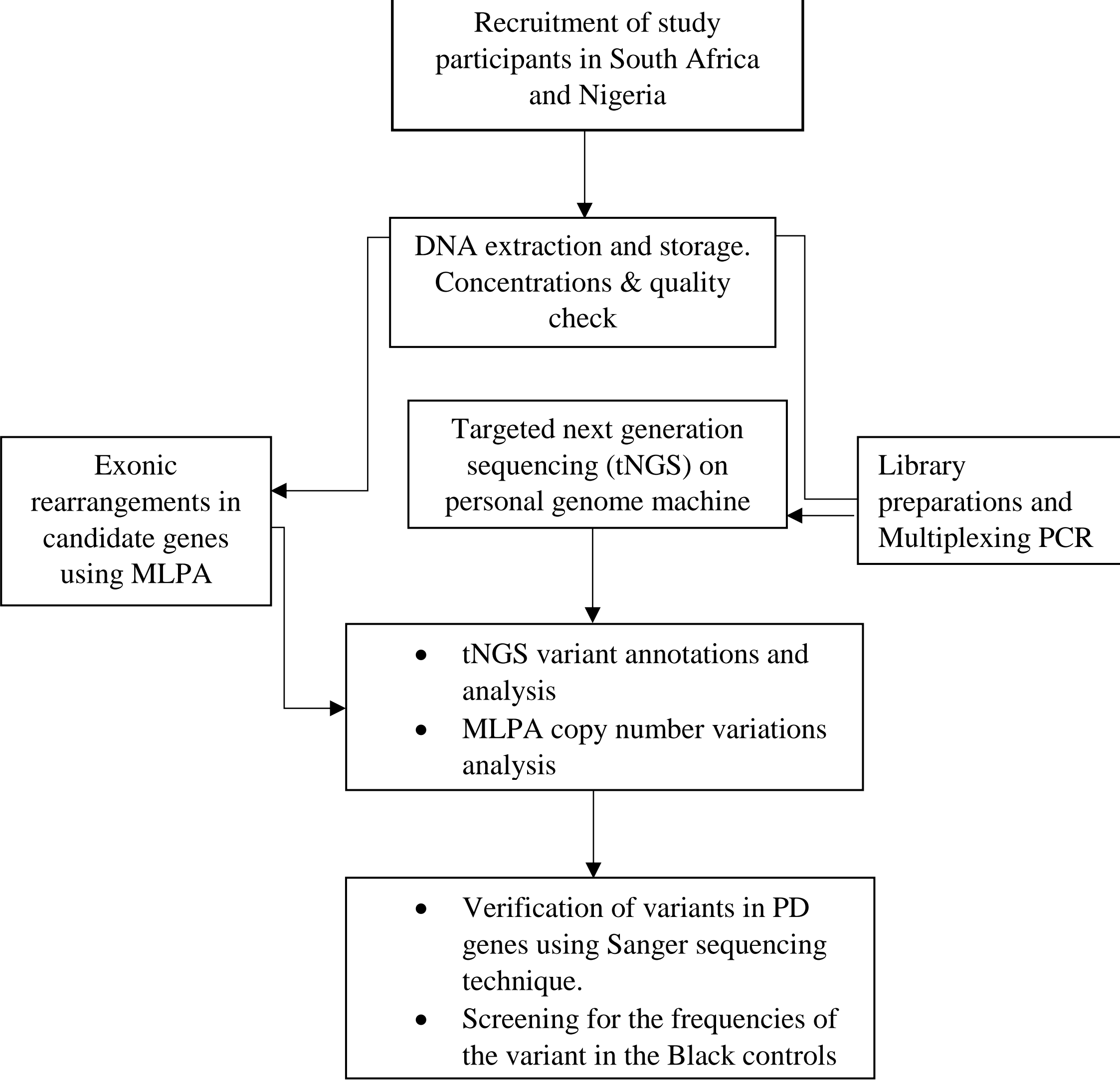

Figure 1.17: An overview of the study framework.

A total of 33 Black South African and 42 Nigerian PD patients were recruited over a two–year period at the Obafemi Awolowo University Ile-Ife and Tygerberg Academic Hospital, Cape Town, South Africa. Extractions of the genomic DNA were carried out in the Division of Molecular Biology and Human Genetics at Stellenbosch University. Copy number variation assays (samples from 15 South African and 42 Nigerian patients) and the tNGS (samples from 33 South African and 14 Nigerian patients) were performed at the Central Analytical Facilities, Stellenbosch University. Data analysis and further studies were also performed at the Division of Molecular Biology and Human Genetics at Stellenbosch University

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CHAPTER TWO

PARKINSON'S DISEASE IN NIGERIA: A REVIEW OF PUBLISHED STUDIES AND RECOMMENDATIONS FOR FUTURE RESEARCH

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CHAPTER TWO

PARKINSON'S DISEASE IN NIGERIA: A REVIEW OF PUBLISHED STUDIES AND RECOMMENDATIONS FOR FUTURE RESEARCH

The following chapter consists a review manuscript submitted to the *Parkinsonism and Related Disorder Journal*, and it is currently under considerations for publication.

My contributions to this chapter were:

- I conceptualised the idea to write a review article on the previous studies on PD in Nigeria to highlight the important findings from the previous studies on PD in the most populated country in Africa, currently understudied for PD. The review also highlighted the challenges of studying PD in Nigeria, which has important implications for the present study.
- I did the literature search of related published articles and retrieved the information used for this review
- I prepared the tables and figures
- I drafted the manuscript.

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Parkinson's disease in Nigeria: A review of published studies and recommendations for future research

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2.1 Abstract

Parkinson's disease (PD) affects 1-2% of individuals above 60 years amounting to over 7 million people worldwide. Thus, PD has become an important contributor to the neurological disease burden. Nigeria is the most populous country in Africa, and alarmingly, approximately 9.1 million Nigerians are above 60 years and are therefore at risk for developing PD. We carried out a critical review of published literature on PD in Nigeria to summarize the findings and to evaluate gaps in knowledge. Seven electronic databases were searched for studies published in English before 18th July 2018. Search terms were ["Parkinson's disease" OR "Parkinson disease" OR "parkinsonian disorders" OR "Parkinsonism"] AND "Nigeria". A total of 44 articles (including eight reviews) published since 1969 were identified and reviewed. Amongst the original research articles, most (23) were on PD symptoms or prevalence. There were only two studies on genetics and two on environmental factors. The estimated crude prevalence of PD in Nigeria was lower (10 to 249/100 000) compared to studies published in Europe (65.6 to 12 500/100 000). Our findings suggest that PD is under-diagnosed in Nigeria. Possible environmental risk factors identified include blacksmithing and well-water contaminated with trace metals. Given the rising numbers of the ageing population in Nigeria, more studies to evaluate the prevalence and causes of PD in this country are urgently needed. To this end, more funding, resources and a workforce of well-trained neurologists and scientists are essential to manage the impending health burden of PD and related disorders in this country.

Keywords: **Parkinson's disease; Parkinsonism; Nigeria; prevalence; symptoms; risk factors**

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2.2 Introduction

Worldwide, approximately 2% of people above the age of 60 and 4% above the age of 80 years are affected with Parkinson disease (PD) (de Lau and Breteler, 2006b) but the prevalence varies widely according to geographic region. In Sub-Saharan Africa (SSA), the prevalence of this condition differs depending on the study cited and ranges from 10 to 235/100 000 in urban populations, (Lekoubou et al., 2014a) compared to the crude prevalence in Europe which ranges from 65.6/100 000 to 12 500/100 000 (von Campenhausen et al., 2005b). Although studies conducted in rural areas in SSA are limited, a door-to-door study in rural Tanzania found a crude prevalence rate of 20/ 100 000, and notably, 78% of the patients were previously undiagnosed (Dotchin et al., 2008b). Based on findings from a systematic review, the prevalence of PD varied from 0.4 to 0.7% of neurological admissions/consultations in hospital-based studies in seven SSA countries (Lekoubou et al., 2014a). Over the past 26 years, the burden of neurological disorders including PD has increased substantially, making PD one of the leading causes of disability and mortality worldwide (Dorsey et al., 2018). To improve health-care planning and health outcomes of people with PD in Nigeria, we must understand not only the number and distribution of people with PD but also how these disorders affect population health.

There is a common misconception that there are few aged individuals (defined here as people aged 60 years and over) in SSA. However, it should be noted that the small increase in the proportion of the aged individuals in SSA masks a large increase in the actual number i.e. the number of people aged 60 and over is predicted to almost double from over 34 million in 2005 to over 67 million in 2030 (Council, 2006; Victoria et al., 2006). Notably, the number of people aged 60 years is currently rising more rapidly in this region than in developed countries and this trend is set to continue in the future (Velkoff and Kowal, 2007).

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Consequently, the number of PD cases is expected to increase significantly especially in the most populous SSA countries such as Nigeria, Ethiopia, Democratic Republic of Congo, Tanzania, South Africa and Kenya (World population review, 2018). Nigeria, the seventh most populous country in the world, with an estimated 182 million people in 2015 (Chapin, 1991.), is a multi-ethnic country inhabited by over 500 ethnic groups (**Supplementary Figure 2.1**). It has a history of slave trade, inter-border trading, intermarriage and cross-border migrations from neighboring countries making Nigerians a heterogeneous group of people (Chapin, 1991). In 2005, Nigeria was ranked among the top 30 countries internationally on the basis of its population aged 60 years and older (Victoria et al., 2006). According to data from the World Bank, approximately 2,75% of Nigerians, corresponding to 5.25 million people, are ≥ 65 years (World Bank, 2015) and these could potentially be considered to be at risk for developing late-onset PD. Also, increasing industrialization in Nigeria without a proper way of handling the effects of industrialization is currently contributing to the environmental degradation mostly caused by carbon emission and other toxicities (Theodore, 2006). The effects of these environmental hazards may be linked to the causes of sporadic forms of PD in Nigeria.

If these predicted trends are realized, then the management and care of patients with PD in Nigeria need urgent attention. It has been noted that current problems to proper care of patients in low-resource settings include lack of access to sustainable, affordable drug treatment and medical supervision (Dotchin and Walker, 2012; Mokaya et al., 2016). Also, the need to raise awareness of PD within the general population and that PD is a condition and not part of general ageing has been highlighted. It has also been suggested that minimal consensus management guidelines for PD should be established to improve the consistency and quality of care to patients in SSA (Williams et al., 2018a).

With this information as a backdrop, we summarized all published studies on PD in Nigeria, and provide recommendations for future studies. Our goal is to highlight not only the research that has been done but also the lack or paucity of studies on this impending health burden in Nigeria.

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It should be noted however that the importance of the implications of this review is not limited to Nigeria but include all SSA countries.

2.3. Methods

We searched seven electronic databases (PubMed, HubMed, BioMedSearch, Ovid, Web of Science®, Scopus and Google Scholar) for articles on PD in Nigeria published on or before 18th July 2018. Search terms were ["Parkinson's disease" OR "Parkinson disease" OR "parkinsonian disorders" OR "Parkinsonism"] AND "Nigeria". Articles selected to be potentially relevant were downloaded to a reference manager, Zotero ("Zotero | Your personal research assistant," 2018). The full text of relevant articles was obtained, and the contents critically evaluated in this review. Exclusion criteria include studies that were not published in English and studies that were not done on Nigerian PD patients or controls. Also, we did not consider articles that did not undergo a formal peer-review process.

2.4. Overview of retrieved articles

A total of 3 496 items were identified in the seven databases searched, and of these 405 were selected for consideration. After duplicates, conference abstracts and non-human studies were removed, 46 articles were considered to be potentially relevant (**Figure 2.1; Supplementary Table 2.1**). The full texts of these articles were retrieved, read and evaluated for content resulting in 44 articles being included in the review. For the two that were excluded; one was not on Nigerian PD patients, and the other was a letter to the editor of a journal. Among the 44 relevant articles, 10 studies investigated prevalence of PD in Nigeria, 13 reported on symptoms and signs in Nigerian PD patients (one study reported on prevalence and on symptoms (Femi et al., 2012), two were genetic reports on PD in Nigerian individuals, two reported on environmental risks factors for PD in Nigeria, seven reported on other diseases mimicking the clinical features of PD, three studied biochemical or pathological findings and eight were review articles (**Supplementary Figure 2.2**).

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Of the 44 articles, 40 (90.9%) articles had either a Nigerian as the first or the last author, and four articles had only non-Nigerian authors. Therefore, most of the studies published on PD in Nigeria were led by Nigerian investigators.

2.5. Prevalence of PD in Nigeria

A total of 10 publications involved estimation of the number of PD patients in Nigeria (**Table 2.1**). Four different study designs were used and included five neurological hospital admissions studies, two neurological out-patient, one hospital-based and one community-based study (Schoenberg *et al* (Schoenberg et al., 1988) re-analyzed the same data set as Osuntokun *et al* (Osuntokun et al., 1987), which was a community-based study, but included only the 3 412 participants aged over 39 years). We calculated crude prevalence ratios of PD cases per 100 000 in each study and this ranged from 10 to 249 (**Table 2.1**). When compared with global PD prevalence per geographic location which ranged from 41 to 2 953 (Pringsheim et al., 2014b), we found that PD prevalence is low in Nigeria.

The five neurological hospital admission studies were carried out in four different locations in Nigeria, and ranged in size from 781 to 9 600 (Chapp-Jumbo et al., 2004; Ekenze et al., 2010b; Osuntokun, 1971; Owolabi et al., 2010; Talabi, 2003). The number of PD cases in these studies varied from 0.4% to 2.2%. The estimated crude prevalence of PD was from 32 to 165/100 000, with the lowest estimate from the Niger Delta area (Chapp-Jumbo EN., 2004) and the highest from Enugu in South East Nigeria (Ekenze et al., 2010b).

Two studies analyzed neurological out-patient populations, one in Lagos in Southwest Nigeria and the other in Kano in Northwest Nigeria (Femi et al., 2012; Okubadejo et al., 2010b). The number of PD patients among patients with neurological disorders varied between the two sites with the Kano study reporting an over four-fold greater number of PD patients (20/1 360 for Lagos vs 80/1 153 for Kano). The reason for this difference is not clear.

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It is, however, plausible to speculate that exposure to environmental hazards such as herbicides and pesticides might be a possible reason for this difference. Similarly, we could not rule out hereditary PD as one of the reasons for the increased PD prevalence in the Kano study. Marriages among related individuals are more frequent in Northwest Nigeria than Southwest, and this might contribute to the increased frequency of Parkinson's Disease. (Theodore, 2006). These two study sites are known to be the most populous places in Nigeria. In addition, Kano is situated in the Northwest Nigeria where blacksmithing and commercial farming are some of the major occupations of the people living in the rural and urban communities, whereas Lagos is more urbanized, and the majority of the people living there are not farmers or involved in blacksmithing.

The hospital-based study investigated the prevalence of PD among all 8 026 hospital admissions, 1 220 of which were neurological admissions (Dada, 1970), and identified 20 PD patients. The crude prevalence estimate from this study was 249/100 000.

The community-based study was a door-to-door survey using simple questionnaires and clinical evaluations of nearly 20 000 individuals. The study identified a total of 699 individuals with neurological conditions, but only two of them had PD (Osuntokun et al., 1987). The crude prevalence estimates for PD from this study was 10/100 000 (Osuntokun et al., 1987) without age-adjustment and 59/100 000 (Schoenberg et al., 1988) when considering only the older population.

In summary, the estimated number of people with PD in Nigeria is low; the reason might be due to under-diagnosis or misdiagnosis of PD. There was evidence of regional variation in the prevalence of PD in Nigeria but the reasons for this are currently not known. The variation may also be linked to the lack of expert neurologists in some areas in Nigeria that can correctly diagnose PD. Also, the lack of adequately informed and trained non-specialists in rural regions is a factor leading to under- and misdiagnosis of PD in SSA. Not all of the epidemiological studies reported on PD in Nigeria described the diagnostic criteria they used (Osuntokun et al., 1987).

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It is noteworthy that there were five neurological hospital admissions studies, two neurological out-patient, one hospital-based and one community-based study reviewed in the present study, each with different limitations that could affect the overall estimates of PD prevalence.

2.6 Clinical profile of PD in Nigeria

Our literature search identified a total of 13 studies cataloguing the symptoms and signs of Nigerian PD patients (**Table 2.2**). In addition, seven Nigerian studies (Adebayo et al., 2013; N.U. et al., 2011; Odeku and Adeloye, 1973; Osuntokun et al., 1972; Owolabi, 2013; Ugoya et al., 2011) reported patients with similar symptoms and signs of PD in other disorders including progressive supranuclear palsy (Owolabi, 2013), drug induced Parkinsonism (Ugoya et al., 2011), typhoid fever (Osuntokun et al., 1972), Lewy body dementia (Adebayo et al., 2013), ischemic cerebrovascular disease (Osuntokun et al., 1969), cranial meningiomas (Odeku and Adeloye, 1973), and essential tremor (Osuntokun et al., 1987). It is important to note that PD symptoms vary widely from patient to patient. One of the Nigerian studies demonstrated clearly the difficulties in diagnosing PD patients stating that "none of the patients had received a definite diagnosis of PD prior to specialist consultation, instead the patient's referral letter stated 'neurological disorder' or `neurological symptoms' (Okubadejo et al., 2010).

2.6.1 Preclinical symptoms of PD

Preclinical symptoms of PD precede the onset of the characteristic motor features of PD, and include olfactory dysfunction, sleep disturbances, depression, anxiety, apathy and constipation (**Table 2.2**). Although, these conditions are not a definite indication to develop PD, cataloguing the presence of these preclinical symptoms in patients is helpful when actual PD is to be diagnosed. There were no published studies that described preclinical symptoms of PD in Nigeria.

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2.6.2 Motor symptoms of PD

Cardinal motor symptoms of PD are rigidity, resting tremor, gait abnormalities and bradykinesia, which have been reported extensively also in Nigerian PD patients (**Table 2.2**) (Femi et al., 2012). These signs are required for PD diagnosis.

One Nigerian study reported that frequent falls with sustained injuries were about three times more common in PD patients than controls, and that the risk for falls increased with increasing age and disease severity (Farombi et al., 2016).

2.6.3 Non-motor symptoms of PD

Non-motor symptoms (NMS), which include cognitive disorders, visual dysfunction related to object and face perception, autonomic impairment, and mood disorders, are seen very frequently in PD patients, and were also reported in many Nigerian PD patients (Akinyemi et al., 2008; Ojagbemi, 2013; Ojagbemi et al., 2013; Ojo et al., 2012; Okubadejo and Danesi, 2017; Okunoye, 2015; Okunoye and Asekomeh, 2013; Owolabi et al., 2016, 2014) (Table 2). Neurobehavioral disorders can occur in PD patients at any stage of the disease, whereas psychotic symptoms occur more commonly during treatment with dopamine agonists. About 90% of PD patients will have at least one neuropsychiatric symptom during the course of the disease (Chaudhuri et al., 2011). Neuropsychiatric symptoms such as depression, visual hallucinations, delusion, aggression and apathy have also been reported in Nigerian PD patients (**Table 2.2**) (Ojagbemi, 2013; Okunoye and Asekomeh, 2013).

NMS greatly impact the quality of life of PD patients and their caregivers (Aarsland et al., 2009; Ojagbemi, 2013). In addition, NMS vary significantly from patient to patient. For example, several Nigerian PD patients had respiratory impairments related to difficulties in breathing, while others had cardiovascular dysfunction or gastrointestinal disorders (Okubadejo and Danesi, 2017; Owolabi et al., 2016, 2014).

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In summary, psychiatric symptoms, gastrointestinal disorders and cardiovascular autonomic dysfunction were the most common NMS observed in Nigerian PD patients (Ojagbemi et al., 2013; Okubadejo and Danesi, 2017; Osuntokun and Bademosi, 1979; Owolabi et al., 2014).

2.7 Biochemical and pathological findings in Nigerian PD patients

Findings from biochemical and pathological studies can provide clues to disease mechanisms. Only three biochemical or pathological studies were identified in our search. One study compared the melanized nigral neuronal count between neurologically normal Nigerians (n=23) and normal British (n=7) individuals as this has been postulated to have a link to PD (Muthane et al., 2006). The results of the study showed no significant differences in the number of melanized neurons between the two groups (Muthane et al., 2006). Another group studied brains (n=94) of neurologically normal Nigerians and found Lewy bodies in four male individuals (Jendroska et al., 1994), suggesting that incidental Lewy body disease is at least as frequent in Nigeria as in industrialized nations. Furthermore, a study investigating the level of homocysteine in plasma samples of 40 Nigerian PD patients and 40 age- and sex-matched healthy controls identified increased levels of homocysteine in nine (22.5%) patients known to be on long-term regimen of levodopa (Ojo et al., 2011). This corroborates the hypothesis that homocysteine levels are significantly increased in levodopa-treated PD patients compared to controls. Also, elevated levels of homocysteine are an emerging risk factor for neurological disorders such as stroke, dementia and Alzheimer's disease (Seshadri et al., 2002). It is possible that further studies on biochemical and pathological findings in Nigerian PD patients could provide unique insights into differences in disease mechanisms between Africans and non-Africans.

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2.8 Environmental risk factors for PD in Nigeria

Regional variation in the incidence of a disease could suggest that there are environmental risk factors contributing to the disease and that long-term exposure to these agents may accelerate the disease process. For example, the use of pesticides that inhibit mitochondrial functions has been associated with PD in the United States (Tanner et al., 2011). Some early reports showed five times higher prevalence of PD in the USA than in Nigeria, and suggested that environmental risk factors could account for this difference in prevalence (Schoenberg et al., 1988). Furthermore, in Nigerian studies, prevalence of PD varied from one environment (region) to another (**Table 2.1**). Although these discrepancies may be due to methodological differences between the studies, it is worthwhile to investigate whether dietary and lifestyle factors such as neurotoxins in certain diets may account for regional variations in the prevalence of PD. One such example is the possible link identified in Guam and New Caledonia between an atypical Parkinsonism phenotype and *Annonaceae's* consumption (Caparros-Lefebvre and Steele, 2005).

Our literature search identified two studies carried out in Nigeria investigating the role of environmental risk factors for PD. In a multi-center case-control study, trace metals identified in well-water were associated with PD (Ogunrin et al., 2012). Also, xenobiotics thought to be associated with PD were studied in Nigerian PD patients but their use was not associated with the disorder (Igbokwe et al., 1993). In one study, blacksmithing, which is a common occupation in Nigeria, mostly among the people from the Northern part of the country (e.g. Kano state), was found to be associated with PD (Akinyemi, 2012; Falope et al., 1992). As well-water is one of the main sources of water supply in Nigeria, it is plausible that environmental factors such as trace metal contamination in well-water as well as blacksmithing may contribute to development of PD but further studies are needed to confirm this.

Nigeria is perceived as a homogeneous group of people. It should be noted, however, that each region in the country has different tribes that make the country quite diverse in terms of culture, language and ancestral origin

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(**Supplementary Figure 2.1**). Also, other factors such as vegetation, climate, topography, lifestyle, diets and the socioeconomic factors differ extensively across different regions of the country; all of these could contribute to the prevalence of PD. For example, people living in South-Western Nigeria are more likely to be exposed to industrial-derived toxins, whereas people living in the oil-rich Southern region could be more exposed to toxic chemicals and aquatic foods contaminations due to oil-spillages.

2.9 Genetic studies on PD in Nigeria

Approximately 5-10% of PD patients have a monogenic form of PD, which is due to highly penetrant, rare pathogenic mutations (Lill, 2016). The genetics of PD is complex as common genetic variants may act in concert with environmental factors. Genome-wide association studies have identified 26 PD risk loci, (Lill, 2016) but none of these loci have been studied in Nigerian PD patients. Familial PD cases in Nigeria have been mentioned (**Table 2.1**), but to date no genetic causes have been identified in these patients. Currently, only one genetic study on Nigerian PD patients has been published (Okubadejo et al., 2008) (**Table 2.3**). The study analyzed mutations in *LRRK2*, *PRKN* and *ATXN3* in 57 Nigerian PD patients who showed cardinal signs of PD such as tremor, rigidity, bradykinesia and gait abnormality, of which nine patients presented with at least one first-degree relative with a history of tremors. No pathogenic mutations in the genes commonly known to cause PD in European, North American or North African populations were found (Okubadejo et al., 2008).

Interestingly, the study identified two heterozygous variants (p.A46T, and p.R334H in *PRKN*) of unknown pathogenicity in two PD patients from different ethnic groups in Nigeria (Okubadejo et al., 2008).

Notably, the fact that nine of the PD cases presented with a first-degree relative with a history of tremor, indicates that an autosomal dominant mode of inheritance is possible in these families.

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The study did not rule out the possibility of mutations in any of the known autosomal dominant PD genes such as *SNCA* and *GBA*.

In another study, Tucci *et al* (Tucci et al., 2012) screened 26 Yoruba (Nigerian) individuals from the Human Genome Diversity Cell Line panel to estimate the frequency and diversity of coding variants in the *EIF4G1* gene (Tucci et al., 2012). No pathogenic mutations were identified.

The limitations of these genetic studies are the small sample sizes and the fact that only and few PD genes were screened. In conclusion, further genetic studies on the Nigerian population are warranted since the frequency of certain pathogenic mutations have been shown to differ widely between different population groups. For example, the frequency of the G2019S mutation in the *LRRK2* gene varies from approximately 40% in North African Arabs, to roughly 28% in Ashkenazi Jews and to only 3% in Caucasian populations from southern Europe (Healy et al., 2008). To date, no Black PD patient has been found to harbor the G2019S mutation (Blanckenberg et al., 2013; Williams et al., 2018) further providing support for the striking differences observed in the genetic etiology between various ethnicities.

2.10 Implications and recommendations

It is important that PD patients are diagnosed early and accurately as epidemiological studies and other clinical studies on PD in Nigeria are dependent on this. Primary care physicians play an important role in this regard. There are a number of differential diagnoses including essential tremor, vascular parkinsonism, normal pressure hydrocephalous, brain tumors, multiple system atrophy, supranuclear palsy, and Huntington's disease, and an initial brain computed tomography (CT) scan should be performed to exclude the major causes of secondary Parkinsonism.

If the history obtained however suggests rarer causes, where possible, appropriate biochemical or genetic tests should be requested. Also, in a SSA setting the management of PD should have a multidisciplinary approach as well as integration into the primary health care systems of each country.

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The team members could include neurologists, nurses, physiotherapists, occupational therapists, speech therapists, neuroscientists, neuropathologists, neuro-geneticists, social workers as well as community health workers. Furthermore, strategies and support services e.g. telemedicine, where patients can be diagnosed and treated remotely via telecommunication technology, should be established to facilitate diagnosis of patients in rural settings. In addition, educational courses focused on non-specialist physicians, nurses and community health officers on the diagnosis and management of neurodegenerative disorders, should be provided as well as country-wide PD awareness campaigns to educate the general public about PD. This may help to alleviate issues of stigmatization, victimization or abandonment experienced by PD patients and their families within their communities. It is also critical to investigate family history of the disorder in PD patients. A positive family history and a young age-at-onset (below 40 years) are both strong indicators of a genetic component underlying the disease in an individual.

Treatment for PD needs to be more accessible and affordable to Nigerian patients. It would be important to conduct surveys on the availability and affordability of anti-parkinsonian medications in SSA countries, similar to the study conducted in Kenya (Mokaya et al., 2016). Anti-parkinsonian drugs should be available and covered under the National Health Insurance Scheme, and the Federal Ministry of Health, policy makers, and politicians need to take action and remove the barriers and obstacles that limit broader availability of these drugs.

In 2012, The International Parkinson and Movement Disorder Society established a Task Force on Africa, and its main mission is assisting with diagnosis of PD in Africa, together with ensuring affordable and sustainable treatment.
(http://www.movementdisorders.org/MDS/Regional-Sections/Task-Force-on-Africa.htm)

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Additional studies on the prevalence of PD are needed to determine the extent of the problem in Nigeria. Problems with the existing prevalence studies include the fact that there were only two door-to-door studies (regarded as the most accurate method for determining prevalence), and that most studies were hospital-based. Since not every individual is taken to a hospital for diagnosis, the true prevalence of PD in the country cannot be ascertained. Worldwide trends indicate rapidly growing numbers of PD cases (Bach et al., 2011) and it is anticipated that this is also true for Nigeria but more studies are required to confirm this. More studies are also needed on the genetic causes of PD in Nigeria since this population is likely to harbor novel mutations. It is possible that future treatments might be based on the genetic etiology identified in overseas populations, which may not be transferable to Nigerian populations. Furthermore, more studies on clinical manifestations of PD in Nigerian PD patients are required, since this might identify clinical symptoms unique to this population. Also, neuroimaging using at least CT, and if available magnetic resonance imaging, can often help in differential diagnosis to exclude other causes of Parkinsonism (Tripathi et al., 2018).

2.7 Study limitations

The limitations of this review include restricting the search to only English articles. It is also possible that even though our search was performed using seven different search engines, some relevant articles were missed because they did not contain the key words used here.

2.8 Conclusion

This review summarizes all the published human studies on PD in Nigeria. It can be concluded that, although 44 studies have been published since 1969, much more research is still needed. Also, the comparability of the existing studies is limited, due to differences in study designs. There was little information on neuroimaging to aid differential diagnosis of PD and there were very few genetic studies on PD in Nigeria.

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It should be noted that Nigeria comprises many different ethnic groups and geographic regions, and this should be taken into account when analyzing epidemiological, genetic, biochemical and pathological findings on PD.

We believe that primary care physicians have an important role in the timeous diagnosis of patients. They need to be aware of prodromal PD and its symptoms, including depression, constipation, hyposmia, and sleep disturbances, which could occur 10-20 years prior to the typical PD signs, as well as non-motor features. Similarly, they need to document the family history, other concomitant diseases, occupational records and history of exposure to environmental hazards for patients as this information may strengthen future etiological studies and the management of PD in Nigeria.

2.13 Conflicts of interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this review.

2.14 Funding

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2.15 Authors' contributions

OGO was the project lead, carried out literature searches, appraised the articles, summarized results, prepared the tables and figures and drafted the manuscript HK carried out literature searches, reviewed the articles and edited the manuscript; SB and MK conceptualized the idea for the research, obtained funding, supervised the project and wrote sections of the manuscript; JC, MOO and OR provided clinical expertise, critically reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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Supplementary Figure S2.2: Distribution of topics covered in the 36 original studies on PD in Nigeria and used in this review. One study reported on both prevalence and symptoms and is included here in both categories. There were eight review articles which are not included in this figure.

APPENDIX IV (Supplementary Table 2.1): List of published articles related to Nigerian studies on PD and used in this review**.**

Table 2.1: Summary on PD prevalence studies in Nigeria

Study year	Study site	Geographic region in Nigeria	Study design	Total hospital/community population	Number of patients with neurological disorders	Number (%) of PD patients	Family history of PD patients	Age at onset for PD (mean +/-SD; years)	Crude prevalence ratio cases/100 000	Study reference
Neurological admission studies										
1970	University of Ibadan	South West	Analysis of neurological admissions during 1957 – 1969	220 000	9 600	90 (0.9)	1	N/A	47	(Osuntokun, 1971)
2003	University of Ibadan	South West	Analysis of neurological admissions during Jan 1998 – Dec 2000	26 355	781	4 (0.5)	N/A	N/A	75	(Talabi, 2003)
2004	University of Port Harcourt	South South	Analysis of neurological admissions during April 1993 – March 2003	92 544	1 393	30 (2.2)	N/A	N/A	32	(Chapp-Jumbo EN., 2004)
2010	University of Nigeria Teaching Hospital Enugu	South East	Analysis of neurological admissions during Jan 2003 – Dec 2007	8 440	1 249	14 (1.1)	N/A	N/A	165	(Ekenze et al., 2010)
2010	Aminu Kano Teaching Hospital	North West	Analysis of neurological admissions during Jan 2005 – July 2007	6 282	980	4 (0.4)	N/A	N/A	63	(Owolabi et al., 2010)
Neurological out-patient studies										
2010	Lagos State University	South West	Analysis of neurological outpatient clinic	N/A	1 360	20 (1.5)	1	61.5 ±10	N/A	(Okubadejo et al., 2010)

	Teaching Hospital		during Jan 2005 – Dec 2006							
2012	Two Tertiary Health Facilities in Kano	North West	Analysis of neurological out-patient clinic during June 2007 – June 2011	N/A	1 153 (96 with parkinsonism)	80 (6.9)	3	58.2±6.72	N/A	(Femi et al., 2012)
Hospital based studies										
1970	Lagos State University Teaching Hospital	South West	Analysis of hospital admissions during 1962-1967	8 026	1 220	20 (1.6)	N/A	N/A	249	(Dada, 1970)
Community based studies										
1987*	Igbo-Ora	South West	Community surveys used questionnaire and simple clinical evaluation in 1982	18 954	699	2 (1.4)	N/A	N/A	10	(Osuntokun et al., 1987)
1988*	Igbo-Ora	South West	Community surveys used questionnaire and simple clinical evaluation in 1982	3 412	N/A	2 (0.05)	N/A	>39	59	(Schoenberg et al., 1988)

N/A, not available Community-based door-to-door survey. Both studies used the same primary data from Nigeria.
Schoenberg et al analyzed data from individuals > 39 years old. *Crude prevalence ratio is given as the number of PD cases/100 000 individuals.

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Table 2.2: Symptoms and signs of PD identified in Nigerian patients

Category	Symptom or sign	Brief description	Reported in Nigerian PD patients	Study reference
Preclinical symptoms	Olfactory problems	Loss of smell	No	
	Gastrointestinal disorders	Indigestion and abdominal pain	No	
	Sleep disorder	Episode sleep	No	
	Mood disorder	Lack of motivation	No	
	Orthostatic hypotension	Low blood pressure when standing up	No	
Primary motor symptoms	Resting tremor	Slight tremor in the hand or foot on one side of the body	Yes	(Osuntokun, 1971), (Akinyemi et al., 2008), (Okubadejo et al., 2008), (Femi et al., 2012), (Okunoye, 2015)
	Bradykinesia	Difficulty with repetitive movements	Yes	(Osuntokun, 1971), (Okubadejo et al., 2008), (Femi et al., 2012), (Okunoye, 2015), (Osuntokun and Bademosi, 1979)
	Rigidity	Stiffness and inflexibility of the limbs, neck and trunk	Yes	(Osuntokun, 1971), (Akinyemi et al., 2008), (Okubadejo et al., 2008), (Femi et al., 2012), (Okunoye, 2015)
	Postural instability	Tendency to be unstable when standing upright	Yes	(Osuntokun, 1971), (Akinyemi et al., 2008), (Okubadejo et al., 2008), (Femi et al., 2012), (Okunoye, 2015)
Secondary motor symptoms	Freezing of gait	Hesitating before stepping and exaggerated first step	Yes	(Osuntokun, 1971), (Femi et al., 2012), (Okunoye, 2015)

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	Micrographia	Shrinkage in handwriting	Yes	(Femi et al., 2012), (Okunoye, 2015)
	Mask-like expression	Decreased unconscious facial movements	No	
	Akathisia	Unwanted accelerations	No	
	Falls	Falling due to instability	Yes	(Farombi et al., 2016)
	Speech problem	Drooling and excess saliva	Yes	(Osuntokun, 1971), (Femi et al., 2012), (Okunoye, 2015), (Osuntokun and Bademosi, 1979)
Non-motor symptoms	Cognitive disorders	Delusion, hallucination, depression, anxiety, apathy and irritability,	Yes	(Osuntokun, 1971), (Ojagbemi, 2013), (Okunoye and Asekomeh, 2013), (Osuntokun and Bademosi, 1979)
		Difficulty remembering events	Yes	(Ojagbemi, 2013), (Okunoye, 2015)
	Poor vision	Sight problem	No	
	Autonomic dysfunction	Cardiovascular disorders	Yes	(Osuntokun, 1971), (Okubadejo and Danesi, 2017)
	Obesity or weight loss	Excessive weight gain or weight loss	No	
	Pulmonary problems	Reduced vital capacity	Yes	(Owolabi et al., 2016)
	Sexual dysfunction	Low libido	No	
	Mood disorder	Persistent low mood	Yes	(Akinyemi et al., 2008)
	Sweating	Excessive night sweating	No	
	Gastrointestinal disorders	Indigestion and abdominal pain	Yes	(Owolabi et al., 2014)

For more information (http://www.pdf.org/en/symptoms).

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Table 2.3: Genetic studies on Nigerian PD patients.

Gene analyzed	Number of patients	Number of controls	Method	Findings	Study reference
Parkin (*PRKN*)	57	51	Sanger sequencing of all exons and exon/intron boundaries	No pathogenic mutations found	(Okubadejo et al., 2008)
Ataxin 3 (*ATXN3*)	57	51	Screen for repeat expansions	No pathogenic mutations found	(Okubadejo et al., 2008)
Leucine-rich repeat kinase (*LRRK2*)	57	51	Sanger sequencing of exons 31 and 41	No pathogenic mutations found	(Okubadejo et al., 2008)

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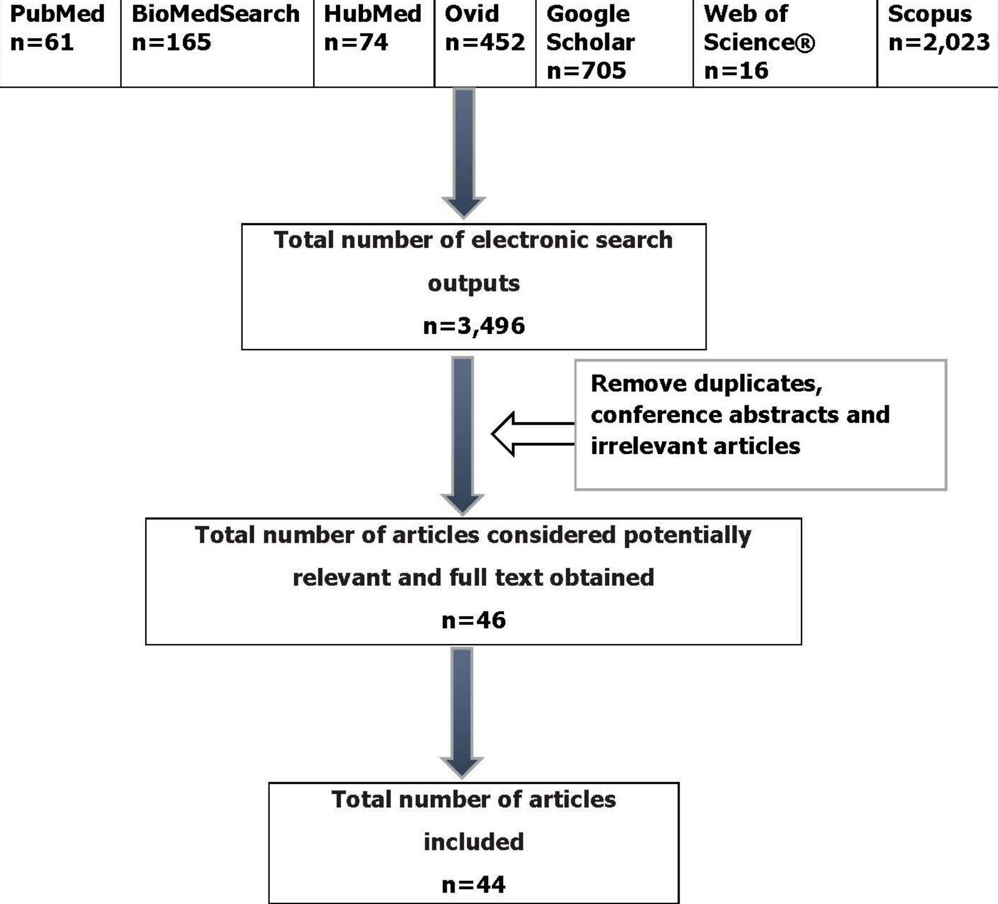

Figure 2.1: Flow diagram of literature search strategy and results. Several electronic databases were searched as described in the methods. See Table S2.1 for full listing of the 46 articles.

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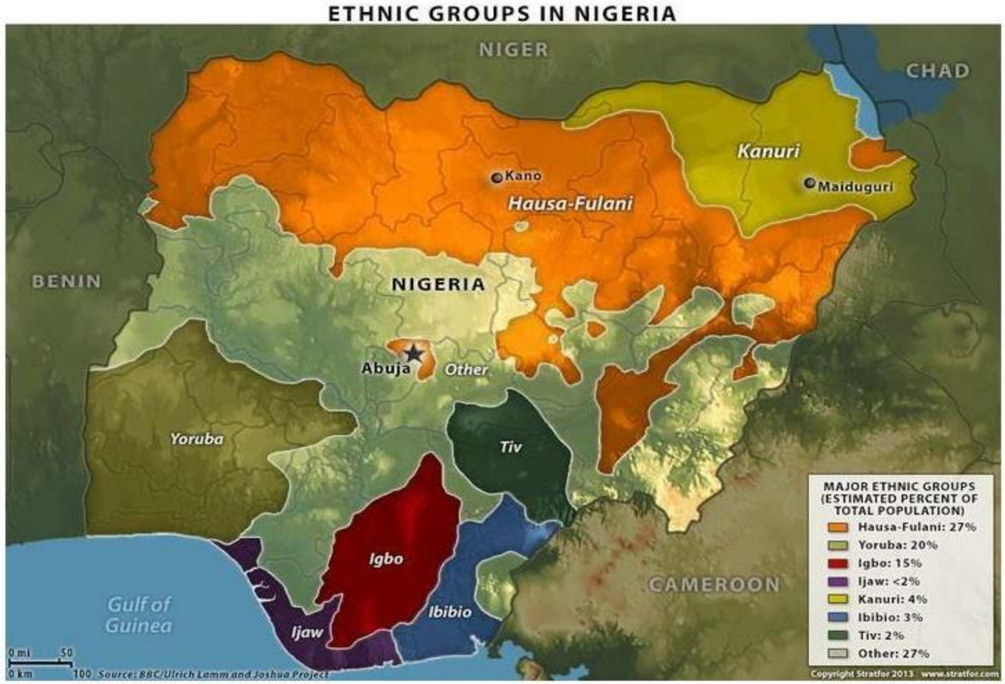

Supplementary Figure 2.1: Map of Nigeria demonstrating significant ethnic diversity of different regions of the country. Permission from Stratfor Worldview (www.stratfor.com) a geopolitical intelligence platform.

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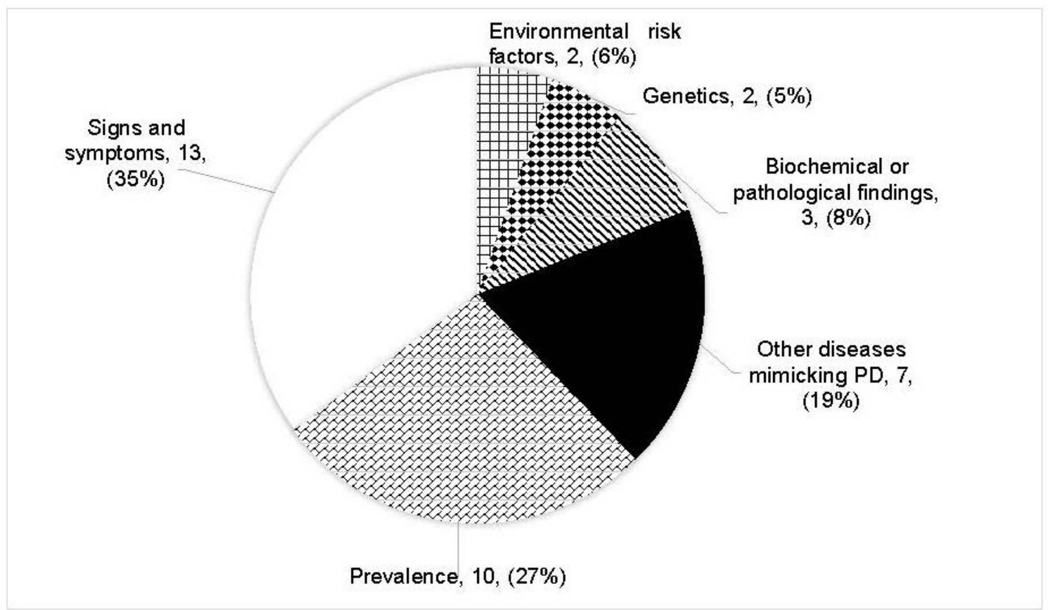

Supplementary Figure 2.2: Distribution of topics covered in the 36 original studies on PD in Nigeria and used in this review. One study reported on both prevalence and symptoms and is included here in both categories. There were eight review articles which are not included in this figure.

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CHAPTER THREE

COPY NUMBER VARIATION ANALYSIS ON 57 BLACK SOUTH AFRICAN AND NIGERIAN PARKINSON'S DISEASE PATIENTS (UNPUBLISHED DATA)

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CHAPTER THREE

COPY NUMBER VARIATION ANALYSIS ON 57 BLACK SOUTH AFRICAN AND NIGERIAN PARKINSON'S DISEASE PATIENTS (UNPUBLISHED DATA)

3.1 Background

Parkinson's disease (PD) is a movement disorder that can be either inherited or sporadic. Patients can have genetic defects such as substitutions, deletions or duplications in the PD genes. Frequently identified mutations in the known PD genes are single base substitutions that lead to the conversion of one amino acid to another. Also, small deletions or insertions have been identified in PD patients and are catalogued in the PD mutation database (https://www.molgen.vib-ua.be/PDMutDB). Most of these mutations are in the *PRKN*, *SNCA*, *LRRK2*, *PINK1* and *PARK7* genes.

The term copy number variation (CNV) refers to structural changes such as large deletions or duplications in the chromosome and in a gene. Studies have shown that in CNVs the rearrangement lengths may vary between 50 and 20000 bases (Frith and Khan 2018). Genomic rearrangements such as multiplications of one or more exons or deletions of exons have been previously reported in the PD genes (Djarmati et al., 2004; Morais et al., 2016; Olgiati et al., 2015; Pankratz et al., 2009). It is, therefore, plausible that CNVs may predispose an individual to developing PD.

These genomic rearrangements are usually not detectable with routine mutation screening techniques such as HRM or Sanger sequencing. Similarly, despite that NGS offers a simultaneous testing of thousands of loci with high reproducibility, high resolution, and scalability to detect mutations in genes, one of the limitations described in the NGS studies in Chapter 1, is the inability to accurately detect large deletions and duplications in a DNA sample.

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Genomic rearrangements such as deletions or duplications can produce a completely different phenotype or lead to a loss of function of a gene (Gamazon and Stranger 2015); hence people affected by CNV in PD could have varied expressions that may impact PD manifestations at any stage.

There are different types of genomic rearrangements. The frequently observed exonic rearrangements (**details in Chapter 1**) are non-tandem duplications of the same strand, non-tandem duplications of the opposite strand, non-tandem double duplication, inversion next to deletion, inversion and tandem multiplication (Frith and Khan 2018).

There are few techniques that can detect CNVs in different diseases. The methods for studying structural variations are difficult, time-consuming and sometimes yield low quality outputs. Some of the popular techniques used include fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH) and genome-wide SNP arrays (Feuk et al 2006). In addition, MLPA assay is a widely used technique for screening of CNVs. The MLPA technique represents a reliable method for a large-scale and cost-effective gene dosage screening. It is efficient and can be used to analyse several CNVs in a single reaction. In a single MLPA reaction, up to 45 different probes can be amplified simultaneously, allowing for the detection of an aberrant copy number of up to 45 genomic sequences including small intragenic rearrangements (Djarmati et al 2007).

The principle of MLPA is based on the simultaneous amplification of the hybridized probes. Each probe is designed to amplify a given exon of a gene. The principles of MLPA and the analyses underlying CNVs have been discussed in **Chapter 1** of this thesis. The control probes located in different chromosomal regions are also added to the same reaction for relative quantification during the analysis. The specific MLPA assay kits for studying CNVs in PD causative genes are available commercially (SALSA MLPA Kit P051/P052 MRC Holland; *www.mrc-holland.com*).

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Table 3.1: List of specific probes included in the SALSA PO51and PO52 probemix

Gene	SALSA PO51 probemix	SALSA PO52 probemix
SNCA	Exons 1,2,3,4,5,6 and A30P mutation	N/A
PRKN /Parkin	Exons 1,2,3,4,5,6,7,8,9,10,11,12	Exons 2,3,4,5,6,7,8,9,10,11,12
PINK1	Exons 1,2,3,4,5,6,7,8	N/A
PARK7	Exons 1,2,3,4,5,6,7	N/A
LRRK2	G2019S mutation	Exons 1,2,10,15,27,41,49 and G2019S mutation
ATP13A2	Exons 2 and 9	14 and 27

Further details available on MLPA website (*https://www.mlpa.com*) and MRC Holland website (*www.mrc-holland.com)*.

The P051 and P052 probe mixes were designed to analyse aberrant CNVs in the PD genes *SNCA, PARK1, PRKN, UCHL1, PINK1, PARK7, LRRK2, GCH1 and ATP13A2*. The probe mixes are designed in such a way that the P051 probe mix contains the probes that can screen each exon in *PARK7, ATP13A2, PINK1*and *PRKN* genes. It also contains a probe for screening each exon in the *SNCA* gene, as well as a probe specific for the *SNCA* A30P mutation. In addition, it contains a probe that will generate a signal when the *LRRK2* G2019S mutation is present **(Table 3.1)**.

Whole exon insertions or deletions are common in the *PRKN* gene in PD patients. These mutations have been identified in autosomal recessive and sporadic forms of the disease. CNV rearrangements involving *PRKN* exons account for 50–60% of all pathogenic exon dosage abnormalities in PD (Kim et al. 2012). Whole-gene multiplications (duplications or triplications) have been identified mostly in *SNCA* in PD patients.

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The whole gene multiplications of SNCA led to the over-expression of the α-synuclein and protein deposits were found in large quantities in the brains of PD patients. It was found that individuals with duplications have a classical PD phenotype, whereas those with triplications have earlier onset and faster disease progression (Wang et al. 2013; Keyser et al. 2010; Sekine et al. 2010; Kojovic et al. 2012; Darvish et al. 2013; Olgiati et al. 2015). CNVs have also been reported in *PINK1* (Cognata et al 2017) and *PARK7* genes. *PARK7* is the third most common gene that is frequently associated with pathogenic CNVs in PD genes, and responsible for early-onset PD (Bonifati et al. 2003; van Duijn et al. 2001). Limited information exists on pathogenic CNVs in *LRRK2* or *ATP13A2 genes*.

The aim of the study

This study was designed to identify possible CNVs in 57 Black South African and Nigerian PD patients. This study used the commercially available MLPA assay kit for PD (SALSA MLPA Kit P051), which comprises 31 exons of the four known PD genes (*SNCA, PARK7, PINK1, and PRKN)* for the exon dosage analysis A subset of the same patients were also screened for mutations in genes using tNGS and described in **Chapter 4** of this study.

Study rationale

Studies on CNVs in PD patients in the SSA are limited. To date one previous study on the CNVs in Black South African PD patients described only one Black PD patient with an heterozygous deletion in *PRKN* exon 4 (Mahne et al 2016). In another study by Haylett et al., only two Black South African patients were found to harbour mutations in the *PRKN* gene (Haylett et al 2012). Equally, the number of studies on CNV assays in the Nigerian PD patients is limited. One previous CNV study on Nigerian PD patients did not identify any CNV mutations (Okubadejo et al 2008).

It is important to understand the roles of CNVs in the PD pathogenesis in SSA. The rationale for the present study was that comprehensive genetic studies on Black PD patients from SSA are limited.

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There is need to use modern techniques and resources to screen a large cohort of patients from this region to ascertain the spectrum of mutations in genes in PD patients. Similarly, CNVs are thought to be a rare cause of PD in the SSA PD patients.

3.2. Methods

3.2.1. Study participants

In this study we used DNA samples from 15 Black South African and 42 Nigerian PD patients (**Supplementary Table 3.1**). The South African patients were recruited at the Neurology Clinic the Tygerberg Academic Hospital, Cape Town, South Africa and the Nigerian PD patients at the Neurology Clinic the Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife, Nigeria. The patients gave written informed consent before participating in the study and ethical committee approvals were obtained from both centres. This study was approved by the Health Research Ethics Committee of Stellenbosch University (HREC S16/08/151), and the Obafemi Awolowo University Teaching Hospital's Ethics and Research Committee (ERC/2015/08/15). The patients fulfilled the diagnostic criteria by the UK Parkinson's Disease Society Brain Bank clinical diagnostic for PD. Patients' baseline demographic data were obtained. Venous blood (20 ml) was collected into two EDTA tubes (10 ml each) for genomic DNA extraction.

Genomic DNA extractions were performed using the Nucleo Spin Blood XL kit (Macherey-Nagel Duren, Germany). The protocol provided by the manufacturer was followed.

The quality and concentrations of the DNA samples were determined using Nanodrop®. DNA samples were prepared according to the instructions by the manufacturer of the MLPA kits (MRC Holland SALSA MLPA Kit P051). The DNA samples included a total of 57 PD patients, three healthy individuals (negative reference controls), no-DNA control, and one PD patient (positive reference) previously tested for exon rearrangements and found to have a deletion in exons 3 and 4 of the *PRKN* gene.

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3.2.2. MLPA assay

The experiments were performed according to the manufacturer's instruction. The MLPA assay was performed in three steps. The first step was the DNA denaturation. On the first day, briefly, 5 µl of the DNA samples (10 ng/µl) were pipetted into 0.2 ml tubes. The DNA samples were denatured in a thermocycler at 98°C for 5 minutes. The samples were cooled down at room temperature.

The DNA samples were hybridized to the SALSA MLPA Kit P051 probe mix by adding to each sample an aliquot of 1.5 µl of probe mix and 1.5 µl of the MLPA buffer. The samples were incubated at 95°C for 1 min, and finally hybridized at 60°C for 16 to 24 hours.

On the second day, the hybridised probes were ligated. The thermocycler temperature was reduced to 54°C, while the samples were still in the thermocycler, 32 µl of Ligase-65 mix were added to each reaction tube, kept at 54°C for 15 min, and finally heated at 98°C for 5 min to inactivate the ligase enzymes.
Thereafter, the samples were cooled down at room temperature for the PCR amplification of the ligated probes. To each reaction mixture, 10 µl of the polymerase master mix were added and immediately transferred into a thermocycler and the PCR was started (33 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds, and a final 20-minute step at 72°C) The reaction was paused at 15°C.

The final reaction products were electrophoresed using an ABI 3100 DNA analyzer (Applied Biosystems) with Liz 500 size standard 0 (Thermo Scientific, Waltham, Massachusetts, USA) and Gene Mapper software v3.0 (Applied Biosystems) at the Central Analytical Facilities of Stellenbosch University.

The data were uploaded on the Coffalyser. Net software (v14072.1958, MRC-Holland, Netherlands) for the copy number analysis. The Coffalyser.Net software provides quality scores for each reaction.

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Coffalyser.Net starts with raw data analysis (baseline correction, and peak identification) and extensive quality control (e.g. DNA quantity used; complete DNA denaturation, degree of sloping) and interpretation of the control fragments that are included in the SALSA MLPA PO51 probe mix. We determined the final probe ratios by comparing the relative probe peak in the DNA sample of interest to all reference samples. Reference DNA samples are expected to have a normal copy number for both the reference and target probes.

3.2.3. Analysis

The MLPA assay was performed on the 57 samples in one reaction according to the experimental procedures. The data were visually analysed to identify peak signal sloping in the eletropherogram graphs. The samples that showed peak signal sloping were repeated. The raw data files (.fsa) were analysed using Coffalyser.Net software. The software required that the fragment MLPA reaction scores (FMRS) for each sample be above the threshold support for a comparative analysis. The score is based on:

- Signal intensity of the median probe fragment
- Maximum probe signal intensity
- Baseline intensity of probe dye
- Signal drop of internal run of probe fragments in combination with signal widening
- Percentage of unused primers
- Probes to peak noise percentage
- DNA concentration check
- DNA denaturation check

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Hence, only the data with FMRS percentage quality scores of 60–100% were included in the comparative analyses between the samples and the references. We corrected errors related to sloping by setting the major outlier ratio filter for low and high peaks at 0.4 and 2.0 respectively to eliminate false negative or false positive results. The pre-ratios and the final ratios generated for the patient samples were compared with the mean values of the corresponding negative reference samples. The typical value for a normal copy number is 1.0. A possible locus rearrangement such as deletion or multiplication with the ratio value of 0.5 or 1.5, respectively. As described in the MLPA manual *(*https://www.mlpa.com*)* the ranges of the final ratio values for a heterozygous deletion of one allele copy is 0.33 to 0.62 (mean 0.52), while the range is 1.27 to 1.41 (mean 1.33) for heterozygous duplications (three allele copies), and 1.76 to 2.0 (mean 1.92) for triplications (four allele copies) **(**https://www.mlpa.com**)**.

3.3. Results and Discussion

In this study, we detected heterozygous deletions in the *PRKN* exons 3 and 4 in the sample which was used as a positive control and previously found to contain these deletions in the Mahne et al 2016 study on a Black South African PD patient. This result proved that the MLPA was working in our laboratory. The analysis of the 57 PD patients for CNV revealed three Nigerian patients with exonic rearrangements. A decrease or increase in the copy number ratio was observed in those three patients. The variations in the ratios indicated possible exonic rearrangements in their DNA (**Table 3.2**). Sample 13.039 had a heterozygous deletion in *PARK7* exon 1. Sample 13.253 had two heterozygous duplications in *PINK1* exon 5 and in *PRKN* exon 1. The third sample 13.334, had two heterozygous duplications in *PRKN* exons 1 and 2 **(Table 3.2**). In each case, the signal peaks in the electropherogram were compared with the reference peaks and showed that variation occurred in the copy number **(Figure 3.1)**.

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Table 3.2: Summary of copy number variations identified in this study

Sample	Ethnicity	CNV identified	Probe length (bp)	CNV ratio
13.039	Yoruba	*PARK7* exon 1 deletion	350	0.5
13.253	Yoruba	*PINK1* exon 5 duplication	143	1.5
13.253	Yoruba	*PRKN* exon 1 duplication	237	1.5
13.334	Yoruba	*PRKN* exon 1 duplication	237	1.5
13.334	Yoruba	*PRKN* exon 2 duplication	287	1.5

See Figure 3.1: For electropherogram results.

The study of CNV mutations is important for the understanding of the pathogenesis of PD. MLPA SALSA PO51 probe mix used in this study could analyse most of the exons of the four known PD genes.

The positive reference used in this study was a Black South African PD patient with deletions in *PRKN* exon 3 and 4 and had PD AAO of 50 years with no family history (**Figure 3.1**).

There are three major mechanisms accountable for genomic rearrangements which have been described in **Chapter 1**: NAHR, NHEJ, and FoSTeS. These three mechanisms are involved in pathogenic genomic rearrangements (Gu et al 2008 and Morais et al 2016). NAHR generates CNVs when genomic segments with high sequence similarity recombine. The double stranded breaks in DNA sequence recruit NHEJ-associated proteins to repair and ligate DNA strands together so that if fragments from different chromosomes ligate together, duplications or deletions of sequence can occur. The FoSTeS serial replication represents a fork disengaging and lagging strand invasion occurring several times during DNA replication processes (La Cognata et al 2017). The CNV aberrations could affect the overall functions of a gene, either by damaging or reducing the effects of a gene. They could also cause an overexpression of a gene if the CNV is a tandem repeat (multiplications). The types of CNVs identified in this study were in these categories.

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The chromosomal regions mostly affected by the CNV are the subtelomeric regions and contain an abundance of segmental duplications. They are more susceptible to non-allelic homologous recombination and are potential regions for genomic instability generating CNVs (Gu et al., 2008; Sharp et al., 2006) . These loci containing many genes including the known PD genes are located in the distal chromosome regions, and are susceptible to high recombination rates

PARK2 spans approximately 1.38 Mb and encodes the protein parkin. The 456-amino acid protein harbors four major functional domains corresponding to its function as an E3 ubiquitin ligase **(Figure 3.2**). Its role in the ubiquitin-proteasome system and mitochondrial maintenance has been described in Chapter 1. Dysregulation of mtDNA homeostasis is a key process in the pathogenesis of neuronal loss in PD (Pinto et al., 2018).

A study of a complete spectrum of mtDNA changes, including deletions, duplications and point mutations in *PRKN* in single neurons from the dopaminergic *substantia nigra* in PD patients and controls showed changes in mtDNA copy number relating to a compensatory mechanism to the accumulated deletions in *PRKN* gene (Dölle et al., 2016). The study suggested that dysregulation of mtDNA homeostasis is a key process in the pathogenesis of neuronal loss in Parkinson disease. They excluded patients with known monogenic causes of PD to ensure that their sample was homogenous and representative for sporadic PD. They ensured that no monogenic mutations were identified in the PD patients in the known PD genes. It is important to relate recent findings in PD genetics research in literature. Considering also, that, the patients studied here are sporadic cases. However, discussing the Dolle *et al* 2016 findings in the present study might not be applicable if the CNVs identified in PRKN gene in the patient are causative or inherited.

The *PRKN* exons 1 and 2 where duplications were identified in this study are part of the *PRKN* coding region and are being expressed during transcription **(Figure 3.2**).

Mutations in *PRKN* gene have been associated with an early-onset PD (Morais et al 2016).

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Parkin mutations are most frequent, explaining 50% of the cases with a clinical diagnosis of familial Parkinson's disease compatible with recessive inheritance and onset <45 years, and 15% of the sporadic cases with onset <45 years (Bonifati, 2012).

Large deletions account for approximately 50%–60% of the *PRKN* disease–causing mutations, only a small number of small deletions or duplications which are a result of exon-exon breakpoints have been identified. Small deletions and duplications occur as well in this region in *PRKN* (Morais et al., 2016).

The CNV mechanism implicated in *PRKN* was NAHR, mediated by Alu elements. Alu-mediated NAHR seems to be the responsible mechanism for the CNVs observed in this study. This mechanism has been previously described in *PRKN* deletions and duplications observed in early onset PD (Jun-Mitsui et al 2010). In contrast, rearrangements that can be explained by NAHR are relatively rare. There is only a limited number of rearrangements (4.3%) in PRKN in patients with early onset PD whose junctions show extended homologies (Mitsui et al., 2010).

In this study, the patient with *PRKN* exon 1 and 2 duplications had AAO of 50 years with no family history of PD (**Supplementary Table 3.1**). The exonic duplications observed may suggest a loss-of-function and possible reduced expression of parkin ubiquitin ligase. CNVs can also regulate the expression of target genes through position effects i.e. through the insertion or deletion of sequences leading to alterations in distance to regulatory elements (Gamazon and Stranger 2015). However, transcriptional analyses will be required to show gains, losses and diversification of functions that characterize the effects of deletion or duplications identified in CNV in this study

It is noteworthy that compound heterozygous duplications of exons 7 and 9 in *PRKN* have also been reported (Hedrich et al., 2002).

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Genomic positions of PRKN exon 1 and 2 [162727771-162443353 GRCh38 coordinates; reverse strand].

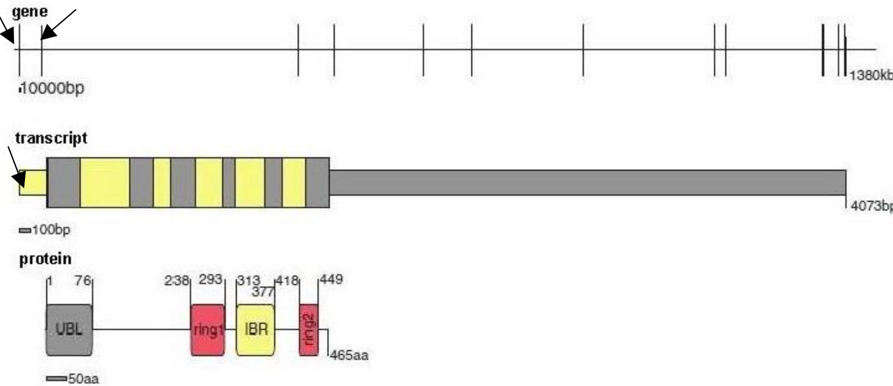

Figure 3.2: Representation of PRKN on genomic and transcript level and the functional domains of the parkin protein. *The PRKN gene spans approximately 1.38 Mb and encodes the protein parkin. It has 12 exons. The 456-amino acid protein harbors four major functional domains corresponding to its function as an E3 ubiquitin ligase. The arrows indicate the locations for PRKN exon 1 and 2 duplications . On transcript level exons are coloured alternately (NM_004562.2). (UBL: ubiquitin-like; IBR: in-between-ring) taken from Nuytemans et al 2010. Permission from John Wiley and Sons.*

The *PARK7* gene has a size of 34 kb with seven exons (**Figure 3.3**). It is among the known PD genes. It accounts for 1.2% of single or multiple exon deletions and duplications reported in PD patients. CNVs in *PARK7* are not as common as point mutations. But, because of the rarity of mutations in *PARK7*, most studies have limited information for *PARK7* CNVs in PD.

In this study, the patient with the *PARK7* exon 1 deletion had AAO of 71 years. The majority of the Nigerian PD patients recruited for this study have late age-at-onset PD. Previous studies from various populations found *PARK7* gene to harbour CNVs in PD. A study on a Dutch family identified CNVs in *PARK7* gene. A homozygous deletion of 14 kb involving the first five of seven exons was found (Bonifati et al. 2003). Further heterozygous CNVs (both deletions and duplication) in *PARK 7* gene have been described in PD studies.

The authors identified two carriers of single heterozygous loss-of-function *PARK7* mutations, including a heterozygous deletion of exons 5 to 7 and an 11-base pair deletion, removing the invariant donor splice site in intron 5.

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Interestingly, both PARK7 mutations identified were found in the heterozygous state only. (Djarmati et al., 2004; Guo et al., 2010; Hedrich et al., 2004). Detailed mechanisms on how *PARK7* contributes to PD have been described in Chapter 1, however, it remains not fully understood how CNVs in *PARK7* develop.

Genome position of PARK7 exon 1 locations [7961692 –7961735 GCHR 38 coordinates-reverse strand]

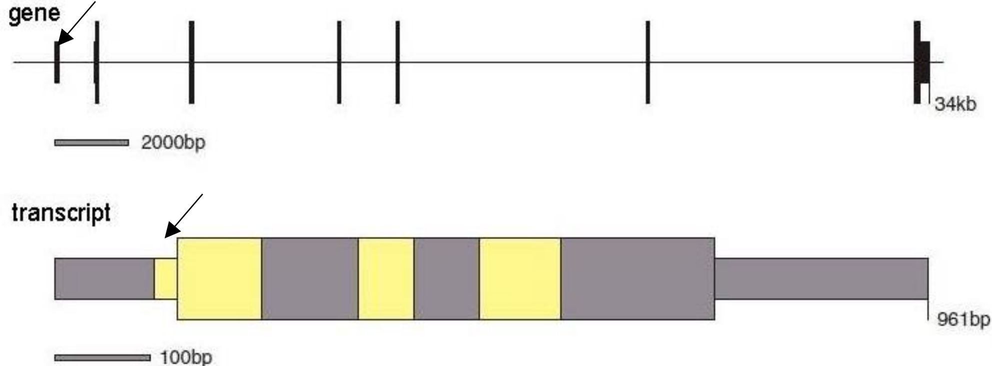

Figure 3.3: Representation of PARK7 on genomic and transcript level. On transcript level exons are coloured alternately (NM_007262.4). *It has 8 Exons and locates at chromosome band 1p36.23. The product of this gene belongs to the peptidase C56 family of proteins. It acts as a positive regulator of androgen receptor-dependent transcription. The arrows indicate the locations where PARK7 exon 1 deletion was located. The diagram is* taken from *Nuytemans et al 2010*. Permission from John Wiley and Sons.

PINK1 gene is 18 kb. PINK1 protein is a putative serine/threonine kinase involved in mitochondrial response to cellular and oxidative stress **(Figure 3.4)**. Approximately 6.5% of known mutations in familial and sporadic patients carry a mutation in *PINK1*, (Nuytemans et al 2010).

A study by Marongiu et al showed that deletions in *PINK1* exons in PD patients are pathogenic. The authors identified an Italian PD patient with a deletion of the entire *PINK1* gene and a splice site mutation in the gene (Marongiu et al., 2007).

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Likewise, homozygous and compound heterozygous deletions involving different combinations of *PINK1* exons 4–8 have been described in both familial and sporadic cases in PD patients from Japan, Brazil, Sudan and Iran (La Cognata et al 2017).

The largest heterozygous deletion known so far is 56 kb and includes the entire *PINK1*. Further heterozygous deletions in *PINK1* involving exons 1, 3–8 and exon 7 have also been described (La Cognata et al 2017). However, while *PINK1* exon dosage variation is rare, and the ones identified were mostly deletions, hence, it is simply not common to identify a duplication in *PINK1* gene, but it is not impossible that *PINK1* exon 5 duplication was identified in a patient in this study, because exon dosage analyses on *PINK1* gene are rare.

Genomic position of PINK1 exon 5 locations [20645560 –20645723, GCHR 38 coordinates-reverse strand]

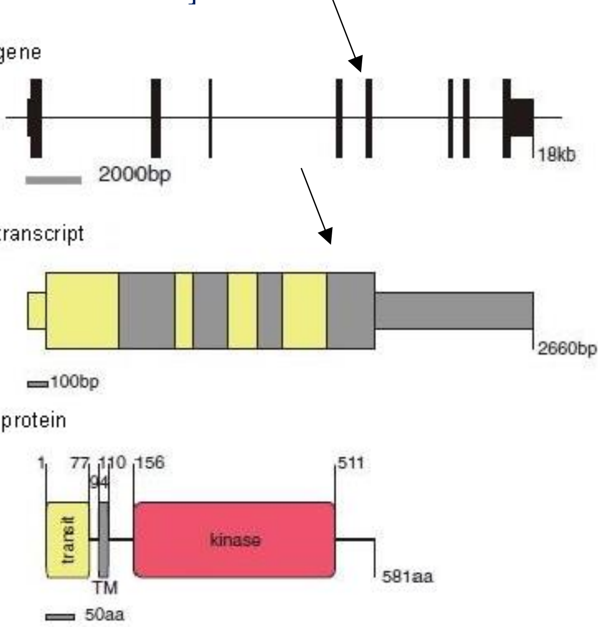

Figure 3.4: Representation of PINK1 on genomic and transcript level and the functional domains of the PINK1 protein. *PINK1 gene is 18 kb and encodes a protein called PTEN induced putative kinase 1.* PINK1 *protein is a putative serine/threonine kinase involved in mitochondrial response to cellular and oxidative stress. The diagram shows the locations of the duplication at gene and transcript levels. On transcript level exons are colored alternately (NM_032409.2). Taken from Nuytemans et al 2010 Permission from John Wiley and Sons.*

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The roles of the CNVs identified in this study need to be investigated further for disease mechanisms and also to identify the specific regions in the DNA susceptible to structural rearrangements, such as tandem multiplication and duplications that often occur due to ambiguity in alignment of repeat sequences (Frith and Khan 2018). CNVs in certain genes like *PRKN* are frequent because of the unstable regions where such genes are located in the genome. The degree of multiplication varies greatly, e.g. duplication (most common) or multiple duplications. (Frith and Khan 2018).

3.4 Study limitations

There were several limitations to this study. It is noteworthy to stress that only one MLPA probemix (PO51) was used for detecting CNVs in this study. In a situation where the CNV electropherogram peaks when compared to the reference probe peak did not confirm the presence of CNV, we did not consider those CNVs further (e.g. *ATP13A2* exon 4 and *PRKN* exon 4 duplications identified in 54 of the 57 patients screened, but the electropherogram peak ratios did not confirm the results. It was therefore, suggested that, they might be artefacts or a random CNVs or maybe polymorphisms that exist in the population screened. In fact, the presence of polymorphisms in this assay could prevent probe ligation, leading to no amplification and wrong identification of CNVs.

It is necessary to mention that the 15 South African and 14 of the 42 Nigerian patients used in this study were also included in the tNGS analysis that screened the coding regions of these genes for pathogenic variants. However, tNGS is limited by its inability to correctly identify CNV especially large deletions or duplications.

In addition, considering that the probemix ligation and amplifications were not quantified especially by using qRTPCR, which makes the study less robust. However, both the internal reference probe and the choice of electrophoresed size markers used, could have detected possible problems related to probe ligations and amplification during the analyses.

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In the panels of the **Figure 3.1 (a-d)**, The variability observed in the reference signal can be introduced by a number of factors, including sample treatments, since all the samples were not screened at the same time. Differences in reaction conditions do not always affect all probes equally, which can lead to changes in relative probe signals. For example, there is a relationship between relative probe signals and polymerase activity. The relative signal of some probes may decrease with reduced polymerase activity, whereas other probes may actually produce higher signals. Variability can also be the result of experimental issues such as pipetting errors. Evaporation can also lead to variability. It is always recommended that the result be validated using other methods.

Moreover, it is important to use an adittional method to verify CNVs identified in this study. It is important therefore, to use two different MLPA probemix to limit errors relating to false positive results.

In conclusion, this study suggests that SSA PD patients could harbour CNVs. The use of the MLPA method to identify CNV was further established in this study. The CNVs identified in this study have not been described in the SSA PD patients in the literature. Since these are recessive genes, there must be two mutations to lead to a disease phenotype. This has not been demonstrated and we are not able to conclude if the CNVs are causative. To identify more CNVs in the patients from SSA origin, sample size must be increased. It will also be necessary to study family members of the PD patients to ascertain the type of mutations, and to determine if there is a founder effect. Finally, it will be essential to establish the mechanisms of pathogenicity of these CNV mutations in PD.

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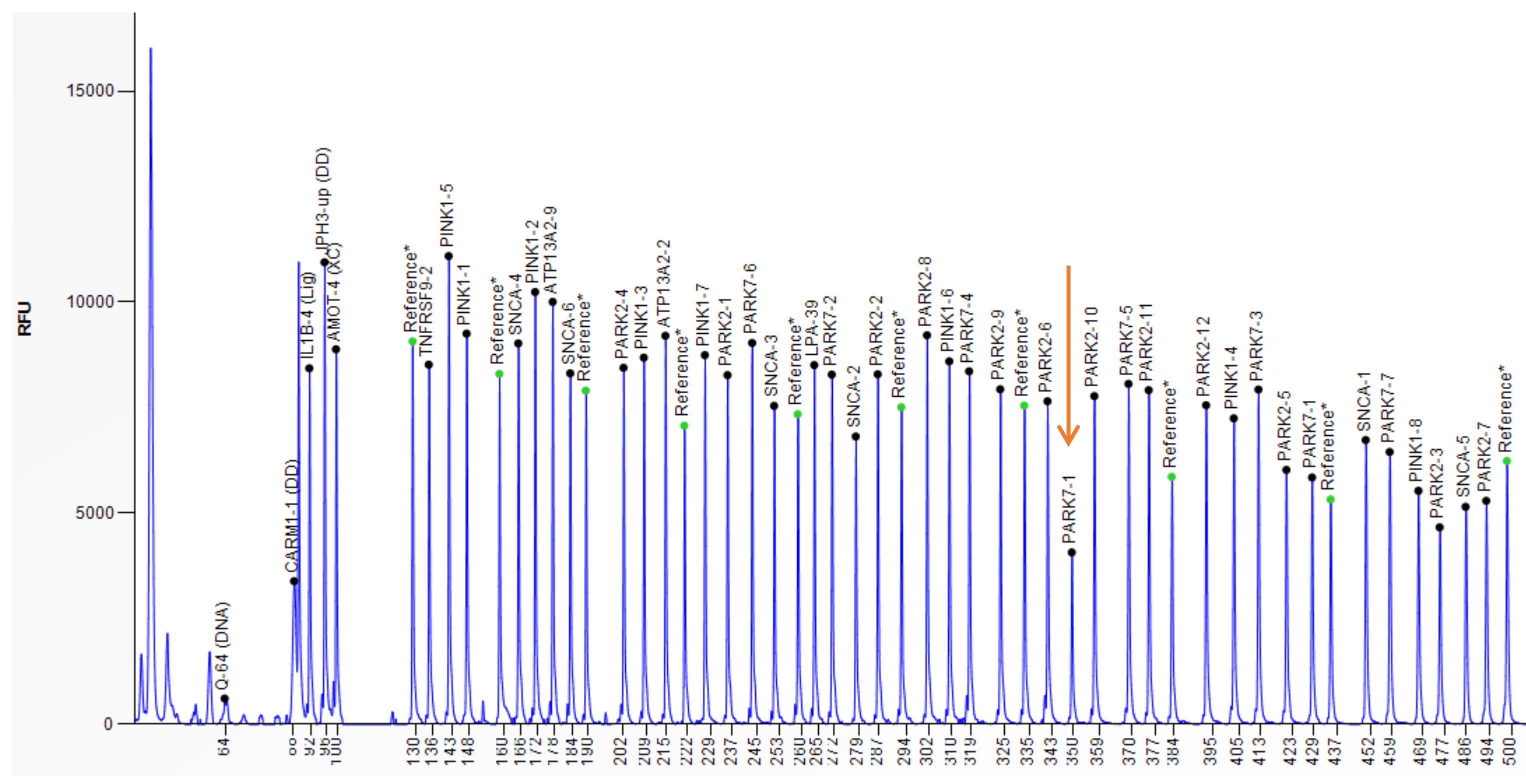

a.

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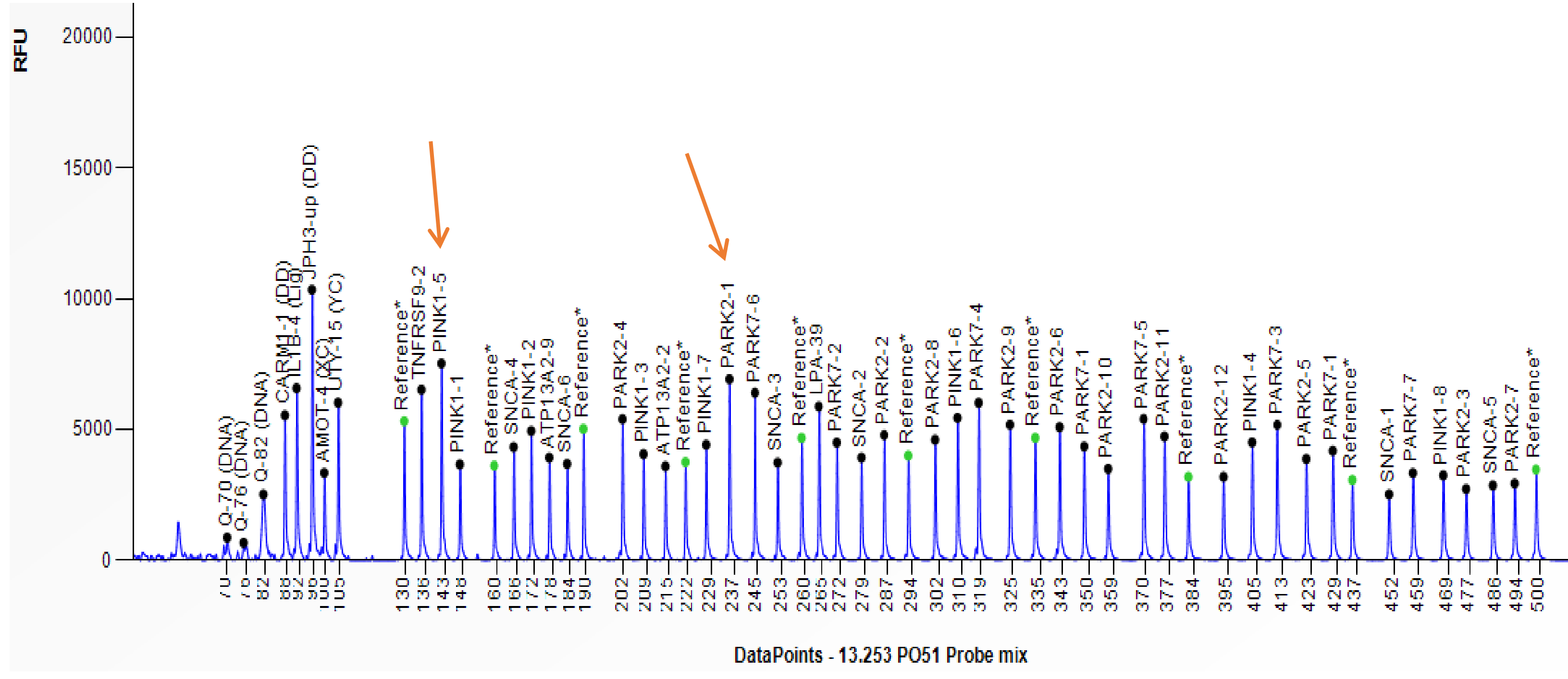
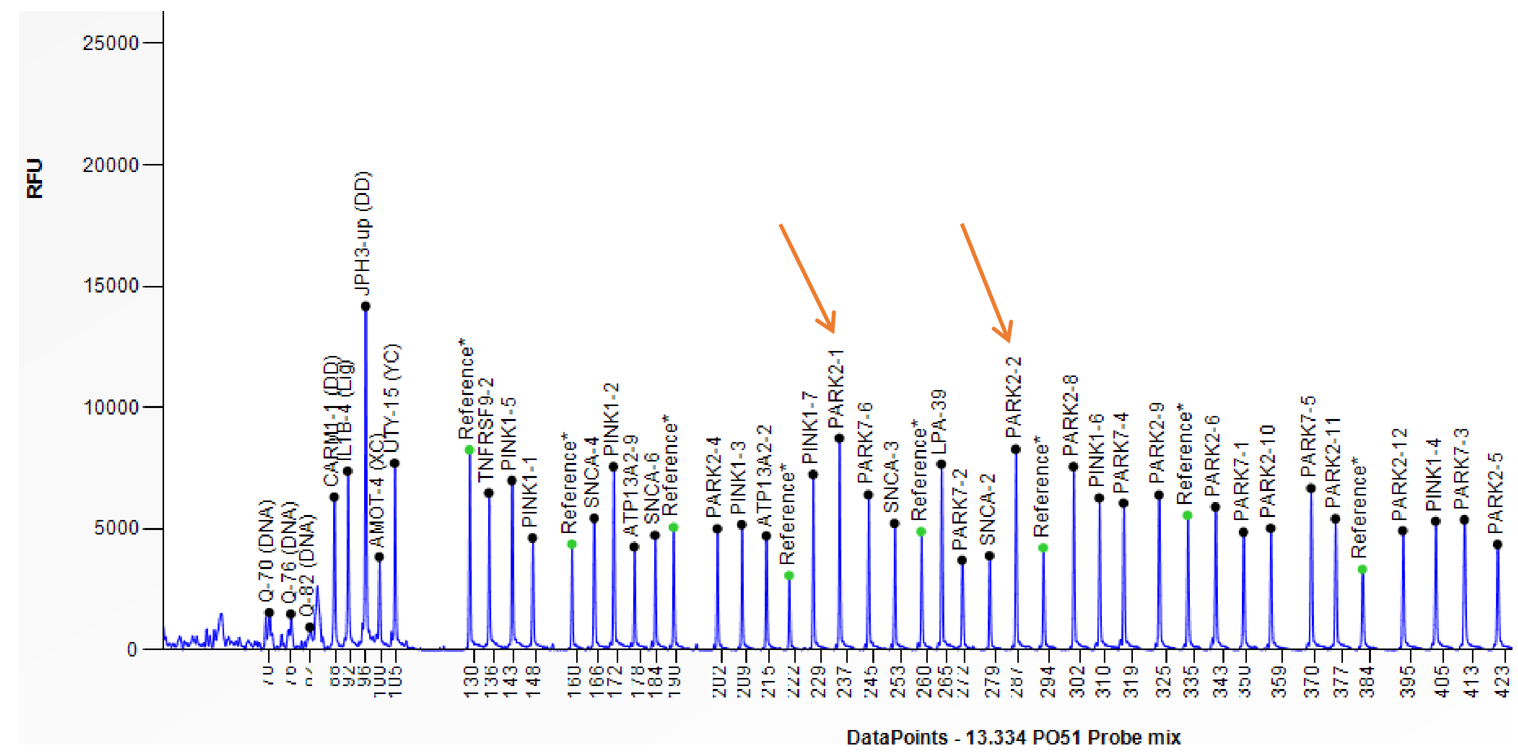


b.



c.

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d.

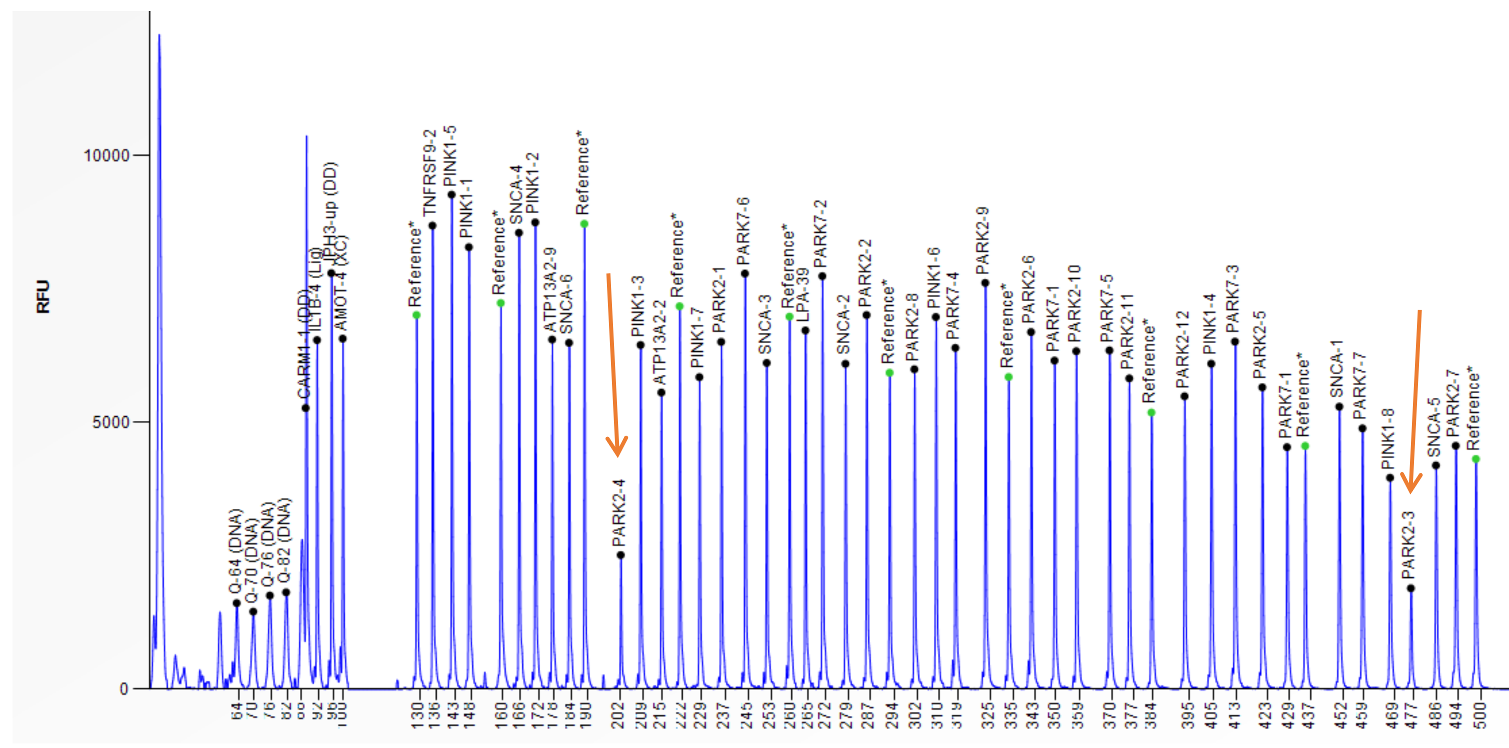

Figure 3.1: Representative electropherograms from MLPA. Figures a-c show results for three Nigerian PD patients (samples 13.039, 13.254 and 13.334) and d shows results for a positive control sample. The figures demonstrate signal peaks of the PD-MLPA fragments obtained by using the SALSA P051 probe mix of the commercially available MLPA kit followed by analysis using Coffalyser. Net software (v14072.1958, MRC-Holland, Netherlands). Red arrows indicate fragments in which CNVs were identified. In sample 13.039 the PARK7-1fragment had a lower peak than expected and is, therefore, likely to contain a deletion. In sample 13.254 two peaks with abnormal heights were observed: PINK1-5 and PRKN-1 fragments had higher than expected peaks and thus are likely to contain insertions. In sample 13.334, two peaks with abnormal heights were observed: PRKN-1 and PRKN-2 fragments had higher than expected peaks and thus are likely to contain insertions

Table S3.0: Demographics of the 57 PD patients.

Sample code	Sex	Ethnicity/ Country	AAO (y)	AAD (y)	Family history of PD	Affected family member	Other diseases
96.87	M	Black/SA	37	38	No	N/A	No
10.308	F	Black/SA	49	77	No	N/A	No
10.309	M	Black/SA	52	59	No	N/A	No
10.313	F	Xhosa/SA	43	49	No	N/A	No
10.314	F	Black/SA	55	57	No	N/A	No
11.781	F	Black/SA	31	35	No	N/A	No
11.830	F	Black/SA	57	60	No	N/A	No
11.833	M	Black/SA	48	50	No	N/A	No
11.834	M	Black/SA	52	56	No	N/A	No
11.835	F	Black/SA	52	60	No	N/A	No
11.894	M	Black/SA	44	49	No	N/A	No
11.895	M	Black/SA	49	53	No	N/A	No
11.962	M	Black/SA	53	77	No	N/A	No
12.177	M	Black/SA	45	55	No	N/A	No
12.178	F	Black/SA	53	55	No	N/A	No
12.975	F	Yoruba/N	80	80	No	N/A	Hypertension
13.007	M	Yoruba/N	52	59	No	N/A	Hypertension
13.008	M	Yoruba/N	62	66	No	N/A	Hypertension
13.036	M	Yoruba/N	57	64	No	N/A	Hypertension
13.037	M	Yoruba/N	79	81	No	N/A	No
13.038	M	Yoruba/N	36	42	No	N/A	No
13.039	F	Yoruba/N	71	73	No	N/A	No
13.090	M	Yoruba/N	59	60	No	N/A	Hypertension
13.091	M	Yoruba/N	79	80	No	N/A	No
13.092	M	Yoruba/N	48	59	No	N/A	No
13.093	M	Yoruba/N	53	64	No	N/A	No
13.094	F	Yoruba/N	62	64	No	N/A	No
13.095	M	Yoruba/N	73	81	No	N/A	No
13.096	M	Yoruba/N	68	71	No	N/A	No
13.117	M	Yoruba/N	60	64	No	N/A	No
13.119	F	Yoruba/N	62	63	No	N/A	No
13.118	M	Yoruba/N	63	64	No	N/A	Hypertension
13.147	F	Yoruba/N	69	71	No	N/A	No
13.167	M	Yoruba/N	72	81	No	N/A	No
13.168	M	Yoruba/N	55	61	No	N/A	No
13.185	M	Yoruba/N	54	57	No	N/A	No
13.186	M	Yoruba/N	60	72	No	N/A	No
13.202	M	Yoruba/N	66	77	No	N/A	No
13.219	M	Yoruba/N	49	49	No	N/A	No
13.220	F	Yoruba/N	70	73	No	N/A	Hypertension
13.241	M	Yoruba/N	84	84	No	N/A	No
13.253	F	Yoruba/N	71	71	No	N/A	No
13.254	M	Yoruba/N	77	78	No	N/A	No
13.275	M	Yoruba/N	69	69	No	N/A	No
13.276	F	Yoruba/N	66	66	No	N/A	No
13.277	M	Yoruba/N	77	78	No	N/A	Hypertension
13.278	M	Yoruba/N	58	60	No	N/A	Hypertension
13.310	M	Yoruba/N	83	84	Yes	Sibling,	No
13.311	M	Yoruba/N	70	74	No	N/A	No

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Sample code	Sex	Ethnicity/ Country	AAO (y)	AAD (y)	Family history of PD	Affected family member	Other diseases
13.321	M	Yoruba/N	63	66	No	N/A	No
13.322	M	Yoruba/N	48	58	No	N/A	No
13.323	M	Yoruba/N	68	74	No	N/A	No
13.324	M	Yoruba/N	66	72	No	N/A	No
13.325	M	Yoruba/N	70	73	No	N/A	No
13.334	F	Yoruba/N	50	54	No	N/A	No
13.335	M	Yoruba/N	59	60	No	N/A	No
13.369	F	Yoruba/N	60	62	No	N/A	No

N/A: Not applicable.

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CHAPTER FOUR

TARGETED NEXT-GENERATION SEQUENCING IDENTIFIES NOVEL VARIANTS IN CANDIDATE GENES FOR PARKINSON'S DISEASE IN BLACK SOUTHAFRICAN AND NIGERIAN PATIENTS

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CHAPTER FOUR

TARGETED NEXT-GENERATION SEQUENCING IDENTIFIES NOVEL VARIANTS IN CANDIDATE GENES FOR PARKINSON'S DISEASE IN BLACK SOUTHAFRICAN AND NIGERIAN PATIENTS

The following chapter consists of a manuscript which has been submitted to the *Frontiers in Genetics Journal*, and it is currently under consideration for publication.

My contributions to this chapter;

- I submitted a proposal used for the ethics approval for this study
- I applied for approval of the importation and exportation of specimen permit
- I retrieved and documented all the patients demographics data used in this study
- I conducted laboratory procedures with other experts in NGS
- I performed preliminary data analyses
- I prepared tables and figures for the results
- I drafted the manuscript

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Word count: 7,644

Number of figures: 4

Running title: Targeted Sequencing of African PD patients

Keywords: Parkinson's disease, next-generation sequencing, scoring of sequence variants, sub-Saharan Africa, South Africa, Nigeria, sequence variants

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4.1 ABSTRACT

BACKGROUND: The prevalence of Parkinson's disease (PD) is increasing in sub-Saharan Africa, but little is known about the genetics of PD in these populations. Due to their unique ancestry and diversity, sub-Saharan African populations have the potential to reveal novel insights into the pathobiology of PD.

METHODS: We recruited 33 Black South African and 14 Nigerian PD patients, and screened them for mutations in 751 genes using an Ion AmpliSeq™ Neurological Diseases panel. We used bcftools to filter variants by quality score > 100 and a minimum read depth > 40, used *annovar* utility software for the annotation, removed variants with minor allele frequency >0.01 in any of the frequency databases, and selected variants using MetaLR and MetaSVM prediction scores. We generated radar plots of these selected variants predicted to be deleterious to illustrate the overall pathogenicity prediction using 17 different prediction scores. We used ConsensusPathDB to generate protein-protein interaction networks, and investigated the effect of *PINK1* variants on the protein structure by molecular modelling.

RESULTS: The mean age-at-onset of PD in the South Africans and Nigerians was 48 ± 8 and 63 ± 13 years, respectively. Sequence data from all samples passed global quality control criteria. We identified 14,655 rare variants with a minor allele frequency ≤0.01, of which, 7,934 were intronic, 5,695 exonic, 27 intergenic, 539 in untranslated regions, and 430 in non-coding RNA. They included 3,175 synonymous and 2,448 missense variants. Altogether 60 variants in 44 genes were considered deleterious and were prioritized based on MetaLR and MetaSVM scores for future follow-up studies. Of the 44 proteins encoded by these genes, 52% (23) were classified as enzymes. We found seven novel variants in three known PD genes *ATP13A2*, *PRKN* and *PINK1*. Protein modelling suggested that PINK1 variant S228F, a site for autophosphorylation, is near the interaction surface with PRKN.

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CONCLUSIONS: We identified variants predicted to be pathogenic in genes not previously known to harbour mutations in PD patients from sub-Saharan Africa. We also identified novel mutations in known PD genes. Further studies are required to validate the findings and ascertain the biological effects of these variants.

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4.2 Background

Parkinson's disease (PD) is a debilitating neurodegenerative disorder that impairs patients' motor skills, and speech coordination. It is one of the leading causes of disability and mortality among neurological disorders globally (GBD 2015 Neurological Disorders Collaborator Group, 2017). Currently, there is no drug that can prevent, reverse, or halt the progression of the disease. The neuropathological hallmark of PD is the progressive loss of predominantly dopaminergic neurons of the *substantia nigra pars compacta* of the midbrain, which regulate voluntary movement. PD affects 1–2% of individuals over the age of 60 years and ~4% of people over the age of 80 years (de Lau and Breteler, 2006a). The diagnosis of PD is largely clinical using criteria such as the UK PD Society Brain Bank criteria (UKPDSBBC) to differentiate ageing related symptoms from PD (Jankovic, 2008).

The pathobiology of the disease is yet to be fully elucidated, but environmental and genetic factors have been linked to PD etiology (Cannon and Greenamyre, 2013; Chin-Chan et al., 2015). PD symptoms usually manifest in the same way in all patients, but the prevalence, incidence and risk factors may vary according to the geographical region (Pringsheim et al., 2014c). Estimates of PD prevalence in sub-Saharan Africa (SSA) vary widely across previous studies and range from 10 to 235/100,000 in urban populations (Lekoubou et al., 2014b; Williams et al., 2018b).

Genetics as an etiologic concept in PD has been well-established (Klein and Westenberger, 2012; Lill, 2016b). Approximately 5–10% of PD patients have a monogenic form of the disease, which is due to highly penetrant, rare pathogenic mutations (Lill, 2016b). For sporadic forms of this disorder, the genetics is complex as common genetic variants may act in concert with environmental factors (Lill, 2016; Polito et al., 2016; Zschiedrich et al., 2009). The genetic discoveries have led to important hypotheses about the mechanisms underlying PD, which include dysfunction of the ubiquitin–proteasome system and mitochondrial dysfunction coupled with oxidative stress (Jain et al., 2005).

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The functional role of the common pathogenic PD mutations is currently being studied in various cellular and animal models.

Most of the studies on the established PD genes (*SNCA, LRRK2, PRKN, PINK1, PARK7 and ATP13A2)* or in the PD-related genes (*GBA, CHCHD2, EIF4G1, SPG11, SYNJ1, TMEM230, MAPT, FBXO7, VPS13C, VPS35, GRN, GIGYF2, HTRA2, DCTN1 and DNAJC6*) were performed in European, North American, North African Arab or Asian populations (Clarimón and Kulisevsky, 2013; Lill, 2016b; Popescu, 2016). Common PD mutations such as *LRRK2* G2019S, have not been studied extensively in SSA populations. Notably, the frequency of *LRRK2* G2019S varies widely across different geographic regions; 1–2% in European PD patients and much higher in Ashkenazi Jews (~20%) and in North-African Arab (41%) PD patients (Bras et al., 2008; Lesage et al., 2006). In general, limited studies exist on the genetics of PD in the Black African populations (Blanckenberg et al., 2013c).

It has been suggested that the common PD mutations are rare among South African PD patients (Blanckenberg et al., 2014b; Haylett et al., 2012b). Similarly, a previous genetic study screened for mutations in *LRRK2*, *PRKN* and *ATXN3* in 57 Nigerian PD patients but did not identify any pathogenic mutations (Okubadejo et al., 2008c). African populations have a diverse ancestry, and have more private alleles than any other populations, suggesting that the genetic etiology of PD in African populations could be unique (Gurdasani et al., 2015).

Next-Generation Sequencing (NGS) provides a way to explore the genetic basis of diseases, and has resulted in the discovery of a large number of disease-associated mutations (Olgiati et al., 2016a). In contrast to whole-genome or whole-exome sequencing (Farlow et al., 2016; Steele et al., 2015), targeted sequencing panels (Gorostidi et al., 2016) focus the analysis on specific genes of interest. Commercially available panels such as the Ion AmpliSeq™ Neurological Diseases Panel were designed to screen genes for mutations in neurological diseases as well as genes involved in brain function.

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This panel targets the exons and RNA splice sites of 751 candidate genes known to harbour mutations in neurological diseases including PD (www.ampliseq.com). In the present study, we used this panel to screen for deleterious sequence variants in Black South African and Nigerian Yoruba PD patients. We identified putative pathogenic mutations in a number of candidate genes in this group of patients.

4.3. Materials and Methods

4.3.1 Study Participants and Sample Collection

The study group consisted of 33 unrelated Black South African PD patients and 14 unrelated Nigerian sporadic PD patients. South African patients were recruited at the Neurology Clinic of Tygerberg Academic Hospital, Cape Town, South Africa, and at the Neurology Clinic of Frere Hospital, East London, South Africa. Nigerian PD patients were recruited at the Neurology Clinic, Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria. All patients were confirmed to have PD by the neurologists, based on the UKPDSBBC diagnostic criteria. All patients provided written informed consent to take part in the study. This study was approved by the Health Research Ethics Committee of Stellenbosch University (HREC 2002/C059, N16/04/041 and S16/08/151), and the Ethics and Research Committee of Obafemi Awolowo University Teaching Hospitals (ERC/2015/08/15). Demographic information and clinical characteristics of the patients are summarized in **Table 4.1**. Individual data on each study participant are available in **Table S4.1**.

Information about co-morbidities was collected, and included information on high blood pressure and diabetes on the Nigerian patients, and information on dementia and arthritis on the Black South African PD patients.

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Blood samples were collected at the recruitment sites into ethylenediaminetetraacetic acid (EDTA) tubes and genomic DNA (gDNA) were extracted using the Nucleo Spin Blood XL kit (Macherey-Nagel Duren, Germany).

4.3.2 Library Construction and Ion Torrent Sequencing

gDNA was quantified with a Qubit 2.0 Fluorometer using the Qubit dsDNA HS assay kit according to the manufacturer's protocol, MAN0002326 REVA.0. Quality scores were determined on the LabChip GXII Touch using the DNA Extended Range LabChip and the gDNA Reagent Kit according to the protocol CLS140166, Rev. C (PerkinElmer, USA). The Ion AmpliSeq™ Neurological Diseases panel and the Ion AmpliSeq™ Library Kit 2.0 (Thermo Scientific, Waltham, Massachusetts, USA) were used for multiplex PCR amplification of 751 genes (**APPENDIX V**) and library construction prior to sequencing on the Ion S5.

In addition to targeting all coding regions, an additional 101 non–coding disease–causing loci were targeted, as well as 14 repeat-expansion regions in genes *AFF2, AR, ATN1, ATXN7, ATXN8OS, ATXN3, CACNA1A, CSTB, DMPK, HTT, JPH3, PABPN1, PPP2R2B,* and *TBP*. Among the 751 neurological genes on the panel, there are 16 genes which have been previously reported to contain mutations in PD patients. These genes are: *SNCA, LRRK2, PRKN, PINK1, PARK7, ATP13A2, EIF4G1, GIGYF2, PLA2G6, FBXO7, VPS35, MAPT, HTRA2, SPG11, GRN* and *DCTN1*.

Using two primer pools with a 20 ng input gDNA template, the target regions were amplified in 13 cycles, consisting of 15 s at 99°C and 16 min at 60°C, on the SimpliAmp Thermal Cycler Kit (Thermo Fisher Scientific, Waltham, MA, USA) using the Ion AmpliSeq™ Neurological Diseases Panel according to the manufacturer's protocol, MAN0006735, REVE.0 (www.ampliseq.com).

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Following amplification with the two primer pools, the products were combined and primer sequences partially digested by the IonShear™ Plus Enzyme Mix II. Barcode adapters were used to generate adapter-ligated libraries. The libraries were purified with Agencourt™ AMPure™ XP reagent and eluted in 50 µl low-Tris-EDTA buffer.

The AmpliSeq™ library was quantified using the Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, MAN0015802 REVA.0. Quantitative PCR amplification was performed using the StepOnePlus™ Real-time PCR system (Thermo Scientific, Waltham, Massachusetts, USA) (**APPENDIX V1**). Libraries were diluted to 60 pM. The diluted, barcoded libraries were combined in equivamolar amounts using the Ion520™ and Ion530™ Chef Kit. In brief, 25 µl of pooled diluted library were loaded on the IonChef liquid handler using reagents, solutions and supplies according to the manufacturer's protocol, MAN0010846, REVD.0. Enriched, template-positive ion sphere particles were loaded onto two Ion530™ Chips. Massively parallel sequencing was performed on the Ion S5™ System using the Ion S5™ Sequencing Solutions and Sequencing Reagent Kits according to the manufacturer's protocol, MAN0010846 REV D.0 (Thermo Fisher Scientific, Waltham, *Massachusetts*, USA) at the DNA Sequencing Unit, Central Analytical Facilities of Stellenbosch University, Stellenbosch, South Africa.

4.3.3 Quality Control, Annotation and Variant Prioritization of Targeted NGS (tNGS) Data

The flow space calibration, base calling, alignment with the reference genome (GRCh38–hg19), coverage analysis and variant calling were performed using standard parameters in the Ion Torrent Suite Version 5.4.0. Sequenced variants, including insertions and deletions (INDELs), splice site variants, single nucleotide variants (SNVs), multiple nucleotide variants (MNVs), as well as variants in the 3' untranslated region (UTR3) and 5' untranslated region (UTR5) were identified.

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The variant call format (VCF) files produced by the Ion Torrent Software Suite (ISS) were filtered using *bcftools* to ensure that:

FILTER = PASS (the ISS internal criteria for setting the PASS filter were met)
QUAL > 100 (thequality score exceeded 100)
FMT/AO ≥ 20 (there were at least 20 read for the alternative allele)
FMT/DP ≥ 40 (there were at least 40 reads)
FMT/SAF ≥ 5 (there were at least 5 reads of the alternative allele in the forward direction)
FMT/SAR ≥ 5 (there were at least 5 reads of the alternative allele in the reverse direction)

These criteria ensured that the observation was made in both directions with at least five reads in either direction, the overall depth was at least 40 and the alternative allele depth was at least 20.

The VCF files were merged into a single file and processed with the utility *annovar* (annovar.openbioinformatics.org/) to produce an annotation file for all the variants that passed the criteria above. We separated the Nigerian and South African subsets of our data set. All available annotations were included. These included conservation scores, allele frequencies and functional predictions (Table S3). Perl (*https://www.perl.org/*) was used to extract data and analyses were carried out in R (R Core Team, 2018). Variants were extracted as homozygous (homo) or heterozygous (hetero) for the alternative allele.

The quality scores for all the variants passing the filters were extracted and analyzed in R. Using the *bam* files, *bedtools* was used to generate statistics on the coverage (depth of sequencing) for the regions in the *Ampliseq* capture panel as defined by the manufacturer's bed file. We read in the quality score for each variant in matrix format. In order to identify novel PD candidate genes, we focused on variants that are rare in control populations as defined by a minor allele frequency (MAF) threshold of 0.01.

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Kaviar allele frequency threshold was set to include only variants that had MAF ≤ 0.01 or no frequency information. We created global classifications of variants and generated a summary of variant types, to encode variants as synonymous, missense or frameshift in the variable amino acid class, insertion, deletion or substitution in the variable mutation type, as well as single or multiple base variants in the variable mutated base*)*.

We merged the variant summary (whether the variant was observed as a *homo*zygous or *hetero*zygous), with the annotation. We also computed how many individuals shared each variant, in total as well as in the regional ethnicities, and displayed the results using Venn diagrams (https://www.r-graph-gallery.com/14-venn-diagramm).

We searched for previously reported mutations in PD patients catalogued in the PDmutDB database (http://www.molgen.vib-ua.be/PDmutDB), from the list of variants identified. The PDmutDB (Cruts et al., 2012; Nuytemans et al., 2010) contains data on mutations in the following genes: *SNCA*, *LRRK2, PRKN, PINK1* and *PARK7*. We accessed the PDmutDB last on 30 September, 2018, and it contained 25 different pathogenic mutations in *SNCA2* in 50 different families, six different pathogenic mutations in *LRRK2* in 940 different families, 127 different pathogenic mutations in *PRKN* in 607 different families, 28 different pathogenic mutations in *PINK1* in 65 different families, and six different pathogenic mutations in *PARK7* in ten different families.

We used the prediction scores MetaLR and MetaSVM for selecting deleterious sequence variants (**Table S4.3**). MetaLR and MKetaSVM are themselves *ensembles* (composite models) of many other scores (Liu et al., 2016). Currently, these two have the best performance on curated data sets (training and test) of non-synonymous variants that contain both deleterious (protein-function altering) and benign variants. We, therefore, used these to prioritize the variants in our data.

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Both metrics were scaled as probabilities [0, 1] with scores close to 1 indicating certainty that the variant is deleterious. We used *score* >0.8 as a cut-off for including the variant into our list of "variants predicted to be deleterious" as recommended by Liu *et al* (Liu et al., 2016). For variants in genes known to harbour mutations in patients with PD in previous studies (*ATP13A2*, *PARK7*, *LRRK2*, *PINK1*, *PRKN*, and *SNCA)*, we used a less stringent filtering threshold >0.45.

The rationale for this was that there is a greater prior probability that a variant in these genes will have an effect on the disease than in genes not previously known to be associated with the PD. We also used 24 other variant scoring algorithms. We plotted the correlation matrix of all 26 mutation scoring algorithm outputs used in our study. We generated Radar plots (http://www.cmap.polytechnique.fr/~lepennec/R/Radar/RadarAndParallelPlots.html) for each variant likely to be deleterious to demonstrate the correlation among 17 different scoring algorithms. All scores were standardized to 0–1 scale with score 1 (furthest from the center of the graph) indicating strongest evidence that the variant is deleterious. See Supplement for details on the scores used in the analyses.

The BAM files of the tNGS data have been deposited to the European Nucleotide Archive (ENA) and can be retrieved with an accession number from https://www.ebi.ac.uk/ena/browse/data-retrieval-rest.

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4.3.4 Sanger Sequencing

We selected four novel variants for verification to confirm the tNGS results. Specific oligonucleotide primers were designed on Ensembl Genome Browser (https://www.ensembl.org/) and Primer3 web version 4.0.0 (primer3.ut.ee/) (Table S4). We used BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) to confirm the primer specificity. PCR was performed in a reaction mixture containing a final volume of 25 µl on a PCR machine (2720 Thermal Cycler, Applied Biosystems, Forster City, California, USA). Clean-up of PCR products was done by adding 0.05 ul of 100 units/µl (at 0.62 µM final concentration) Shrimp Alkaline Phosphatase and 0.5 µl of 20 units/µl (at 1.25 µM final concentration) Exonuclease 1 enzymes (New England Biolabs UK Ltd). Sequencing of the purified DNA samples was carried out using the BigDye® Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Forster City, California, USA) on an ABI Prism™ 3730 XL automated sequencer (Applied Biosystems, Forster City, California, USA), at the DNA Sequencing Unit, Central Analytical Facilities of Stellenbosch University.

4.3.5 High Resolution Melt (HRM) Analysis

Two novel variants were selected for HRM screening in 150 Black South African controls to assess their frequency. DNA samples were amplified using the same primers as for Sanger sequencing (**Table S4.3**) to screen for the sequence variants *ATP13A2* S960R and *PRKN* D245E. Briefly, a reaction mixture containing 1 µl of 2 µM (at 0.08 µM final concentration) SYTO9 fluorescence dye (ThermoFisher Life Technology, Waltham, Massachusetts, USA) was added, to make a final reaction volume of 25 µl. The samples were placed in a Rotor-Gene 6000 Series real-time analyzer HRM machine (Corbett Research UK Ltd, Cambridge, United Kingdom) for the real-time PCR and HRM. The range of melting temperatures selected was 65–90°C. The melting curves were viewed on the Rotor-Gene Q series software (https://www.qiagen.com) for the analysis of the normalised and the difference graphs.

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4.3.6 Protein Structures and Modelling for Functional Prediction

To understand the changes introduced by the variants predicted to be deleterious in the protein structure, we selected novel variants identified in this study in *PINK1*, a known PD gene, for this analysis. We extracted protein information from the Protein Data Bank (https://www.rcsb.org/) (Kouranov et al., 2006).

The human PINK1 structure was aligned to the *Pediculus humanus corporis* structure reported by Schubert et al (Schubert et al., 2017), using PyMOL (pymol.org) Structural alignment was possible because PINK1 is highly conserved between phyla. Exceptions to the conservation occur in limited regions that form loops. The most divergent regions are the insertion sequence 1, which is poorly conserved and in *P. humanus corporis* is about 20 amino acids shorter, and in insertion sequence 2, which is weakly conserved, but about the same length (Schubert et al., 2017). A previously reported pathogenic *PINK1* mutation L347P was used as a reference (Schubert et al., 2017).

We used Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) to construct a protein secondary and tertiary structure to extend known crystal structure for PINK1. Phyre2 is a suite of tools available on the web to predict and analyse protein structure, function and mutations (Kelley et al., 2015).

We then used Mobyle (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#welcome) to predict stability of β-turn secondary structure in *PINK1 S228F*. This method can predict both the presence and the type of β-turns, using propensities and multiple alignments (Fuchs and Alix, 2005).

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4.3.7 Network Analysis

We used Ingenuity Pathway Analysis® (IPA) tool version 9.0 (Qiagen's Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com) to determine the functional classes, cellular localization and drug targets of the 44 genes harboring variants predicted to be deleterious.

To determine the functional connections that exist between the proteins encoded by the known PD genes and other genes that we identified to harbor putative pathogenic mutations in the PD patients, a network analysis was carried out using ConsensusPathDB 33 (11.04.2018) (http://cpdb.molgen.mpg.de/CPDB)188-191). The list of 44 genes was submitted to the ConsensusPathDB web-based tool for generating an inferred network. ConsensusPathDB–human integrates interaction networks in *Homo sapiens* including binary and complex protein–protein, genetic, metabolic, signalling, gene regulatory and drug–target interactions, as well as biochemical pathways. Currently the data originate from 32 public resources for interactions and interactions that have been curated from the literature.

When the ConsensusPathDB analysis as carried out (4 September, 2018), the database contained the following annotations: 170,276 unique physical entities, 603,543 unique interactions, 17,410 gene regulations, 397,088 protein interactions, 1,738 genetic interactions, 23,482 biochemical reactions, 163,825 drug-target interactions and a total of 5,359 pathways. ConsensusPathDB infers a network to include proteins or metabolites that are not in the user-supplied input list, but associate two or more nodes (gene/protein/metabolites) on the input list with each other. These nodes are termed intermediate nodes and are ranked according to the significance of association with the input nodes given their overall connectivity in the background network. This is quantified by a z-score calculated for each intermediate node with the binomial proportion test. We used only interactions with "high confidence" evidence as implemented in a study by Kamburov et al. (Kamburov et al., 2013).

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4.4 RESULTS

4.4.1 Characteristics of Study Participants

In this study, 33 Black South African and 14 Nigerian PD patients were screened for mutations in 751 candidate genes as part of a neurological diseases gene panel. The South African patients were of Sotho, Xhosa, Zulu or Ndebele origins, and the Nigerian patients were from the Yoruba ethnic group. The age-at-onset (AAO) of PD in patients varied between the two study groups. The estimated mean AAO for the South African PD patients was 48±8 years while the mean AAO for the Nigerian patients was 63±13 years (**Table 4.1** and **Table S4.1**). Recruitment in South Africa was prioritized so that predominantly PD patients with earlier AAO (30–59 years) were recruited. In Nigeria, the PD patients recruited were mostly sporadic cases and had later onset of the disease (60–85 years). One Nigerian patient had an AAO of only 36 years, and five others were <60 years.

Two of the South African PD patients had a positive family history of the disease. PD is known to affect males more often than females. In the South African patients, 45% were female, but in the Nigerian patients, only 21% were female. Numbers of patients with comorbidities were 2/33 and 8/14 among the South African and Nigerian patients, respectively (**Table 4.1**).

4.4.2 Quality of NGS Data

The sequencing data on the 47 samples were of good quality. We plotted a graph for the target region coverage using the *bamfiles* generated by the Ion Torrent Variant Caller (**Figure S4.1**). This graph showed that 41 samples were above 80% for the fraction of capture target bases at an average read depth of 40X, three samples had a coverage of 78–79%, and another three samples had a coverage of 61–76% (**Figure S4.1**).

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The mean and the median scores of the variant calls were standardised and plotted as a smooth density histogram. The histograms showed the quality versus depth for the variants called in each sample. The two peaks observed in the histogram per sample showed adequate overall distribution of quality and depth density (**Figure S4.2**).

We assessed separately the quality of sequence data on the six known PD genes *ATP13A2*, *LRRK2*, *PARK7, PINK1*, *PRKN*, and *SNCA*. As can be seen in Figure S3, there was quite a large variation in the quality scores for the exonic regions of these genes. Many regions were below the 100 threshold set for the analyses.

4.4.3 Identification of Sequence Variants

We applied stringent threshold criteria for the filtering and annotation of the variants to exclude low quality variants. Altogether 25,917 sequence variants passed quality control. We then removed from the list all variants with MAF>0.01 in any of the sequencing databases used as reference databases for the study (Table S3) and were left with 14,655 rare variants. These rare variants could be classified into 7,934 intronic and 5,695 exonic variants (Figure 4.1; an interactive html-version of the figure is at https://www.frontiersin.org). They included 198 UTR5 and 341 UTR3 variants, as well as 32 frameshift, 3,175 synonymous and 2,448 missense variants. Altogether 14,057 were SNVs and 598 MNVs. There were 261 insertions, 600 deletions and 13,794 substitutions (**Figure 4.1**).

From the list of previously described pathogenic mutations in known PD genes and linked to PD (*LRRK2, PARK7, PINK1, PRKN, and SNCA*) and catalogued in the PDmutDB database (http://www.molgen.vib-ua.be/PDmutDB/), or variants reported in *ATP13A2,* we did not find any of the known PD mutations in the study groups. A subset of the South African samples had been screened for mutations in these genes previously by other methods (Haylett et al. 2012).

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In these previous studies one patient (78_074) was found to have two mutations in the *PRKN* gene: G430D and exon 4 deletion (Haylett et al. 2012). These variants did not pass quality control in the current study. Quality score was 50.1 (only variants with score of >100 were considered), depth was 31 (had to be at least 40), number of reads in reverse 19 (had to be at least 20), number of reads for the alternative allele: 12 (had to be at least 20); number of reads for in forward for the alternative allele: 5 (had to be at least 5), and number of reads in reverse for the alternative allele: 7 (had to be at least 5)

We also assessed how often each variant was found in common between the two study groups. The shared variants in the study groups included 5899 substitutions, 189 deletions and 110 insertions **(Figure 4.2**).

4.4.4 Pathogenicity Prediction of Variants

To determine which variants were likely to be pathogenic and contribute to the PD pathobiology in the study groups, we present data on 26 different scoring systems (**Table S4.3 and Figure S4.4**). For the final selection of variants predicted to be deleterious, we used **MetaLR** and **MetaSVM** as these two have shown the best performance on curated data sets (Liu et al., 2016).

We focused on identifying homozygous and heterozygous rare (MAF ≤ 0.01) or novel (not seen in any of the databases listed in Table S3) exonic variants predicted to be pathogenic, to identify novel genes that could have an association with the PD pathobiology. The goal was to minimize the number of false positives by applying stringent filtering criteria. We realize that this would lead to false negatives, but in a study to identify novel genes for disease pathobiology, it is better to apply stringent threshold. Altogether, 58 exonic, heterozygous missense variants and two exonic homozygous rare (MAF ≤0.01) missense variants were predicted to be deleterious (**Table 4.2** and **Table 4.3**), in 44 genes.

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In Figure 3 we present the radar plots demonstrating 17 different pathogenicity scores for each of these 60 variants. The deleteriousness scores were standardised for uniformity before making the radar plots. The observations of the variants identified in the known PD genes *PRKN* and *PINK1*, and in the *ARSA, ATP2A1, CP, DST, FLNA, GNE, PC, PSEN2*, *RYR1, SAMHD1, SLC12A6, and TMEM67* genes indicated limited concordance between the deleteriousness prediction tools.

The 60 variants predicted to be deleterious based on **MetaLR** and **MetaSVM** scores included seven novel heterozygous missense variants in three known PD genes: *ATP13A2 (S960R)*, *PRKN* (*P153R and D245E*) and *PINK1* (*S73L, S228F, S284Y and P305A*) (**Table 4.2**). Most (53/60) of the sequence variants predicted to be deleterious were not in any of the known PD genes and included 41 genes not previously associated with PD genetics (**Table 4.2**).

The *ATP13A2 (S960R)* substitution was among the variants that passed the threshold of >0.8 for deleteriousness using MetaLR and MetaSVM scores. However, because the known PD genes already have adequate supporting evidence for their role in the pathobiology of PD, it is therefore, plausible to identify candidates with a lower deleteriousness score (i.e., because of prior evidence, the chances of false positives is lower at the same threshold). Hence, we relaxed the MetaLR and MetaSVM deleteriousness threshold to >0.45 to allow six additional deleterious variants in *PRKN (P153R and D245E)* and *PINK1 (S73L, S228F, S284Y and P305A*) to be included in the list of variants predicted to be deleterious (**Figure S4.5**, **Table 4.2**, and **Table 4.3**).

Altogether, 21% (7/33) of the South African patients and 29% (4/14) of the Nigerian PD patients did not have any deleterious variants in the 751 genes studied here (Table 3).

Some patients had more than one deleterious variant (Table 4.3). They included 22 (67%) South African and six (43%) Nigerian patients. Twelve (36%) South African and five (36%) Nigerian PD patients had two pathogenic variants, of which compound heterozygosity occurred in four SA PD patients and as well two homozygotes identified in the South African PD patients (Table 4.3).

Ten (30%) South Africans had 3-5 variants predicted to be deleterious, which probably means that some of these variants are not associated with PD. Only one Nigerian patient had three variants predicted to be deleterious, and no Nigerian patient had more than three variants predicted to be deleterious.

Thirteen variants were found in more than one study patient (**Table 4.2**, **Table 4.3**). One variant (*SCN4A E81Q*) was found in four patients, two of whom were South African and two Nigerian. This variant, therefore, had an estimated MAF of 0.043 among Black SSA PD patients. Three variants (*GALC T445S*, *GNE D203E*, and *PINK1* P305A) were found in three South African patients (Table 2 and Table 3) yielding an estimated MAF of 0.045 among Black South African PD patients. *RYR1 P1632S* was found in two South African and one Nigerian patient. Altogether eight variants (*CAPN3 R441Q, CLN6 S257G, NDUFAF5 G294A, PC R732G, PRNK D245E, PSEN1 V191A, RYR1 D2943N*, and *RYR1 H3642Q*) were found in two study patients yielding an estimated MAF of 0.021 among Black SSA PD patients.

4.4.5 Follow-up Studies

Four novel variants (*PRKN D245E, ATP13A2 S960R, PINK1 S228F* and *PINK1 P305A*) were selected for verification using Sanger sequencing. As shown in **Figure S6**, all four were confirmed.

The frequencies of two of these variants (*PRKN D245E* and *ATP13A2 S960R*) were assessed in 150 controls of Black South African ancestry using HRM. The HRM normalised and the difference graphs showed that the variants were present only in the two PD patients analysed by tNGS and Sanger sequencing, and were not found in any of the controls (**Figure S4.7**). The estimated frequency among Black South Africans controls was, therefore, < 0.0033.

Functional classification of the 44 genes revealed that 23 (52%) of them were enzymes (**Table S4.5**). Other functional classes included transporter, ion channel and transmembrane receptor. The cellular localization was in the

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cytoplasm for 26, nucleus for six, plasma membrane for eight, and extracellular for four of the proteins encoded by the 44 genes. Altogether 43 of 44 genes (97.7%) were mapped to 66 distinct proteins in ConsensusPathDB (**Figure S4.7**). Interactions were shown between the 44 proteins, on which 20 proteins were intermediate nodes and 24 proteins are seed nodes involved in the control of biological processes. The ConsensusPathDB analyses also revealed interactions between the three known PD genes (*ATP13A2*, *PRKN* and *PINK1*) and some of the 41 other genes not previously associated with PD. The PRKN protein appeared to be at the center of several interactions, suggesting that autophagy and mitochondrial dysfunction, as well as, oxidative stress could be plausible mechanisms of PD pathobiology for the 41 novel genes connected to PRKN gene (**Figure S4.7**). Also, PINK1 protein demonstrated interaction with PRKN in this analysis. We highlighted signs and symptoms of the neurological diseases that are associated with the 41 non-PD genes and their potential phenotypic overlap with PD **(Table S4.6).**

The variants S228F, S284Y and P305A identified in *PINK1*, which is an established PD gene, were subjected to protein modelling to predict the effects of these variants on the function of the PINK1 protein. For this analysis the human PINK1 was aligned to the *Pediculus humanus corporis* structure reported by Schubert et al (Schubert et al., 2017). In the models the *PINK1* mutation S228F (red) is seen in the interface between parkin (blue and green) and ubiquitin (beige) in the complex (**Figure 4.4**). PINK1 S228 is also highly conserved across different species and is a known site for autophosphorylation, which is important for the kinase activity (Rasool et al., 2018; Schubert et al., 2017). The *PINK1* mutation S284Y (yellow) is seen on the surface of PINK1 but not at a binding interface. Although, it is the carboxy-terminal residue in a beta sheet (β4) and tyrosine is not frequently found in the carboxy-terminal position of a beta sheet, it is unlikely to have a large destabilizing effect.

PINK1 P305A (orange) mutation is in a loop not directly interacting with ubiquitin (**Figure 4.4**).

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It is located in an insertion sequence 3 (Schubert et al., 2017), which comprises 27 amino acids that in many species contains 5 or 6 prolines. P305 is conserved among the eight species aligned by Schubert et al. (Schubert et al., 2017). The conservation among species suggests that it might be required for efficient folding of PINK1. Substitution of alanine for proline is likely to make the loop at the top of the molecule (**Figure 4.4**) more flexible and possibly less ordered. Not observed in the current study, but shown as a reference in **Figure 4.4** is a previously identified *PINK1* mutation L347P (magenta), which has been shown to disrupt an α-helix in the core of the carboxyl-terminal lobe of PINK1 destabilizing the structure (Schubert et al., 2017). This mutation has been reported in three different studies (Doostzadeh et al., 2007; Hatano et al., 2004; Rogaeva et al., 2004), including in a study on a patient diagnosed with juvenile parkinsonism, which was inherited in an autosomal recessive manner (Hatano et al., 2004).

4.5 DISCUSSION

In this study, we recruited 47 Black South African and Nigerian PD patients and screened them for rare pathogenic mutations in 751 genes involved in neurological diseases. The study groups represented SSA population understudied in PD genetics. Altogether 60 potentially deleterious sequence variants with MAF ≤ 0.01 in 44 different genes were identified.

4.5.1 tNGS of 47 Black African PD Patients Identifies Novel Variants in Three PD Genes

To date, 34 genes have been directly implicated in the etiology of PD (Jansen et al., 2017; Michelle K Lin and Farrer, 2014; Quadri et al., 2018). Six of these genes have been consistently and robustly associated with PD pathology (*ATP13A2*, *LRRK2*, *PARK7, PINK1*, *PRKN*, and *SNCA*). However, most PD patients do not harbor mutations in these genes.

The 751-gene array used in the current study contains only 16 of the 34 known PD genes, but it does have all six genes (*ATP13A2*, *LRRK2*, *PARK7, PINK1*,

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PRKN, and *SNCA)* with the strongest prior evidence of being involved in PD pathobiology.

The panel covers 100% of the exonic regions of these six genes, but the quality of the sequence data obtained on them was variable. We identified pathogenic sequence variants in only three (*ATP13A2*, *PRKN* and *PINK1*) of these 16 genes in the SSA study patients.

Previous studies found mutations in these genes in patients with an early onset PD (Morais et al., 2016). A study by Sironi et al (Sironi et al., 2008) analysed the *PRKN* gene in 146 unrelated early AAO PD patients and identified twelve cases (8.2%) with homozygous or compound heterozygous point mutations and/or exon rearrangements. In our study, the AAO varied between different patients with the same variants. The *PRKN D245E* variant was found in two Black South African male PD patients, one of whom had an AAO of 49, and the other 35 years, whereas P153R, the second *PKRN* variant predicted to be deleterious, was identified in a single Nigerian male patient who was diagnosed at the age of 36 years. The South African male PD patient with the *ATP13A2* S960R variant had AAO of 39 years. The three South African patients (two female and one male patient) with the *PINK1* P305A variant had AAO of 30, 53 and 55 years. Patients with three different *PINK1* variants (S73L, S228F and S284Y) were all 53 years when diagnosed with PD. Since the recruitment in South Africa targeted younger patients, it is difficult to make any generalizations of the AAO and type of variant.

The protein structure and modelling analysis of the *PINK1* S228F found in the 53-year old Nigerian patient suggested that the variant is functionally important. The S228F mutation occurs at the interface between PINK1 and ubiquitin (**Figure 4.4**). Substitution of serine with phenylalanine changes both the physical size of the side chain as well as its properties, changing from polar to hydrophobic.

The size alone has the potential to sterically hinder the binding, and the change in hydrophobicity will likely change the rates of association and dissociation should the S228F not sterically hinder the binding.

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More importantly, a recent study (Rasool et al., 2018) demonstrated that autophosphorylation of S228 is required for ubiquitin binding. Together, these observations make it highly probable that S228F causes, or contributes to, PD in this patient.

We did not identify any of the previously reported mutations in *SNCA, LRRK2, PARK7, PINK1* or *PRKN* associated with PD and catalogued in the PDmutDB database (https://www.molgen.vib-ua.be/PDMutDB/database) in the SSA patients. There are two possible explanations for this. One is that as seen in previous genetic studies on SSA PD patients, common mutations such as *LRRK2* G2019S are a rare cause of PD in these populations (Blanckenberg et al., 2013). The other possible explanation is that the variants in these genes did not pass our strict quality control.

4.5.2 The Majority of variants predicted to be deleterious are in genes not previously associated with PD pathobiology

Out of the 60 missense variants predicted to be deleterious in the current study, 53 were in 41 genes not previously known to harbour mutations in PD patients. More than half of the 44 genes with deleterious variants identified in this study encode enzymes. Previous studies have pointed to neuroinflammation and immune dysfunction as important mediators in PD development (Poewe et al., 2017). Enzymes are key regulators in neuro-inflammation processes and could also be associated with other enzyme-dependent-biomedical pathways. Inheritance pattern for mutations in genes encoding enzymes is usually autosomal recessive. Hence, these genes serve as important candidate genes in further studies to understand PD pathobiology.

The novel PD genes with deleterious variants identified in this study have important physiological functions (http://www.uniprot.org). Many of them are part of the basement membrane and have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signalling, neurite outgrowth and metastasis.

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Some of them are part of calcium channels responsible for muscle fibres and nerve impulses and the regulation of other genes. Below we highlight some of the deleterious variants found in these 41 genes.

We identified a hemizygous deleterious variant in *AR* in one 55-year old male Black South African PD patient (**Table S4.1**, **Table 4.2** and **Table 4.3**). *AR* is located on the X chromosome. AR protein functions as a steroid-hormone activated transcription factor and also stimulates transcription of androgen-responsive genes. Mutations in this gene have been attributed to many forms of cancer, and recently mutations in AR were associated with spinal and bulbar muscular atrophy (Lieberman, 2018). The same patient also had a deleterious variant in the *GNE* gene.

We identified two different deleterious variants in *FLNA* (A666V and G1698S) in the Black South African PD patients (**Table 4.2** and **Table 4.3**). A 37-year old man was homozygous for the A666V variant, whereas a 52-year old woman was heterozygous for the G1698S variant (Table S1 and Table 3). The male patient also had a deleterious variant in *ALDH7A1*, and the female patient had two other deleterious *RYR1* variants. *FLNA* gene encodes a protein named filamin A, which binds to many other proteins in the cell to carry out various functions, including the attachment of cells to one another, cell movement, determination of cell shape, and cell survival. It is involved in skeletal muscle and brain development.

We also identified four deleterious variants in the *RYR1* gene (**Table 4.2** and **Table 4.3**), which encodes a protein involved in the formation of calcium channels. Physiologically, calcium regulates the dopamine release during exocytosis, suggesting that this protein could have a potential role in PDpathobiology. Two of the *RYR1* variants (*H3642Q* and *P1632S*) were present in two PD patients.

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We found a heterozygous deleterious variant *SCN4A E81Q* in four patients, two of whom were South African and two Nigerian. The sodium channel genes are highly conserved with few amino acid substitutions among some 65 mammalian proteins (Zakon, 2012). A number of pathogenic mutations which give rise to a variety of muscle disorders including various myotonias and periodic paralyses, have been characterized in *SCN4A* (Platt and Griggs, 2009). Almost all of the consequential mutations occur in the transmembrane helices of the pore loops. Only two mutations have been characterized in the N-terminal cytoplasmic domain. One of these, P72L, showed very little functional effect, and was thought to be a modifier. The other, R104H, caused complete loss of function when in recessive form (sodium channel is composed of two mutated subunits). The E81Q mutation identified in the current study in four PD patients scored as deleterious by prediction algorithms. It is difficult to add further interpretation to the predicted consequence of an E81Q substitution. It is possible that decreased functionality of the sodium channel could aggravate tremor caused by, and characteristics of PD.

The *AARS* gene catalyses the attachment of alanine to tRNA. The *Y535F* mutation was found in *AARS* in one female Nigerian PD patient, who was diagnosed with PD at the age of 80 (**Table S4.1**, **Table 4.2** and **Table 4.3**).

We also found a deleterious variant (G5585S) in the *TTN* gene in one female South African PD patient who developed PD at the age of 31 years (**Table S4.1, Table 4.2** and **Table 4.3**). The same patient had two other deleterious variants, one in *NDUFS2* and the other in *SAMHD1*. *TTN* encodes the protein called titin, which plays an important role in muscle movement. Mutations in this gene have been linked to severe limb girdle muscular dystrophies (Harris et al., 2017).

The role for the *POLG* gene in PD is still debatable. A study published in 2008 identified two mutations in 31 unrelated patients with autosomal recessive parkinsonism (Wong et al., 2008).

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Moreover, it is interesting that in our study a heterozygous deleterious variant in *POLG* was found in one Nigerian patient, who also had a deleterious variant in the *CAPN3* gene (**Table 2** and **Table 3**). The patient had an AAO of PD at 71 (Table S1) and had a history of exposure to environmental toxins that could damage the mitochondria, and potentially make him more susceptible for developing PD.

4.5.3 Study Limitations

Our study has several limitations. First, the sample size was small making it difficult to estimate the actual contribution of genetic factors to PD in the SSA populations. The belief that PD is caused by witchcraft and does not have a genetic link among Black SSA populations (Mokaya et al., 2017), may have contributed to difficulty in recruiting more patients for the study. More awareness of PD is needed in this region, which can be achieved by organizing educational programs and PD support groups.

Second, we used a commercially available targeted gene panel for sequencing and were able to analyse primarily the protein-coding regions of the 751 genes of the panel. It is possible that intronic variants could produce aberrant RNA splicing of exons and cause premature decay of mRNA, resulting in no protein synthesis (Aneichyk et al., 2018; Cartegni et al., 2002). Furthermore, 18 of the 34 previously identified PD genes were not on the panel.

Third, the Ion Torrent sequencing platform used in the study has difficulty in detecting the length of homopolymer stretches with precision. We observed this in the annotation where INDELs appeared as one or more codons at a time. Also, tNGS might not be able to correctly detect large deletions that span entire exons and other structural changes. It is important to mention that 15 of the 33 South African and all of the 14 Nigerian patients used in this study were also included in the MLPA analysis. The results showed that three Nigerian PD patients had four CNVs in three known PD genes (*PINK*, *PRKN* and *PARK7*). The role of heterozygous and homozygous structural changes in PD is well-established (La Cognata et al., 2017), and future studies in this SSA sample should take this into consideration.

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It is important therefore, to screen the Black South African PD patients with early AAO for copy number variations especially in the *PINK1* and *PRKN* genes as copy number variations in these genes have been linked to PD (Pankratz et al., 2011).

Fourth, annotation of sequence variants in terms of effect on the protein remains problematic, as demonstrated by the discrepancy between predictions from different algorithms. We chose to use the MetaLR and MetaSVM algorithms that currently appear to perform best, but also present results from 26 different scoring algorithms.

Fifth, due to the small sample sizes, we were not able to carry out a replication study for the variants identified among the SSA PD patients. As numerous previous genetic studies have shown (Elbaz et al., 2006), replication is an essential next step for our study. This will, however, require long-term recruitment efforts to achieve a larger sample size.

Sixth, variants identified in our study as potentially deleterious might not be involved in PD pathobiology. It is plausible that some of the patients had other clinical conditions, in addition to PD. It is, therefore, possible that the discovered deleterious variants may contribute to that other disease. On the other hand, it is also possible that having only one mutant allele was not sufficient to manifest as a disease since many of the genes encode for enzymes. Most (58/60) of the deleterious variants were found in a heterozygous state in the study patients and these patients could be carriers of rare, non-PD diseases. Previous studies have shown that each human being has approximately "110-120 predicted highly deleterious alleles" in his/her genome (Henn et al., 2016). Only extensive follow-up studies, which are beyond the scope of this study, will be able to address this limitation.

Seventh, we did not carry out any functional follow-up studies. We acknowledge that identification and prioritization of PD candidate genes requires additional work, including functional cell-based assays (Jansen et al., 2017) and use of animal models to characterize mutations and understand their true significance in PD. Other groups have used induced pluripotent stem cell

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(iPSC) models to assess the functional significance of sequence changes in neuronal cell lines (Djelloul et al., 2015; Holmqvist et al., 2016; Russ et al., 2018).

In conclusion, the use of tNGS to screen for pathogenic mutations in the understudied SSA populations for PD genetics in this study provided a list of novel candidate genes. Further studies are required to ascertain their roles in PD among SSA patients.

4.6 Authors' contributions

OG led the project, obtained ethics approvals for the study, did literature searches, carried out Sanger sequencing and HRM, performed data analyses using Ion Torrent software, prepared some of the tables and figures and drafted the manuscript. HK supervised the project, critically appraised the results in this study and edited the manuscript. GT co-supervised the project, performed data analysis, wrote results, generated figures and tables, and contributed to knowledge. AV and CvH performed tNGS. SB and MK conceptualised the idea for this research, obtained ethics approvals, invited other collaborators, obtained funding, supervised part of the work and revised the manuscript. MF, TS, MK, ACR, LvH and JC recruited patients in Nigeria and South Africa, provided clinical expertise and revised the manuscript. AA, MO, OR, SA, WH and DT revised the manuscript and contributed to knowledge. All authors approved the final version of the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org.

4.9 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4.10 Tables and Figures

Table 4.1: Characteristics of the 47 PD patients

Characteristic	**Black South African N = 33**	**Nigerian N = 14**
Sex, males, n (%)	18 (54)	11 (78)
Average age-at-onset ± SD (range), years	48 ± 8 (30-59)	63 ± 13 (36-80)
Average age at recruitment ± SD (range), years	55 ± 11 (35-78)	67 ± 11 (42-81)
Positive family history for PD, n (%)	2 (6)	0
History of trauma/head injury, n (%)		4 (29)
Co-morbidities,[a] n (%)	2 (6)	8 (57)

[a]Co-morbidities on which information was collected on the Nigerian patients included high blood pressure and diabetes, whereas information on dementia and arthritis was collected on the Black South African PD patients.
See Table S4.1 for additional individual clinical information on the patients.

Table 4.2. Variants predicted to be deleterious identified using MetaLR and MetaSVM

Chr	Start	Symbol	Ref	Alt	AAfrom	AAPos	AAto	Sanger sequencing verification[1]	HRM screening[1]	Known PD gene	FreqNGR	FreqSA	FreqAll
16	70296316	AARS	T	A	Y	535	F				1	0	1
22	40755001	ADSL	G	T	A	206	S				0	1	1
5	125919688	ALDH7A1	C	T	R	110	Q				0	1	1
X	66863156	AR	A	T	T	559	S				0	1	1
22	51063778	ARSA	T	C	N	442	S				0	1	1
22	51065288	ARSA	G	T	P	220	T				1	0	1
1	17313614	ATP13A2	T	G	S	960	R	Yes	Yes	Yes	0	1	1
16	28912085	ATP2A1	G	A	D	525	N				0	1	1
11	62458888	BSCL2	A	G	L	290	P				1	0	1
15	42693950	CAPN3	G	A	R	441	Q				1	1	2
10	50828566	CHAT	T	G	M	84	R				0	1	1
1	154544030	CHRNB2	C	T	P	244	L				1	0	1
15	68500645	CLN6	T	C	S	257	G				1	1	2
21	47544826	COL6A2	G	A	G	588	S				0	1	1
2	238258801	COL6A3	G	A	R	1683	C				0	1	1
3	148899821	CP	T	C	E	842	G				1	0	1
19	10893647	DNM2	G	T	V	234	L				0	1	1
6	56765318	DST	A	C	S	106	R				1	0	1
6	56765371	DST	C	T	A	89	T				0	1	1
X	153583294	FLNA	C	T	G	1698	S				0	1	1
X	153592919	FLNA	G	A	A	666	V				0	1	1
14	88414158	GALC	G	C	T	445	S				0	3	3
9	36236974	GNE	A	C	D	203	E				0	3	3
17	10443936	MYH2	T	C	D	328	G				0	1	1
17	10314218	MYH8	A	C	L	488	R				0	1	1
12	4763994	NDUFA9	G	A	R	75	H				1	0	1
20	13797783	NDUFAF5	G	C	G	294	A				0	2	2
1	161182208	NDUFS2	C	G	P	352	A				0	1	1
11	66618540	PC	G	C	R	732	G				0	2	2
1	20960259	PINK1	C	T	S	73	L			Yes	0	1	1
1	20966392	PINK1	C	T	S	228	F	Yes		Yes	1	0	1

1	20971057	PINK1	C	A	S	284	Y			Yes	0	1	1
1	20971119	PINK1	C	G	P	305	A	Yes		Yes	0	3	3
15	89868793	POLG	G	A	H	613	Y			Yes	1	0	1
3	64133345	PRICKLE2	T	G	Q	274	P				0	1	1
6	161781223	PRKN	A	C	D	245	E	Yes	Yes	Yes	0	2	2
6	162622239	PRKN	G	C	P	153	R			Yes	1	0	1
14	73659375	PSEN1	T	C	V	191	A				0	2	2
1	227073297	PSEN2	G	A	V	139	M				0	1	1
1	227079048	PSEN2	A	G	Y	319	C				0	1	1
19	12921137	RNASEH2A	C	T	R	186	W				0	1	1
19	38974116	RYR1	C	T	P	1632	S				1	2	3
19	38998362	RYR1	G	A	D	2943	N				0	2	2
19	39019242	RYR1	C	G	H	3642	Q				0	2	2
19	39025421	RYR1	C	T	A	3769	V				0	1	1
20	35533822	SAMHD1	T	G	N	452	T				0	1	1
17	62019123	SCN4A	C	T	V	1507	I				0	1	1
17	62036686	SCN4A	C	A	S	653	I				0	1	1
17	62049961	SCN4A	C	G	E	81	Q				2	2	4
4	52895854	SGCB	T	C	N	140	S				0	1	1
15	34534333	SLC12A6	C	A	G	696	V				0	1	1
15	34542869	SLC12A6	A	C	I	503	M				0	1	1
6	152809602	SYNE1	A	G	W	333	R				0	1	1
20	2376062	TGM6	T	A	L	135	H				0	1	1
11	61160781	TMEM216	A	G	E	38	G				1	0	1
8	94827551	TMEM67	G	A	S	928	N				0	1	1
16	2130346	TSC2	G	T	G	949	V				0	1	1
16	2134230	TSC2	C	T	S	1092	L				0	1	1
16	2138318	TSC2	C	T	R	1507	C				1	0	1
2	179590564	TTN	C	T	G	5585	S				0	1	1

Start refers to GRCh38 coordinates. Chr, chromosome; Ref, reference allele based on GRCh38; Alt, alternative (variant) allele found in this study; Symbol, Gene Symbol available at the Gene database at NCBI (https://www.ncbi.nlm.nih.gov/); AAfrom, amino acid in reference sequence; AAPos, amino acid position; AAto, amino acid in variant sequence; CountNGR, number of variant alleles in the Nigerian sample; CountRSA, number of variant alleles in the South African sample; CountAll, number of variant alleles in the entire study sample.

[1]Four variants were selected for Sanger sequencing and two variants for HRM analysis.

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Table 4.3: Variants predicted to be deleterious identified in study patients.

Subject	Coverage	AARS:Y535F	ADSL:A206S	ALDH7A1:R110Q	ARSA:N442S	ARSA:P220T	AR:T559S	ATP13A2:S960R	ATP2A1:D525N	BSCL2:L290P	CAPN3:R441Q	CHAT:M84R	CHRNB2:P244L	CLN6:S257G	COL6A2:G588S	COL6A3:R1683C	CP:E842G	DNM2:V234L	DST:A89T	DST:S106R	FLNA:A666V
South Africa																					
s10_308	0.81													het							
s10_309	0.79																				
s10_310	0.84																				
s10_313	0.85																				
s10_314	0.83																				
s11_781	0.79																				
s11_830	0.88															het					
s11_833	0.83										het										
s11_834	0.88																				
s11_835	0.91																				
s11_894	0.80																				
s11_895	0.83		het															het			
s11_910	0.87																				
s11_962	0.85											het									
s12_170	0.76																				
s12_171	0.87							het													
s12_172	0.78																				
s12_176	0.72																		het		
s12_177	0.80																				
s12_178	0.80																				
s12_179	0.78																				
s12_180	0.85																				
s12_486	0.82																				
s43_059	0.85																				
s52_023	0.92																				
s55_052	0.89																				
s55_065	0.86																				
s60_039	0.85						hom														
s61_081	0.85														het						
s78_074	0.87																				
s84_052	0.85								het												
s94_069	0.83				het																
s96_087	0.88			het																	hom
Nigeria																					
s12_975	0.88	het																			
s13_007	0.86																				
s13_008	0.61																				
s13_090	0.86																				
s13_036	0.87																				
s13_037	0.84													het							
s13_038	0.80									het											
s13_039	0.86										het										

Subject	Coverage	FLNA:G1698S	GALC:T445S	GNE:D203E	MYH2:D328G	MYH8:L488R	NDUFA9:R75H	NDUFAF5:G294A	NDUFS2:P352A	PC:R732G	PINK1:P305A	PINK1:S228F	PINK1:S284Y	PINK1:S73L	POLG:H613Y	PRICKLE2:Q274P	PRKN:D245E	PRKN:P153R	PSEN1:V191A	PSEN2:V139M	PSEN2:Y319C
South Africa																					
s10_308	0.81																				
s10_309	0.79																				
s10_310	0.84							het												het	
s10_313	0.85		het			het															
s10_314	0.83																				
s11_781	0.79								het												
s11_830	0.88																				
s11_833	0.83																				
s11_834	0.88																				
s11_835	0.91	het																			
s11_894	0.80																				
s11_895	0.83			het													het				
s11_910	0.87																				
s11_962	0.85													het							het
s12_170	0.76																				
s12_171	0.87																		het		
s12_172	0.78							het													
s12_176	0.72			het																	
s12_177	0.80																				
s12_178	0.80										het		het								
s12_179	0.78																				
s12_180	0.85										het										
s12_486	0.82																het				
s43_059	0.85		het							het											
s52_023	0.92				het																
s55_052	0.89																				
s55_065	0.86		het																		
s60_039	0.85			het																	
s61_081	0.85																		het		
s78_074	0.87																				
s84_052	0.85																				
s94_069	0.83									het	het					het					
s96_087	0.88																				
Nigeria																					
s12_975	0.88						het														
s13_007	0.86																				
s13_008	0.61																				
s13_090	0.86																				
s13_036	0.87																				
s13_037	0.84																				
s13_038	0.80																	het			
s13_039	0.86														het						

Subject	Coverage	RNASEH2A:R186W	RYR1:A3769V	RYR1:D2943N	RYR1:H3642Q	RYR1:P1632S	SAMHD1:N452T	SCN4A:E81Q	SCN4A:S653I	SCN4A:V1507I	SGCB:N140S	SLC12A6:G696V	SLC12A6:I503M	SYNE1:W333R	TGM6:L135H	TMEM216:E38G	TMEM67:S928N	TSC2:G949V	TSC2:R1507C	TSC2:S1092L	TTN:G5585S
South Africa																					
s10_308	0.81							het						het							
s10_309	0.79																				
s10_310	0.84																				
s10_313	0.85																				
s10_314	0.83																				
s11_781	0.79						het														het
s11_830	0.88																				
s11_833	0.83																				
s11_834	0.88																				
s11_835	0.91			het	het																
s11_894	0.80																				
s11_895	0.83							het													
s11_910	0.87																				
s11_962	0.85																				
s12_170	0.76																				
s12_171	0.87																				
s12_172	0.78	het		het	het																
s12_176	0.72								het												
s12_177	0.80																				
s12_178	0.80																				
s12_179	0.78									het					het		het				
s12_180	0.85																				
s12_486	0.82												het								
s43_059	0.85																			het	
s52_023	0.92																	het			
s55_052	0.89		het			het															
s55_065	0.86											het									
s60_039	0.85																				
s61_081	0.85																				
s78_074	0.87										het										
s84_052	0.85					het															
s94_069	0.83																				
s96_087	0.88																				
Nigeria																					
s12_975	0.88																				
s13_007	0.86							het													
s13_008	0.61																				
s13_090	0.86							het													
s13_036	0.87					het															
s13_037	0.84																		het		
s13_038	0.80																				
s13_039	0.86																				

s13_091	0.85																																											
s13_092	0.87										het				het		het																											
s13_093	0.83																										het														het			
s13_094	0.86																																											
s13_095	0.86																																											
s13_096	0.83				het																																							

Subject, sample code; Coverage, tNGS data coverage for the listed sample; other column headers indicate gene and variant for which data are provided. Hemi, patient was hemizygous for the variant; het, patient was heterozygous for the variant; hom, patient was homozygous for the variant. SA, South African; NGR, Nigerian. See Table S1 for additional clinical information on the patients.

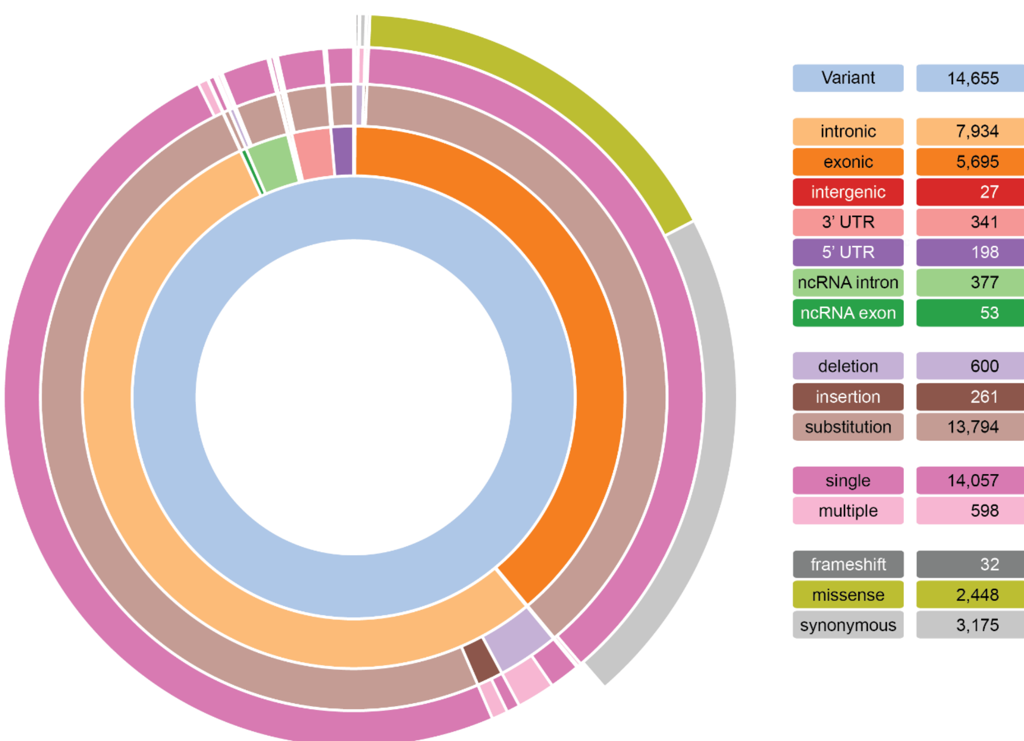

Figure 4.1: Sunburst diagram showing the functional classes of 14,655 rare (MAF≤0.01) sequence variants identified in 33 Black South African and 14 Nigerian PD patients. An interactive HTML-version of the figure is available at Frontiers website

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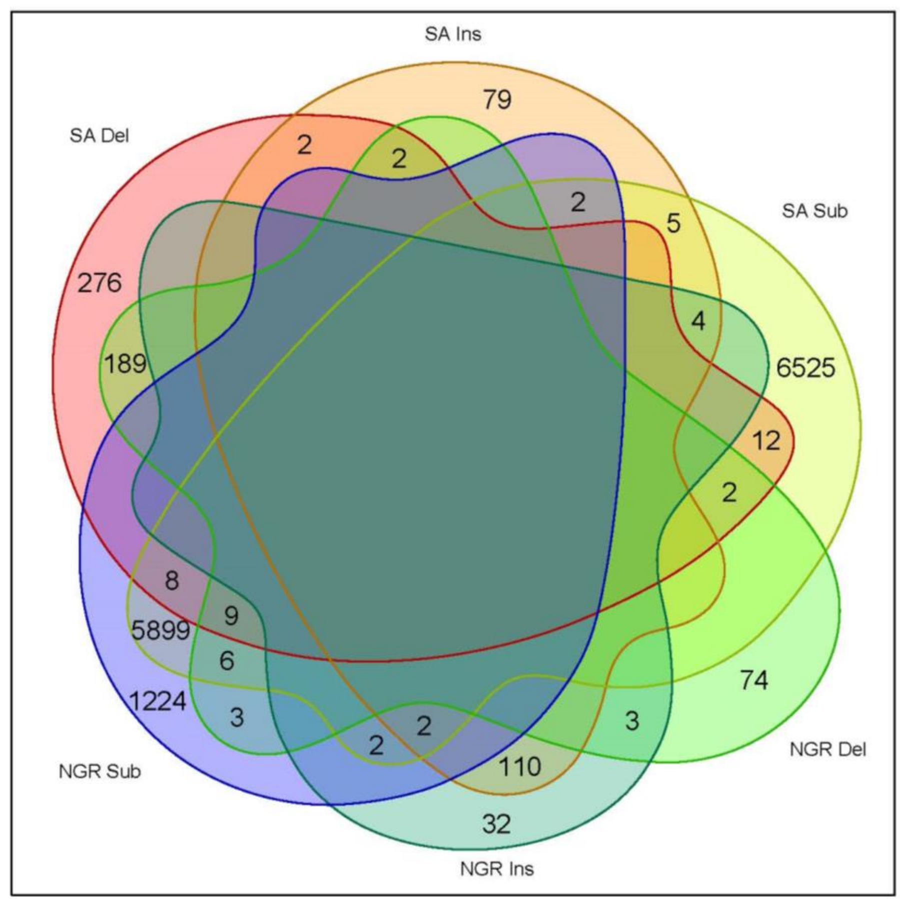

Figure 4.2: Venn diagram showing the distributions of the shared variants (substitution, insertion and deletions) between South African (SA) and Nigerian *NGR) PD patients. The number of deletion, insertion and substitution variants shared between the Nigerian and South African PD patients in this study is shown using Venn diagram. Of which, 5899 substitutions, 189 deletions and 110 insertions were shared.*

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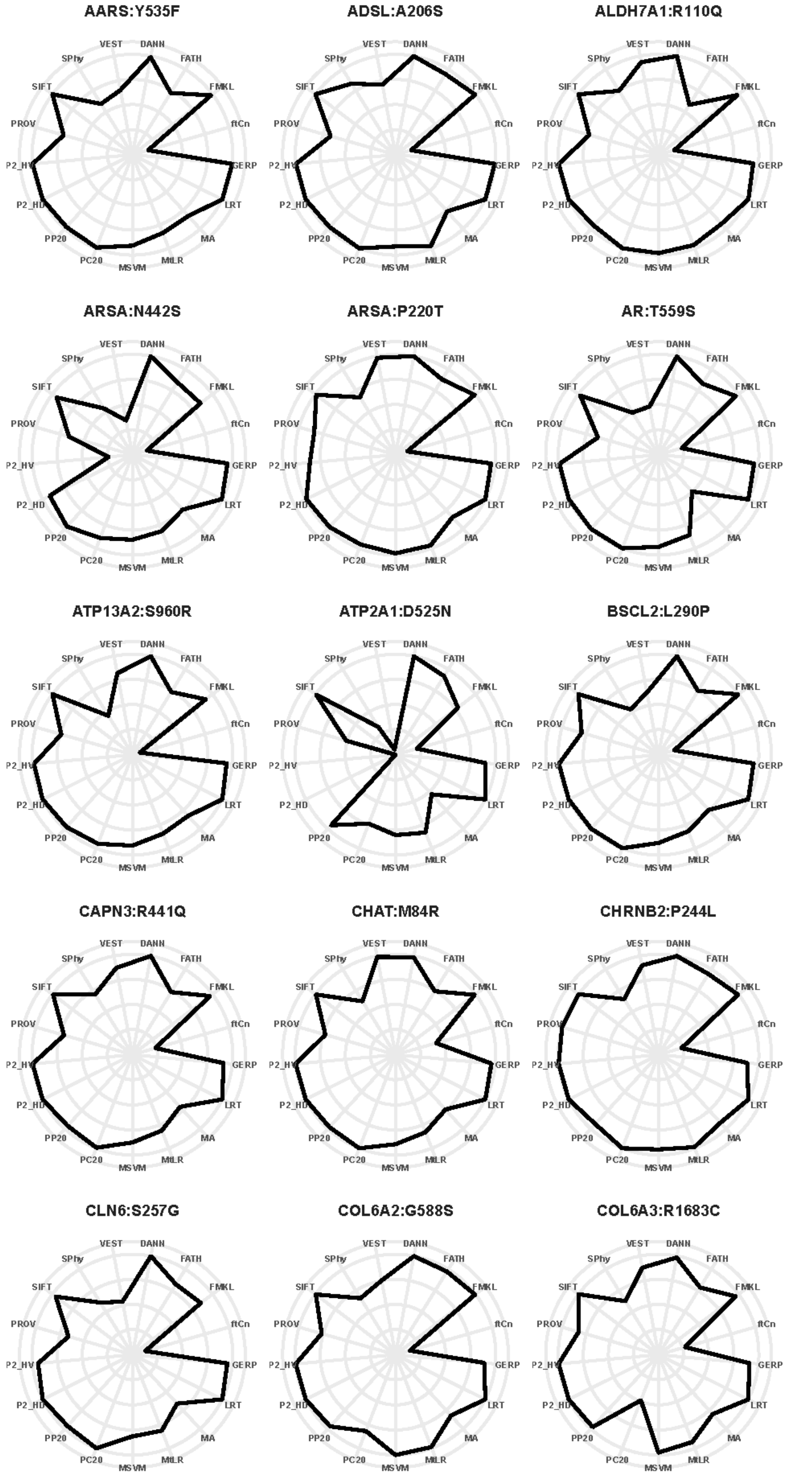

Figure 4.3. Radar plots of 60 selected missense variants. Plots are identified by symbol and amino acid change and illustrate 17 scores for each variant considered deleterious. All scores were standardized to 0–1 scale with score 1 (furthest from the center of the graph) indicating strongest evidence that the variant is deleterious. See Supplement for details on the scores used in the analyses.

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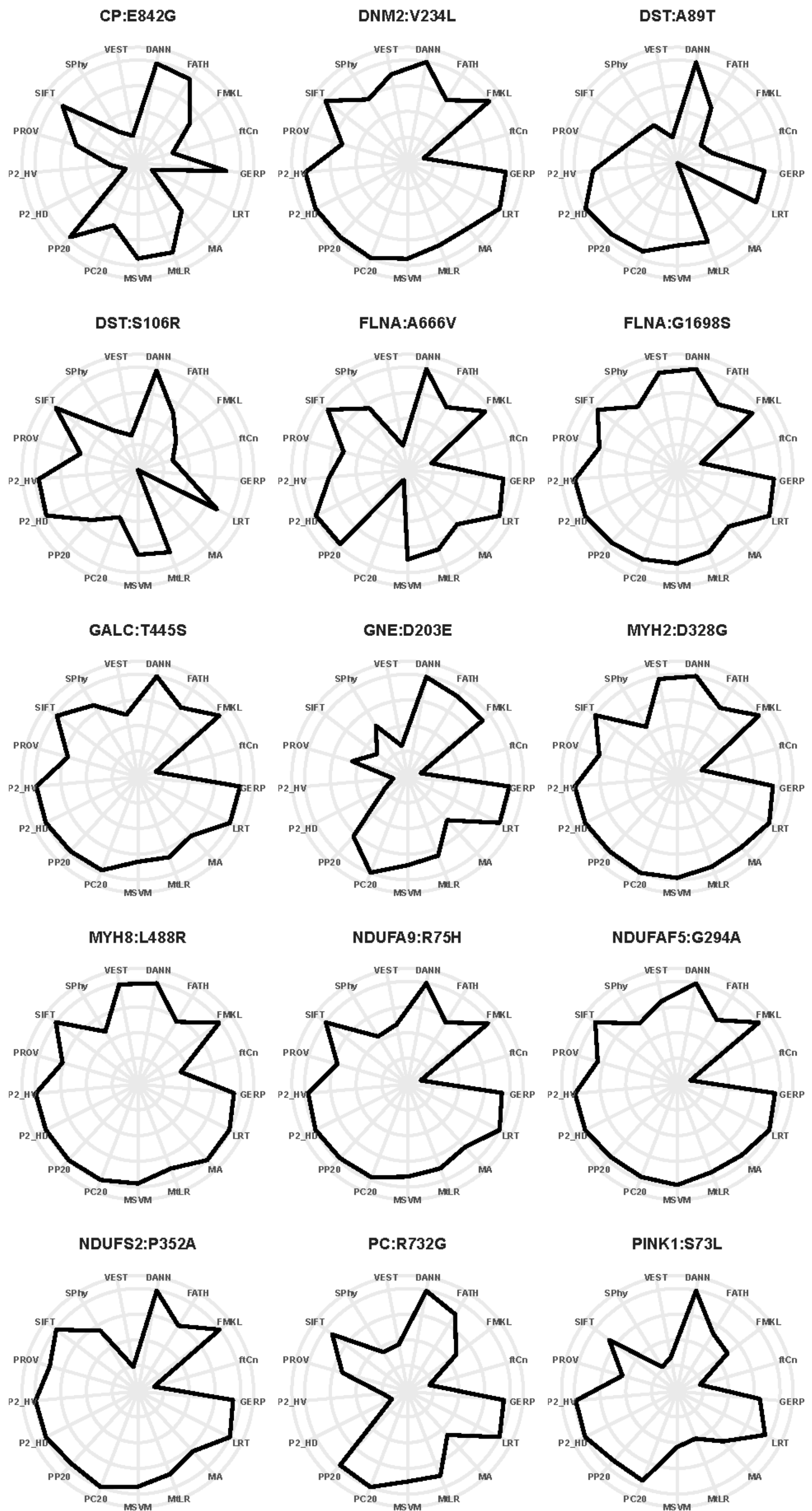

FIGURE 3 (continued)

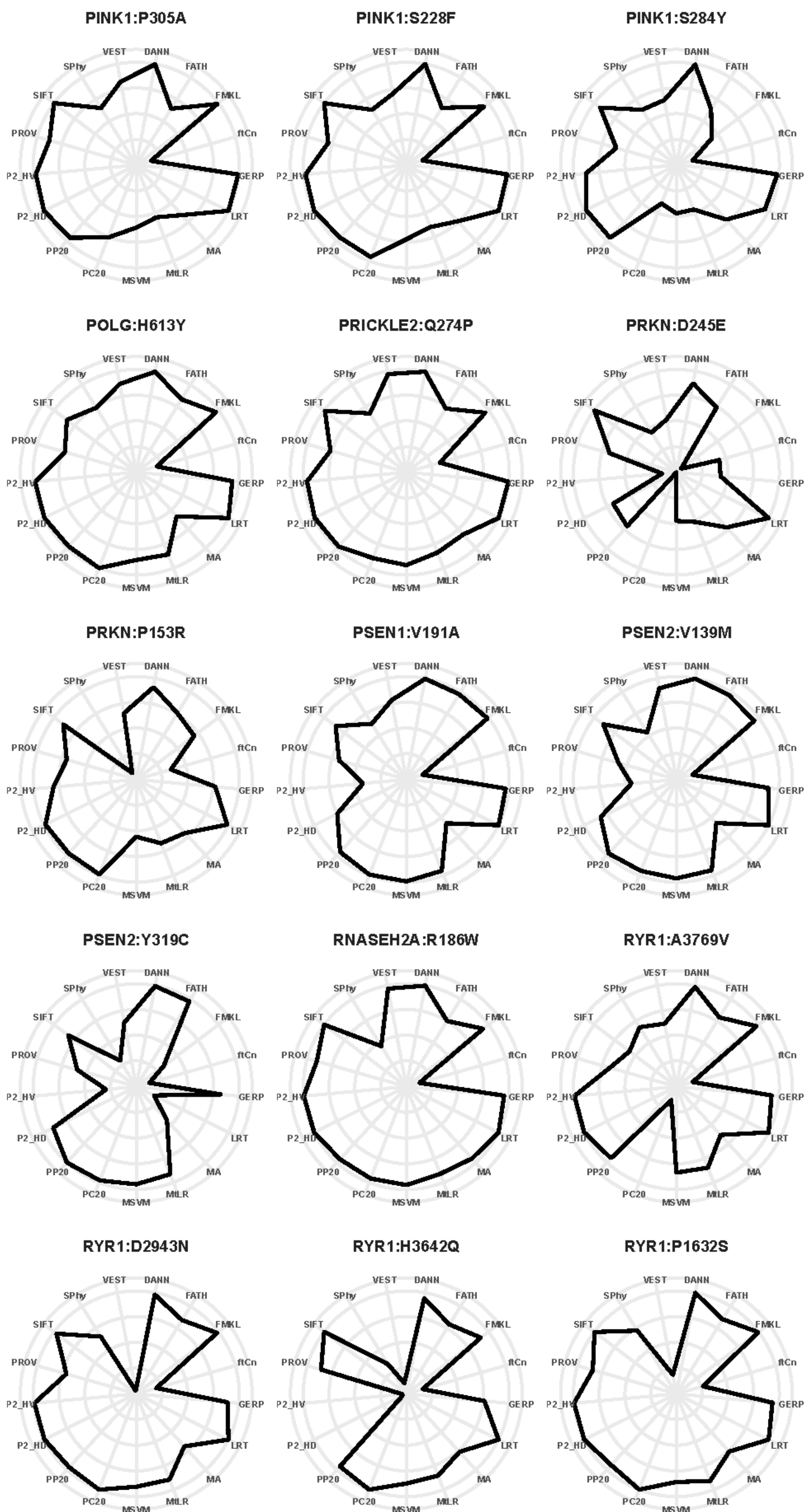

FIGURE 3 (continued)

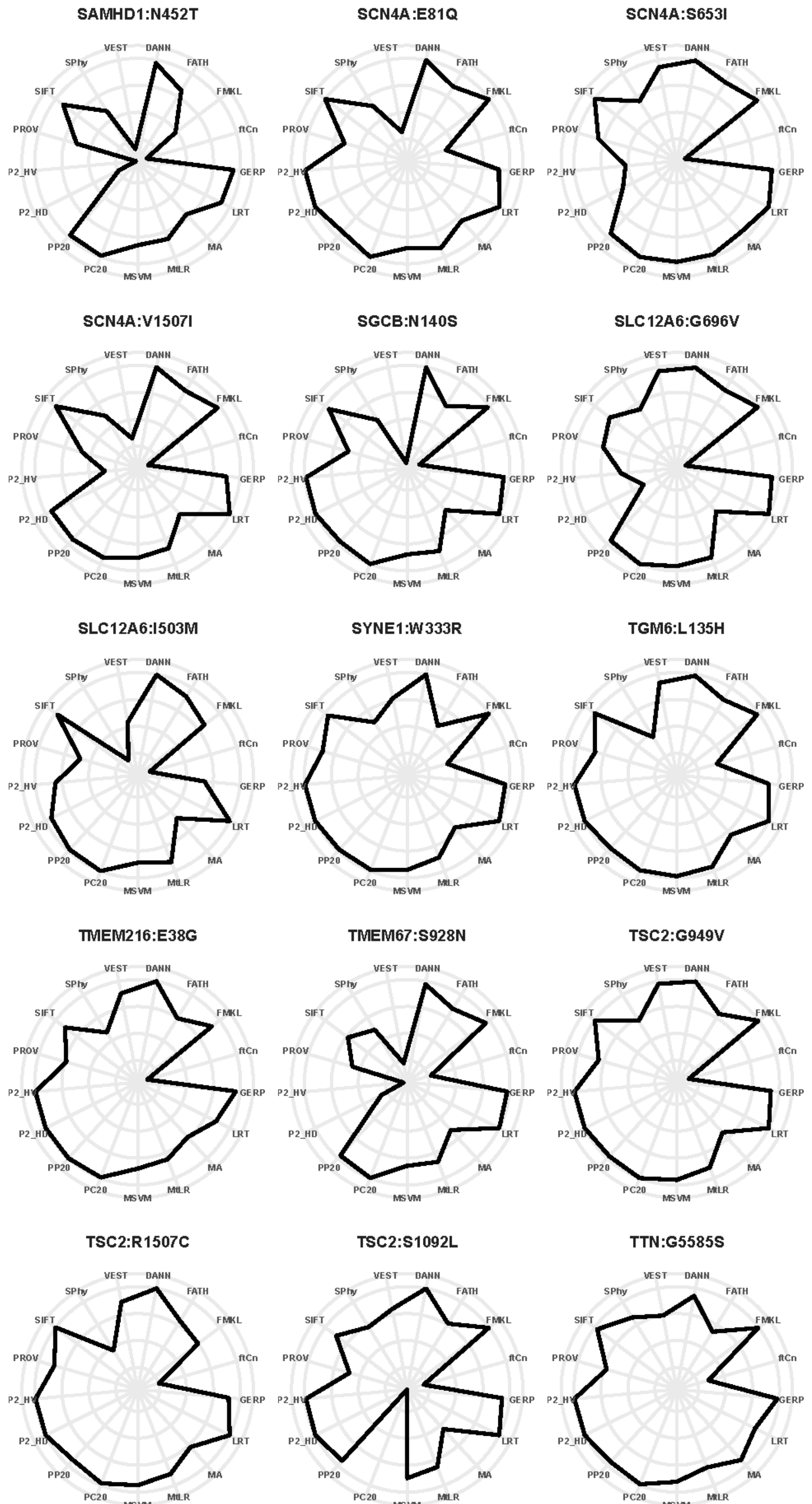

Figure 4.3 (continued)

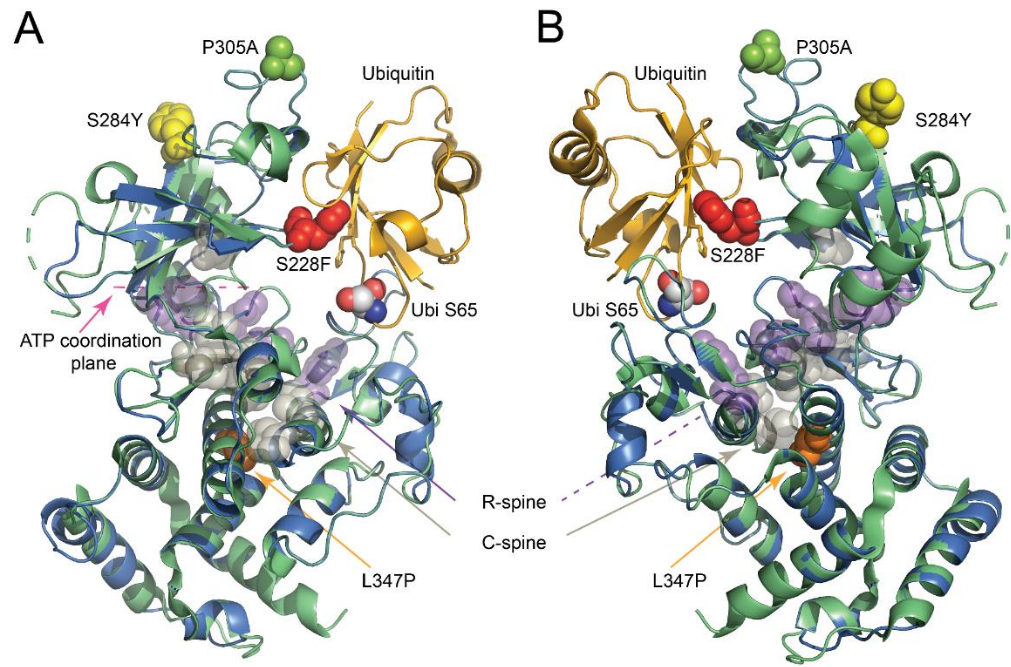

FIGURE 4.4. Stereoscopic crystal structure models of PINK1 and its substrate ubiquitin demonstrating the potential effects of sequence variants S228F, S284Y, and P305A on the function of PINK1 protein. *Crystal structure of PINK1 bound to ubiquitin showing position of sequence variants in PINK1 as well as the position of serine 65 in ubiquitin. Structures are represented in ribbon format. Ubiquitin is shown in gold. PINK1 is shown in light green as the crystal structure derived from the human louse (Schubert et al. 2017) with the predicted structure of the human PINK1 overlayed in light blue. PINK1 sequence variants shown are L347P (orange) located centrally in the helical domain of the C-lobe; S228F (red) in the centre, S284Y (yellow) near the top, and P305A (green) at the top. The ubiquitin S65 is show in CPK atom colors. In addition, the catalytic spine (C-spine) is shown in faded grey and the regulatory spine is shown in faded purple. The ATP necessary for phosphorylating S65 has to be coordinated between two hydrophobic amino acids of the C-spine. The approximate position of the plane of ATP coordination (red arrow) is indicated by the dashed red line.*

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Table S4.1. Clinical information on the 47 PD patients and their deleterious sequence variants

Sample code	Sex	Ethnicity/Country	AAO (y)	AAD (y)	Family history of PD
s43_59	M	Black/SA	51	61	Yes
s52_23	M	Black/SA	50	57	
s55_52	M	Black/SA	42	49	
s55_65	M	Black/SA	40	42	
s60_39	M	Black/SA	55	56	
s61_81	M	Black/SA	55	57	
s78_74	F	Black/SA	56	63	
s84_52	F	Black/SA	57	61	
s94_69	F	Black/SA	30	36	Yes
s96_87	M	Black/SA	37	38	
s10_308	F	Black/SA	49	77	
s10_309	M	Black/SA	52	59	
s10_310	M	Black/SA	45	48	
s10_313	F	Black/SA	43	49	
s10_314	F	Black/SA	55	57	
s11_781	F	Black/SA	31	35	
s11_830	F	Black/SA	57	60	
s11_833	M	Black/SA	48	50	
s11_834	M	Black/SA	52	56	
s11_835	F	Black/SA	52	60	
s11_894	M	Black/SA	44	49	
s11_895	M	Black/SA	49	53	
s11_910	F	Black/SA	59	62	
s11_962	M	Black/SA	53	77	
s12_170	M	Black/SA	52	62	
s12_171	M	Black/SA	39	78	
s12_172	F	Black/SA	55	56	
s12_176	F	Black/SA	51	55	
s12_177	M	Black/SA	45	55	
s12_178	F	Black/SA	53	55	
s12_179	F	Black/SA	30	35	
s12_180	M	Black/SA	55	55	
s12_486	M	Black/SA	35	49	
s12_975	F	Yoruba/N	80	80	
s13_007	M	Yoruba/N	52	59	
s13_008	M	Yoruba/N	62	66	

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s13_036	M	Yoruba/N	57	64
s13_037	M	Yoruba/N	79	81
s13_038	M	Yoruba/N	36	42
s13_039	F	Yoruba/N	71	73
s13_090	M	Yoruba/N	59	60
s13_091	M	Yoruba/N	79	80
s13_092	M	Yoruba/N	48	59
s13_093	M	Yoruba/N	53	64
s13_094	F	Yoruba/N	62	64
s13_095	M	Yoruba/N	73	81
s13_096	M	Yoruba/N	68	71

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Table S4.2: Conservation scores, allele frequencies and functional prediction tools used in the annotation of sequence variants

Annovar Name	Description	Source
RefSeq Annotation		
Gene.refGene	RefGene gene Symbol	https://www.ncbi.nlm.nih.gov/refseq
GeneDetail.refGene	Variant in HGNC Notation per transcript	https://www.ncbi.nlm.nih.gov/refseq
ExonicFunc.refGene	Type of mutation (synonymous, frameshift, etc.)	https://www.ncbi.nlm.nih.gov/refseq
AAChange.refGene	Variant at protein level in HGNC Notation	https://www.ncbi.nlm.nih.gov/refseq
cytoband	Cytological Bands	http://www.software.broadinstitute.org/software/igv/cytoband
genomicSuperDups	Known Large Duplications	http://varianttools.sourceforge.net/Annotation/GenomicSuperDups
SNP databases		
snp142	SNP name in dbSNP v142	http://www.ncbi.nlm.nih.gov/snp
avsnp142	SNP name	http://www.ncbi.nlm.nih.gov/snp
Frequencies		
X1000g2015aug_all	1000 Genomes Frequencies All	http://www.internationalgenome.org/category/population
X1000g2015aug_afr	1000 Genomes Frequencies African	http://www.internationalgenome.org/category/population
X1000g2015aug_eas	1000 Genomes Frequencies East Asian	http://www.internationalgenome.org/category/population
X1000g2015aug_amr	1000 Genomes Frequencies American	http://www.internationalgenome.org/category/population
Kaviar_AF	Kaviar Frequencies	http://www.db.systemsbiology.net/kaviar
Kaviar_AC	Kaviar Variant Count	http://www.db.systemsbiology.net/kaviar
Kaviar_AN	Kaviar Total Allele Count	http://www.db.systemsbiology.net/kaviar
cg69	Cancer Genome Frequencies	http://www.completegenomics.com/public-data/69-genomes/
gnomAD_genome_ALL	Genome Aggregation Database (gnomAD) Genome Frequencies All	http://gnomad.broadinstitute.org/
gnomAD_genome_AFR	gnomAD Genome Frequencies African	http://gnomad.broadinstitute.org/
gnomAD_genome_AMR	gnomAD Genome Frequencies American	http://gnomad.broadinstitute.org/
gnomAD_genome_ASJ	gnomAD Genome Frequencies Ashkenazi Jewish	http://gnomad.broadinstitute.org/
gnomAD_genome_EAS	gnomAD Genome Frequencies East Asian	http://gnomad.broadinstitute.org/
gnomAD_genome_FIN	gnomAD Genome Frequencies Finnish	http://gnomad.broadinstitute.org/

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Annovar Name	Description	Source
gnomAD_genome_NFE	gnomAD Genome Frequencies Non-Finnish European	http://gnomad.broadinstitute.org/
gnomAD_genome_OTH	gnomAD Genome Frequencies Other	http://gnomad.broadinstitute.org/
esp6500siv2_all	esp 6500 Frequencies All	http://www.evs.gs.washington.edu
esp6500siv2_aa	esp 6500 Frequencies African American	http://www.evs.gs.washington.edu
esp6500siv2_ea	esp 6500 Frequencies European American	http://www.evs.gs.washington.edu
gnomAD_exome_ALL	gnomAD Exome Frequencies All (as above)	http://gnomad.broadinstitute.org/
gnomAD_exome_AFR	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_AMR	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_ASJ	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_EAS	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_FIN	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org//
gnomAD_exome_NFE	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_OTH	gnomAD Exome Frequencies	http://gnomad.broadinstitute.org/
gnomAD_exome_SAS	gnomAD Exome Frequencies South Asian	http://gnomad.broadinstitute.org/
ExAC_ALL	ExAC Frequencies All	http://exac.broadinstitute.org/
ExAC_AFR	ExAC Frequencies African	http://exac.broadinstitute.org/
ExAC_AMR	ExAC Frequencies American	http://exac.broadinstitute.org/
ExAC_EAS	ExAC Frequencies East Asian	http://exac.broadinstitute.org/
ExAC_FIN	ExAC Frequencies Finnish	http://exac.broadinstitute.org/
ExAC_NFE	ExAC Frequencies Non-Finnish European	http://exac.broadinstitute.org/
ExAC_OTH	ExAC Frequencies Other	http://exac.broadinstitute.org/
ExAC_SAS	ExAC Frequencies South Asian	http://exac.broadinstitute.org/
Functional Annotation		
cosmic70	Cosmic database	https://cancer.sanger.ac.uk/cosmic/
CLINSIG	Clinical significance in ClinVar	www.openbioinformatics.org/annovar
CLNDBN	Variant disease name	www.openbioinformatics.org/annovar
CLNACC	Variant Accession and Versions	www.openbioinformatics.org/annovar
CLNDSDB	Variant disease database name	www.openbioinformatics.org/annovar
CLNDSDBID	Variant disease database ID	www.openbioinformatics.org/annovar
ICGC_Id	International cancer genome consortium ID	www.openbioinformatics.org/annovar

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Annovar Name	Description	Source
ICGC_Occurrence	International cancer genome consortium occurrence	*www.openbioinformatics.org/annovar*
nci60	60 human cancer cell lines	*www.openbioinformatics.org/annovar*
Prediction Scores		
CADD13_RawScore	Combined annotation dependent depletion score	https://cadd.gs.washington.edu/
CADD13_PHRED	Combined annotation dependent depletion prediction	https://cadd.gs.washington.edu/
SIFT_score	sorting intolerant from tolerant score	http://sift.bii.a-star.edu.sg/
SIFT_pred	sorting intolerant from tolerant prediction	http://sift.bii.a-star.edu.sg/
Polyphen2_HDIV_score	Pholyphen2 score based on HDIV.	http://genetics.bwh.harvard.edu/pph2/
Polyphen2_HDIV_pred	Pholyphen2 prediction based on HDIV	http://genetics.bwh.harvard.edu/pph2/
Polyphen2_HVAR_score	Polyphen2 score based on HVAR.	http://genetics.bwh.harvard.edu/pph2/
Polyphen2_HVAR_pred	Polyphen2 prediction based on HVAR.	http://genetics.bwh.harvard.edu/pph2/
LRT_score	LRT score	*http://www.doclogica.com/*
LRT_pred	LRT prediction	http://www.doclogica.com/
MutationTaster_score	MutationTaster score	*www.mutationtaster.org*
MutationTaster_pred	MutationTaster prediction.	*www.mutationtaster.org*
MutationAssessor_score	MutationAssessor score	http://mutationassessor.org/r3/
MutationAssessor_pred	MutationAssessor prediction	http://mutationassessor.org/r3/
FATHMM_score	FATHMM score	*http://www.fathmm.biocompute.org.uk*
FATHMM_pred	FATHMM prediction	*http://www.fathmm.biocompute.org.uk*
PROVEAN_score	PROVEAN score	http://provean.jcvi.org/index.php
PROVEAN_pred	PROVEAN prediction	http://provean.jcvi.org/index.php
VEST3_score	VEST V3 score	*https://omictools.com/vest-tool*
CADD_raw	CADD raw score.	*https://cadd.gs.washington.edu/info*
CADD_phred	CADD phred-like score	*https://cadd.gs.washington.edu/info*
DANN_score	DANN score.	*https://omictools.com/dann-tool*
fathmm.MKL_coding_score	fathmm-MKL score for one coding variant	*http://www.fathmm.biocompute.org.uk/fathmmMKL.htm*
fathmm.MKL_coding_pred	fathmm-MKL prediction for one coding variant	*http://www.fathmm.biocompute.org.uk/fathmmMKL.htm*
MetaSVM_score	MetaSVM score.	*www.openbioinformatics.org/annovar*
MetaSVM_pred	MetaSVM prediction	*www.openbioinformatics.org/annovar*
MetaLR_score	MetaLR score	*www.openbioinformatics.org/annovar*

Annovar Name	Description	Source
MetaLR_pred	MetaLR prediction	*www.openbioinformatics.org/annovar*
Eigen	Eigen	*https://eigen.tuxfamily.org/dox/*
Dann	Dann score	*https://omictools.com/dann-tool*
FATHMM_noncoding	FATHMM score for one noncoding variant	*http://www.fathmm.biocompute.org.uk*
FATHMM_coding	FATHMM score for one noncoding variant	*http://www.fathmm.biocompute.org.uk*
GWAVA_region_score	GWAVA region score	*https://www.sanger.ac.uk/sanger/StatGen_Gwava*
GWAVA_tss_score	GWAVA score	*https://www.sanger.ac.uk/sanger/StatGen_Gwava*
GWAVA_unmatched_score	GWAVA unmatched score	*https://www.sanger.ac.uk/sanger/StatGen_Gwava*
Conservation Scores		
integrated_fitCons_score integrated_confidence_value	Fitness Consequences	*https://compgen.cshl.edu/fitCons/*
GERP++_RS	GERP++ Genomic Evolutionary Rate Profiling	*https://mendel.stanford.edu/SidowLab/downloads/gerp*
phyloP7way_vertebrate	PhyloP score for 7 vertebrate species (Phylogenetic Hidden Markov Model)	*www.openbioinformatics.org/annovar*
phyloP20way_mammalian	PhyloP score for 20 mammalian species	*www.openbioinformatics.org/annovar*
phastCons7way_vertebrate	PhastCons score for 7 vertebrate species	*www.openbioinformatics.org/annovar*
phastCons20way_mammalian	PhastCons score for 20 mammalian species	*www.openbioinformatics.org/annovar*
SiPhy_29way_logOdds	SiPhy score for biased substitutions (SIte-specific PHYlogenetic)	*www.openbioinformatics.org/annovar*

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Table S4.3: Primers used in Sanger sequencing

Gene	Variant	Primer	Primer sequence	Annealing temperature (ºC)	Product size *(bp)*
ATP13A2	c.3010A>C	S001F*	5'-GGCAGATCAACACCAACCTG-3'	55	248
		S002R*	5'-CAGTCAGCTCCCTACTCACC-3'		
PRKN	c.1182T>G	S011F*	5'-TCTTTGTTTCCCCAGGCCTA-3'	60	300
		S012R*	5'-GCTCAGCACAGACTCACCAT-3'		
PINK1	c.88G>C	S017F	5'-AAGTTTGTTGTGACCGGCG-3'	55	278
		S018R	5'-GAGACGGTTAGGGAGCCC-3'		
PINK1	c.683C>T	S021F	5'-AGGCAGGGCTTACAAGGAAC-3'	55	220
		S022R	5'-TGCTCTCAAAGAAGTCCCAGT-3'		

*These primers were also used in HRM (for results, see Figure S4.6)

Table S4.4: Information on the 44 selected genes with variants predicted to be deleterious based on the Ingenuity Pathway Analysis

Gene	Description	Location	Family	Drugs
AARS	alanyl-tRNA synthetase	Cytoplasm	Enzyme	
ADSL	adenylosuccinate lyase	Cytoplasm	Enzyme	
ALDH7A1	aldehyde dehydrogenase 7 family member A1	Cytoplasm	Enzyme	
AR	androgen receptor	Nucleus	ligand-dependent nuclear receptor	estradiol valerate/testosterone enanthate, estradiol cypionate/testosterone cypionate, BMS-641988, cyproterone acetate/ethinyl estradiol, enzalutamide, galeterone, ostarine, 1-testosterone, flutamide/goserelin, nandrolone phenpropionate, androgen receptor antagonist, apalutamide, darolutamide, AZD3514, APC-100, EPI-506, bicalutamide/leuprolide, bicalutamide/goserelin, dexamethasone/enzalutamide, SHR3680, LY2452473, enzalutamide/exemestane, drospirenone/ethinyl estradiol, nilutamide, TRC253, bicalutamide, SXL01, proxalutamide, hydroxyflutamide, testolone, flutamide, nandrolone decanoate, testosterone cypionate, cyproterone acetate, nandrolone, drospirenone, medroxyprogesterone acetate, oxandrolone, danazol, dihydrotestosterone, fluoxymesterone, stanozolol, spironolactone, methyltestosterone, testosterone, oxymetholone, 7alpha-hydroxytestosterone, testosterone propionate, testosterone enanthate
ARSA	arylsulfatase A	Cytoplasm	Enzyme	
ATP13A2	ATPase 13A2	Cytoplasm	Transporter	
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	Cytoplasm	Transporter	
BSCL2	BSCL2, seipin lipid droplet biogenesis associated	Cytoplasm	Other	
CAPN3	calpain 3	Cytoplasm	Peptidase	

CHAT	choline O-acetyltransferase	Nucleus	Enzyme	
CHRNB2	cholinergic receptor nicotinic beta 2 subunit	Plasma Membrane	transmembrane receptor	varenicline, ABT-089, ABT 418, isoflurane, mecamylamine, succinylcholine, rocuronium, doxacurium, amobarbital, mivacurium, pipecuronium, rapacuronium, metocurine, atracurium, cisatracurium, acetylcholine, nicotine, D-tubocurarine, arecoline, lobeline, enflurane, pancuronium, vecuronium
CLN6	CLN6, transmembrane ER protein	Cytoplasm	Other	
COL6A2	collagen type VI alpha 2 chain	Extracellular Space	Other	collagenase clostridium histolyticum
COL6A3	collagen type VI alpha 3 chain	Extracellular Space	Other	collagenase clostridium histolyticum
CP	ceruloplasmin	Extracellular Space	Enzyme	
DNM2	dynamin 2	Plasma Membrane	Enzyme	
DST	dystonin	Plasma Membrane	Other	
FLNA	filamin A	Cytoplasm	Other	
GALC	galactosylceramidase	Cytoplasm	Enzyme	
GNE	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	Cytoplasm	Kinase	
MYH2	myosin heavy chain 2	Cytoplasm	Enzyme	
MYH8	myosin heavy chain 8	Cytoplasm	Enzyme	
NDUFA9	NADH:ubiquinone oxidoreductase subunit A9	Cytoplasm	Enzyme	
NDUFAF5	NADH:ubiquinone oxidoreductase complex assembly factor 5	Cytoplasm	Other	
NDUFS2	NADH:ubiquinone oxidoreductase core subunit S2	Cytoplasm	Enzyme	

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PC	pyruvate carboxylase	Cytoplasm	Enzyme	
PINK1	PTEN induced putative kinase 1	Cytoplasm	Kinase	
POLG	DNA polymerase gamma, catalytic subunit	Cytoplasm	Enzyme	lamivudine/nelfinavir/stavudine, lamivudine/stavudine, lamivudine/nevirapine/stavudine, stavudine, vidarabine, zalcitabine
PRICKLE2	prickle planar cell polarity protein 2	Nucleus	Other	
PRKN	parkin RBR E3 ubiquitin protein ligase	Cytoplasm	Enzyme	
PSEN1	presenilin 1	Plasma Membrane	Peptidase	Tarenflurbil
PSEN2	presenilin 2	Cytoplasm	Peptidase	Tarenflurbil
RNASEH2A	ribonuclease H2 subunit A	Nucleus	Enzyme	
RYR1	ryanodine receptor 1	Cytoplasm	ion channel	Dantrolene
SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1	Nucleus	Enzyme	
SCN4A	sodium voltage-gated channel alpha subunit 4	Plasma Membrane	ion channel	diclofenac/omeprazole, diclofenac/misoprostol, diclofenac, flecainide, riluzole, zonisamide
SGCB	sarcoglycan beta	Plasma Membrane	Other	
SLC12A6	solute carrier family 12 member 6	Plasma Membrane	Transporter	
SYNE1	spectrin repeat containing nuclear envelope protein 1	Nucleus	Other	
TGM6	transglutaminase 6	Cytoplasm	Enzyme	
TMEM216	transmembrane protein 216	Extracellular Space	Other	
TMEM67	transmembrane protein 67	Plasma Membrane	Other	
TSC2	TSC complex subunit 2	Cytoplasm	Other	
TTN	titin	Cytoplasm	Kinase	

Source: QIAGEN 2000-2018 (*www.qiagenbioinformatics.com*). All rights reserved.

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Table S4.5. Signs and symptoms of genetic diseases associated with the 44 selected genes harbouring variants predicted to be deleterious, and their overlap with cardinal PD signs and symptoms

Gene Symbol	Disease	OMIM#	Signs & symptoms	Possible clinical overlap with PD
AARS	Charcot-Marie-Tooth disease, axonal, type 2N	613287	Distal muscle weakness and atrophy; mild sensory loss	Distal muscle weakness
AARS	Epileptic encephalopathy, early infantile, 29	616339	Seizure	
ADSL	Adenylosuccinase deficiency	103050	Psychomotor retardation; aggressive behaviour; epileptic seizures	
ALDH7A1	Epilepsy, pyridoxine-dependent	266100	Seizures	
AR	Androgen insensitivity	300068	Anxiety; reduced masculinity	
AR	Breast cancer	312300	Pain; red or swollen lymph nodes	
AR	Hypospadias 1, X-linked	300633	Abnormal male reproductive system	
AR	Spinal and bulbar muscular atrophy of Kennedy	313200	Muscle wasting in the arms and legs resulting in cramping; leg muscle weakness and fall	Leg muscle weakness and fall
AR	Prostate cancer	176807	Difficulty with urination	Difficulty with urination

ARSA	Metachromatic leukodystrophy	250100	Abnormal muscle movement; decreased muscle tone; difficulty walking; frequent falls	Abnormal muscle movement; difficulty walking; frequent falls
ATP13A2	Kufor-Rakeb syndrome	606693	Bradykinesia; rigidity; tremors	Bradykinesia; rigidity; tremors
ATP13A2	Spastic paraplegia, 78, autosomal recessive	617225	Weakness in the legs; increased muscle tone and stiffness (spasticity); urinary problems; lack of sensation in the feet	Weakness in the legs; increased muscle tone and stiffness (spasticity); urinary problems
ATP2A1	Brody myopathy	601003	Muscle cramping and stiffening	Muscle cramping and stiffening
BSCL2	Encephalopathy, progressive, with or without lipodystrophy	615924	Problems with coordination; rhythmic muscle contractions; muscle spasm; fainting or fatigue	Rhythmic muscle contractions; muscle spasm
BSCL2	Lipodystrophy, congenital generalized, type 2	269700	Fat accumulation around the neck and jaw	
BSCL2	Neuropathy, distal hereditary motor, type VA	600794	Difficulty walking; muscle weakness; problems with coordination; loss of muscle	Difficulty walking; muscle weakness
BSCL2	Silver spastic paraplegia syndrome	270685	Weakness in the legs; increased muscle tone and stiffness; urinary problems; lack of sensation in the feet	Weakness in the legs; increased muscle tone and stiffness

CAPN3	Muscular dystrophy, limb-girdle	618129 (AD); 253600 (AR)	Abnormality walking; muscle weakness; loss of muscle	Abnormality walking; muscle weakness
CHAT	Myasthenic syndrome, congenital, 6, presynaptic	254210	Muscle weakness; trouble walking; tingling sensation in the hands or feet	Muscle weakness; trouble walking
CHRNB2	Epilepsy, nocturnal frontal lobe, 3	605375	Rhythmic muscle contractions; muscle spasms; seizures, amnesia	Rhythmic muscle contractions; muscle spasms
CLN6	Ceroid lipofuscinosis, neuronal, 6	601780	Abnormal retinal vasculature; mental deterioration; intellectual disability	
CLN6	Ceroid lipofuscinosis, neuronal, Kufs type, adult onset	204300	Difficulties with muscle coordination; involuntary movements such as tremors and dysarthria.	Difficulties with muscle coordination; involuntary movements such as tremor
COL6A2	Myosclerosis, congenital	255600	Muscle weakness; poor motor control; inability to sit or stand without support	Muscle weakness; poor motor control
COL6A2	Bethlehem myopathy 1	158810	Muscle weakness; joint stiffness (contractures) in fingers, wrists, elbows, and ankles	Muscle weakness; joint stiffness
COL6A2	Ullrich congenital muscular dystrophy 1	254090	Muscle weakness; difficulty walking; contractures (neck); joint looseness	Muscle weakness

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COL6A3	Bethlem myopathy 1	158810	Muscle weakness; joint stiffness (contractures) in fingers, wrists, elbows, and ankles	Muscle weakness; joint stiffness
COL6A3	Dystonia 27	616411	Difficulty with jaw movement, swallowing or speech; pain and fatigue due to constant contraction of muscles	Difficulty with jaw movement, swallowing or speech; pain and fatigue due to constant contraction of muscles
COL6A3	Ullrich congenital muscular dystrophy 1	254090	Muscle weakness; difficulty walking; contractures	Muscle weakness
CP	Cerebellar ataxia	604290	Impaired coordination in the arms and legs; frequent stumbling; unsteady gait; motor disability; slurred speech; vocal changes; headaches	Unsteady gait; motor disability; slurred speech
CP	Hemosiderosis, systemic, due to aceruloplasminemia	604290	Fatigue; severe exercise limitation	
CP	Hypoceruloplasminemia, hereditary	604290	Tremors; chorea; ataxia; eyelid twitching; grimacing	Tremors; ataxia
DNM2	Centronuclear myopathy 1	160150	Muscle weakness	Muscle weakness
DNM2	Charcot-Marie-Tooth disease, axonal type 2M	606482	Distal muscle weakness and atrophy; mild sensory loss	Distal muscle weakness
DNM2	Charcot-Marie-Tooth disease, dominant intermediate B	606482	Distal muscle weakness and atrophy; mild sensory loss	Distal muscle weakness

DNM2	Lethal congenital contracture syndrome 5	615368	Akinesia; micrognathia; pulmonary hypoplasia; pterygia; multiple joint contractures	Akinesia
DST	Neuropathy, hereditary sensory and autonomic, type VI	614653	Cramping; difficulty walking; muscle weakness; problems with coordination, or loss of muscle	Difficulty walking; muscle weakness
DST	Epidermolysis bullosa simplex, autosomal recessive 2	615425	Blisters inside the mouth and throat; thickened skin on the palms and soles of the feet; scalp blistering, scarring and hair loss	
FLNA	FG syndrome 2	300321	Mild to severe hypotonia; constipation; a distinctive face	
FLNA	Cardiac valvular dysplasia, X-linked	314400	Chest pain; shortness of breath; light-headedness	
FLNA	Congenital short bowel syndrome	300048	Diarrhea; poor appetite; fatigue; vomiting	Diarrhea
FLNA	Frontometaphyseal dysplasia 1	305620	Hearing loss	Hearing loss
FLNA	Heterotopia, periventricular, 1	300049	Mild intellectual disability including difficulties with reading and spelling	Difficulties with spelling
FLNA	Intestinal pseudoobstruction, neuronal	300048	Nausea; vomiting; abdominal bloating or swelling; constipation	

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FLNA	Melnick-Needles syndrome	309350	Short stature; skeletal abnormalities; characteristic facial features	
FLNA	Otopalatodigital syndrome, type I	311300	Hearing loss; chest deformities; abnormal fingers and toes	Hearing loss
FLNA	Otopalatodigital syndrome, type II	304120	Hearing loss	Hearing loss
FLNA	Terminal osseous dysplasia	300244	Brachydactyly; camptodactyly; clinodactyly, severe limb deformities; joint contractures	
GALC	Krabbe disease	245200	Changing muscle tone and rigidity; hearing loss that leads to deafness; failure to thrive; feeding difficulties; irritability and sensitivity to loud sounds; severe seizures (may begin at a very early age); unexplained fever; vision loss	Changing muscle tone and rigidity; vision loss
GNE	Nonaka myopathy	605820	Muscle weakness and degeneration	Muscle weakness and degeneration
GNE	Sialuria	269921	Failure to thrive; slightly prolonged neonatal jaundice; hepatomegaly; microcytic anemia	
MYH2	Proximal myopathy and ophthalmoplegia	605637	Difficulty swallowing; general muscle weakness	Difficulty swallowing; general muscle weakness
MYH8	Carney complex variant	608837	Weight gain; high blood pressure; diabetes; easy bruising	

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MYH8	Trismus-pseudocamptodactyly syndrome	158300	Inability to open mouth wide; shortened muscles, including hamstrings and calf muscles	
NDUFA9	Leigh syndrome due to mitochondrial complex I deficiency	256000	Generalized weakness; lack of muscle tone; lactic acidosis	Generalized weakness; lack of muscle tone
NDUFAF5	Mitochondrial complex I deficiency, nuclear type 16	616238	Hypotonia; myalgia; exercise intolerance	Hypotonia
NDUFS2	Mitochondrial complex I deficiency	252010	Hypotonia; myalgia; exercise intolerance	
PC	Pyruvate carboxylase deficiency	266150	Abdominal pain; vomiting; tiredness; muscle weakness	Muscle weakness
PINK1	Parkinson disease 6, early onset	605909	Stiff muscles; difficulty standing; difficulty walking; difficulty with bodily movements; involuntary movements; muscle rigidity; problems with coordination; rhythmic muscle contractions; slow bodily movement; slow shuffling gait	Stiff muscles; difficulty standing; difficulty walking; difficulty with bodily movements; involuntary movements; muscle rigidity; problems with coordination; rhythmic muscle contractions; slow bodily movement; slow shuffling gait
POLG	Mitochondrial DNA depletion syndrome 4A (Alpers type)	203700	Hypotonia; lactic acidosis, failure to thrive; tubulopathy; microcephaly; psychomotor delay; sensorineural hearing loss	Hearing loss
POLG	Mitochondrial DNA depletion syndrome 4B (MNGIE type)	613662	Hypotonia; lactic acidosis, failure to thrive; tubulopathy; microcephaly; psychomotor delay; sensorineural hearing loss	Hearing loss

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POLG	Mitochondrial recessive ataxia syndrome (includes SANDO and SCAE)	607459	Hypotonia; lactic acidosis, failure to thrive; tubulopathy; microcephaly; psychomotor delay; sensorineural hearing loss	Hearing loss
POLG	Progressive external ophthalmoplegia, autosomal dominant 1	157640	Drooping eyelids (ptosis) and paralysis of the eye muscles ophthalmoplegia	Ptosis
POLG	Progressive external ophthalmoplegia, autosomal recessive 1	258450	Drooping eyelids (ptosis) and paralysis of the eye muscles ophthalmoplegia.	Ptosis
PRICKLE2	N/A	N/A	N/A	
PRKN	Parkinson disease, juvenile, type 2	600116	Stiff muscles; difficulty standing; difficulty walking; difficulty with bodily movements; involuntary movements; muscle rigidity; problems with coordination; rhythmic muscle contractions; slow bodily movement; slow shuffling gait	Stiff muscles; difficulty standing; difficulty walking; difficulty with bodily movements; involuntary movements; muscle rigidity; problems with coordination; rhythmic muscle contractions; slow bodily movement; slow shuffling gait
PRKN	Adenocarcinoma of lung, somatic	211980	Shortness of breath, persistent cough, coughing up blood. unexplained weight loss. chest pain	
PRKN	Adenocarcinoma, ovarian, somatic	167000	Abdominal or pelvic pain, increased abdominal size. excessive fatigue or lethargy, needing to urinate often or urgently, appetite loss.	

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PRKN	Leprosy	607572	Blister, loss of colour, rashes, ulcers, or redness	
PSEN1	Acne inversa, familial, 3	613737	Lumps; rash; ulcers	
PSEN1	Alzheimer disease, type 3	607822	Mental decline, difficulty thinking and understanding, confusion, delusion, disorientation, forgetfulness, and apathy.	Apathy, delusion and forgetfulness
PSEN1	Alzheimer disease, type 3, with spastic paraparesis and unusual plaques	607822	Mental decline, difficulty thinking and understanding, confusion, delusion, disorientation, forgetfulness, and apathy. Weakness in the legs. Muscle stiffness and lack of sensation in the feet.	Apathy, delusion and forgetfulness. Weakness in the legs. Muscle stiffness and lack of sensation in the feet
PSEN1	Pick disease	172700	Mood changes, compulsive, behaviour, depression.	Mood changes.
PSEN1	Alzheimer disease type 3 with spastic paraparesis and apraxia	607822	Memory loss; behavioural and personality change; progressive loss of language ability; gait disturbances; seizures	Gait disturbances
PSEN1	Cardiomyopathy, dilated, 1U	613694	Arrhythmia; dyspnea; fatigue; syncope; swelling of the legs and feet	

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PSEN1	Dementia, frontotemporal	600274	Loss of empathy and other interpersonal skills; lack of judgment and inhibition; apathy; repetitive compulsive behaviour	Apathy
PSEN2	Alzheimer disease-4	606889	Mental decline, difficulty thinking and understanding, confusion, delusion, disorientation, forgetfulness, and apathy.	Apathy, delusion and forgetfulness
PSEN2	Cardiomyopathy, dilated, iV	613697	Shortness of breath, swelling of legs, fatigue, weight gain, fainting, palpitations, dizziness or light headedness and blood clots in the dilated left ventricle	
RNASEH2 A	Aicardi-Goutières syndrome 4	610333	Microcephaly; early progressive encephalopathy; lack of motor and social skills; feeding difficulties; irritability; vomiting; spasticity	Lack of motor and social skills
RYR1	Central core disease	117000	Muscle weakness	Muscle weakness
RYR1	King-Denborough syndrome	145600	Progressive weakness; short stature; kyphoscoliosis; pectus carinatum or excavatum; facial and skeletal deformities; susceptibility to malignant hyperthermia	

RYR1	Minicore myopathy with external ophthalmoplegia	255320	Severe muscle weakness; generalized myopathy; high-arched palate; respiratory insufficiency	
RYR1	Neuromuscular disease, congenital, with uniform type 1 fiber	117000	Muscles weakness; fatigue; mild proximal weakness	Muscles weakness
RYR1	Malignant hyperthermia susceptibility 1	145600	Rapid heart rate, rapid breathing, acidosis, muscle rigidity, and breakdown of muscle tissue	Muscle rigidity
SAMHD1	Chilblain lupus 2	614415	Rash mainly on acral surfaces most exposed to cold	
SAMHD1	Aicardi-Goutieres syndrome 5	612952	Microcephaly; early progressive encephalopathy	
SCN4A	Hyperkalemic periodic paralysis, type 2	170500	Muscle stiffness and pain; irregular heartbeat.	Muscle stiffness
SCN4A	Hypokalemic periodic paralysis, type 2	613345	Extreme muscle weakness beginning in childhood or adolescence	Extreme muscle weakness
SCN4A	Myasthenic syndrome, congenital, 16	614198	Weak muscles; trouble walking; tingling sensation in hands or feet; eyelid drooping; fatigue; dry mouth.	
SCN4A	Myotonia congenita, atypical, acetazolamide-responsive	608390	Muscle weakness in legs, hands, neck and face; stiffness and tightness of muscles	Stiffness and tightness of muscles
SCN4A	Paramyotonia congenita	168300	Muscle stiffness and weakness	Muscle stiffness and weakness

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SGCB	Muscular dystrophy, limb-girdle, autosomal recessive 4	604286	Gait abnormality	Gait abnormality
SLC12A6	Agenesis of the corpus callosum with peripheral neuropathy	218000	Peripheral neuropathy	
SYNE1	Emery-Dreifuss muscular dystrophy 4, autosomal dominant	612998	Head and neck muscle weakness; muscle atrophy	
SYNE1	Spinocerebellar ataxia, autosomal recessive 8	610743	Ataxia; uncoordinated walk; poor hand-eye coordination; dysarthria; involuntary eye movement; vision problems; difficulty processing, learning, and remembering information	Ataxia; uncoordinated walk; poor hand-eye coordination; dysarthria; involuntary eye movement; vision problems
TGM6	Spinocerebellar ataxia 35	613908	Limb ataxia; gait ataxia; hyperreflexia; dysarthria; hand tremor; saccadic pursuit	Limb ataxia; gait ataxia
TMEM216	Joubert syndrome 2	608091	Hypotonia; abnormal breathing patterns; abnormal eye movements; ataxia; distinctive facial features; intellectual disability	Hypotonia
TMEM216	Meckel syndrome 2	603194	Eye abnormalities including microphthalmia; under-development of the nerves of the eyes	
TMEM67	COACH syndrome	216360	Mental retardation; liver fibrosis; ataxia; abnormal eye movements	Abnormal eye movements
TMEM67	Joubert syndrome 6	610688	Ataxia; abnormal eye and tongue movements; low muscle tone	Low muscle tone leading to movement disorder
TMEM67	Meckel syndrome 3	607361	Eye abnormalities	

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TMEM67	Nephronophthisis 11	613550	Polyuria; polydipsia; fatigue	
TMEM67	Barder-Biedl syndrome 14, modifier of	615991	Progressive retinal degeneration, postaxial polydactyly, obesity, learning difficulties, and renal tract and genital anomalies	
TSC2	Focal cortical dysplasia, type II, somatic	607341	Seizure	
TSC2	Lymphangioleiomyomatosis, somatic	606690	Shortness of breath, especially during physical activity	
TSC2	Tuberous sclerosis-2	613254	Behavioural changes, such as temper tantrums, anxiety, or sleep problems; nausea; headache; autism; developmental delays	
TTN	Cardiomyopathy, dilated, 1G	604145	Arrhythmia; dyspnea; swelling of the legs and feet; fatique	
TTN	Cardiomyopathy, familial hypertrophic, 9	613765	Chest pain; dyspnea especially with physical exertion; palpitations; light-headedness; dizziness; fainting	
TTN	Muscular dystrophy, limb-girdle, autosomal recessive 10	608807	Progressive weakness of muscles of the pelvic girdle, legs, arms and shoulders	Progressive weakness of muscles of the pelvic girdle, legs, arms and shoulders
TTN	Myopathy, proximal, with early respiratory muscle involvement	603689	Distal leg weakness; nocturnal respiratory symptoms due to respiratory muscle weakness	Distal leg weakness

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TTN	Salih myopathy	611705	Facial muscle weakness; scoliosis; delayed motor development	
TTN	Tibial muscular dystrophy, tardive	600334	Muscle weakness	Muscle weakness

Information was collected from OMIM at www.omim.org/.

*Since data on the *PRICKLE2* gene in OMIM are limited, we carried out a literature search. *PRICKLE2* missense mutations were found to segregate with Autism Spectrum Disorders (ASDs) in a study by Sower and colleagues (PMID: 23711981). They identified two different heterozygous missense variants in the *PRICKLE2* gene (E8Q and V153I) in two unrelated families. The variants were not found in 192 controls and have low frequencies in databases. Similarly, the frequent association of ASDs with epilepsy suggests a shared underlying genetic susceptibility; several genes, when mutated, can contribute to both disorders, including *PRICKLE2* (PMIDs: 24938409 22037766). The PRICKLE2 protein functions in the non-canonical WNT signalling pathway, which regulates intracellular calcium release and Planar cell polarity in dopaminergic neurons (PMID: 24431302). *PRICKLE2* gene is expressed mainly in the adult brain where it is associated with the postsynaptic density (PMID: 21324980). In addition, Tao and colleagues found missense mutations (R104Q, R144H, Y472H, R148H; V153I V605F) in both male and female patients with myoclonic seizures, epilepsy, generalized electroencephalography pattern, and ataxia (PMID: 21276947). They also identified a heterozygous deletion of the *PRICKLE2*gene in a male patient with developmental delay, epilepsy, and autistic disorder. In a GWAS, SNPs in *PRICKLE2* gene were shown to affect the vitamin B6, vitamin B12, folate, and homocysteine concentrations (PMID:19303062).

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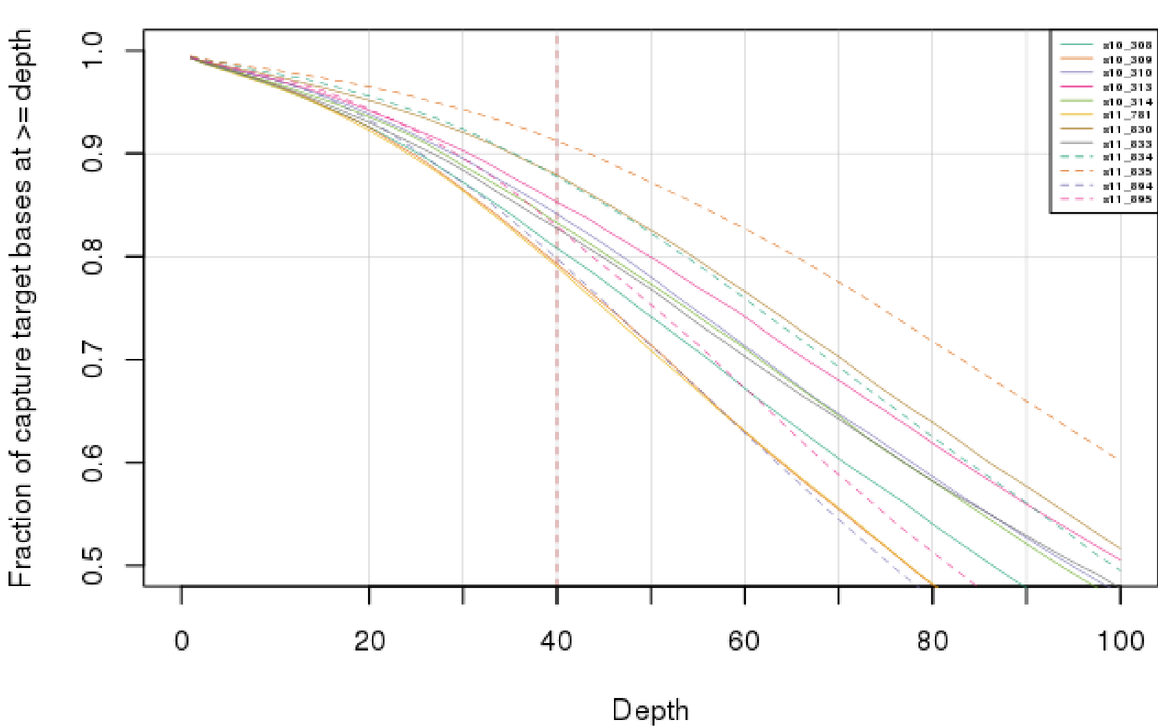

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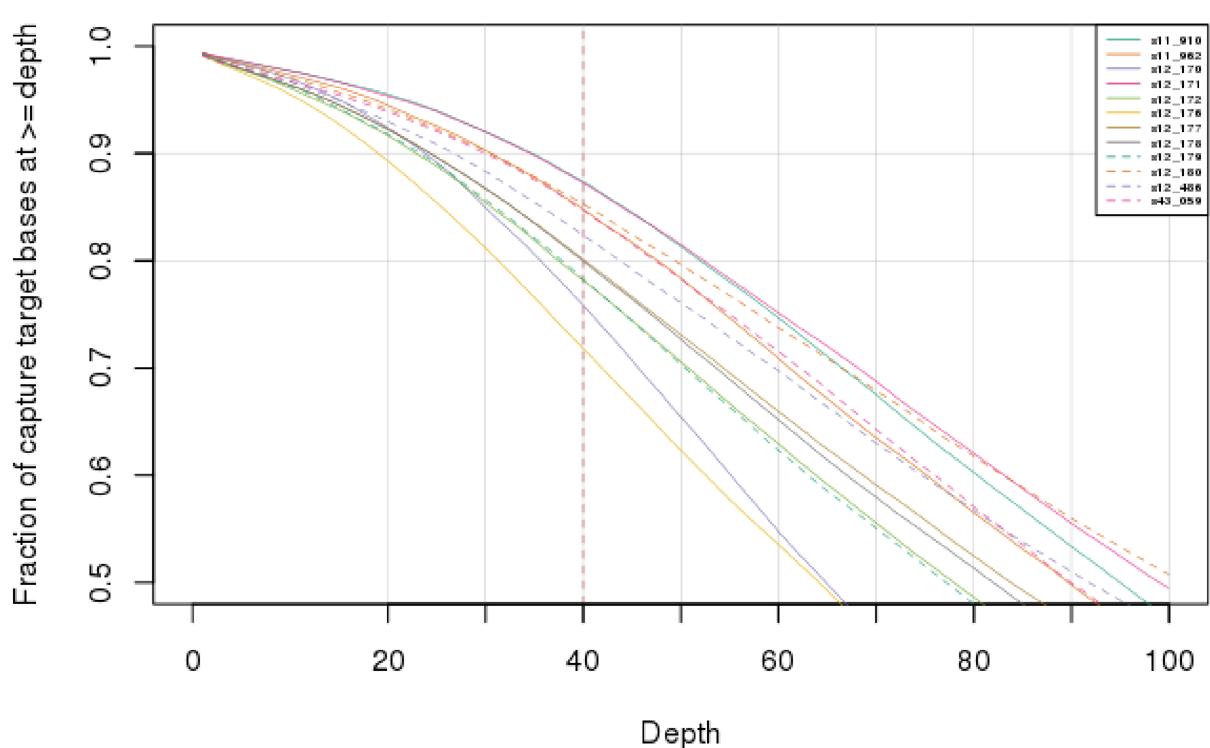

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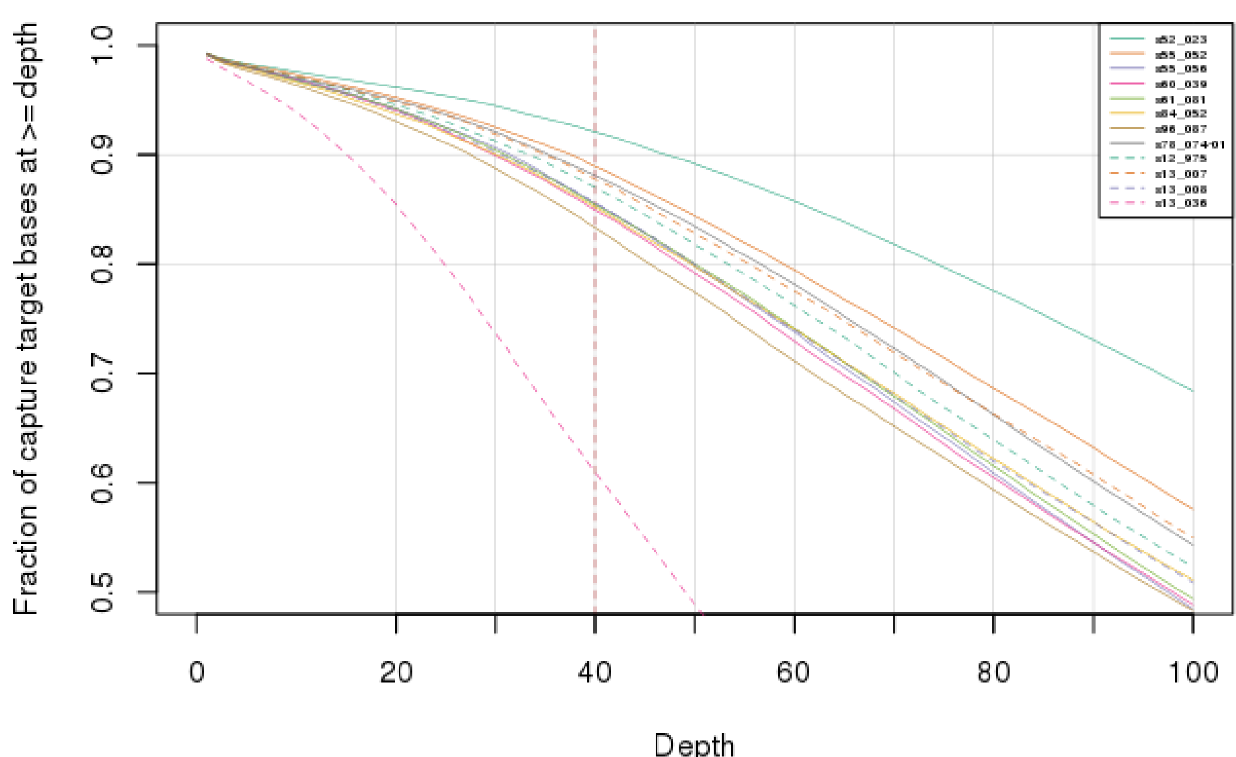

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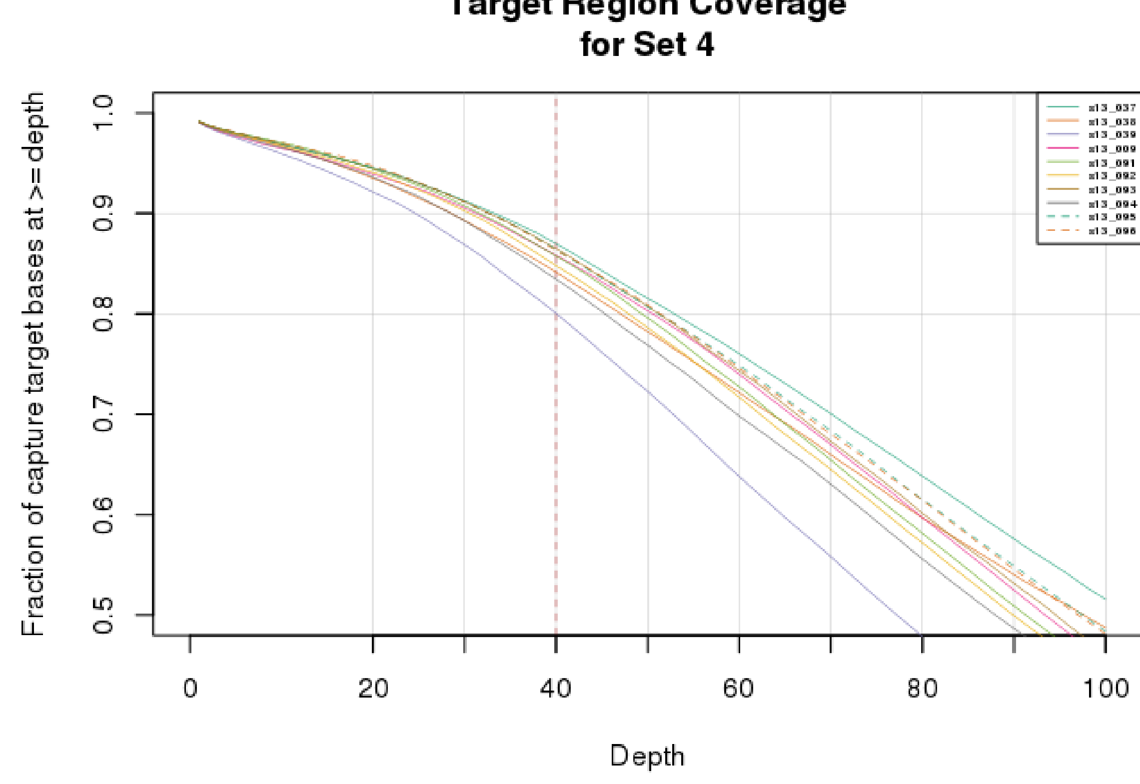

d.

Figure S4.1. Target region coverage for the 47 samples. Sets 1-4(a-d) represent the samples binned into sets of up to12 samples each. Most (42/47) of the samples show coverage of 80% of the target region at a depth of 40x. Sample codes are shown in the insert.

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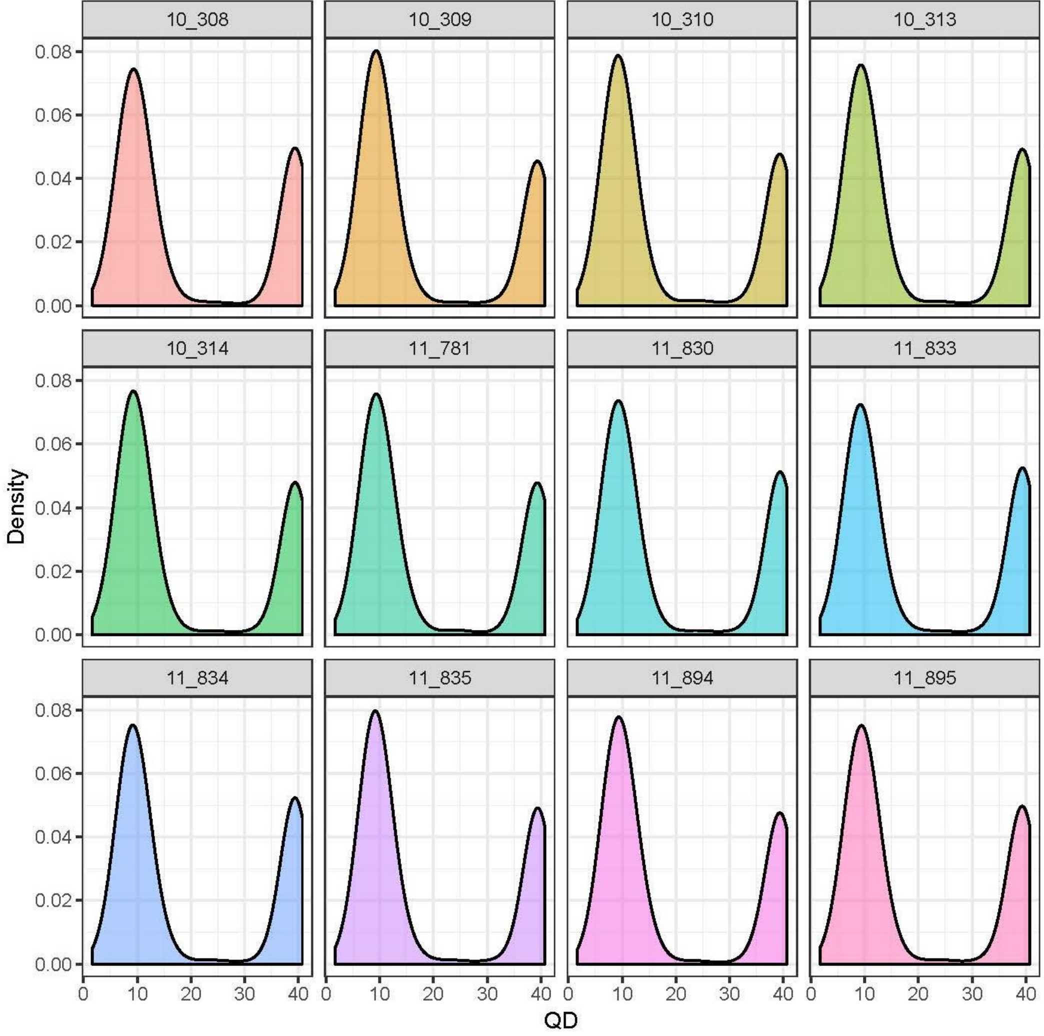

a.

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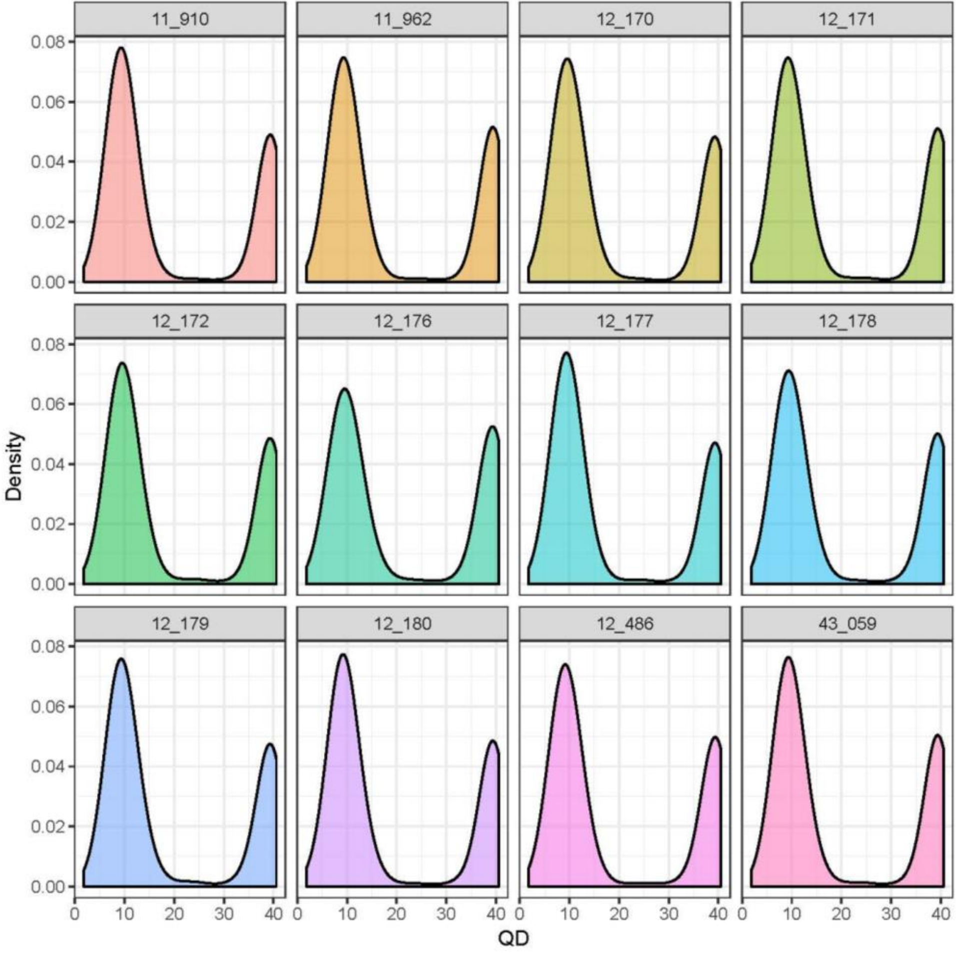

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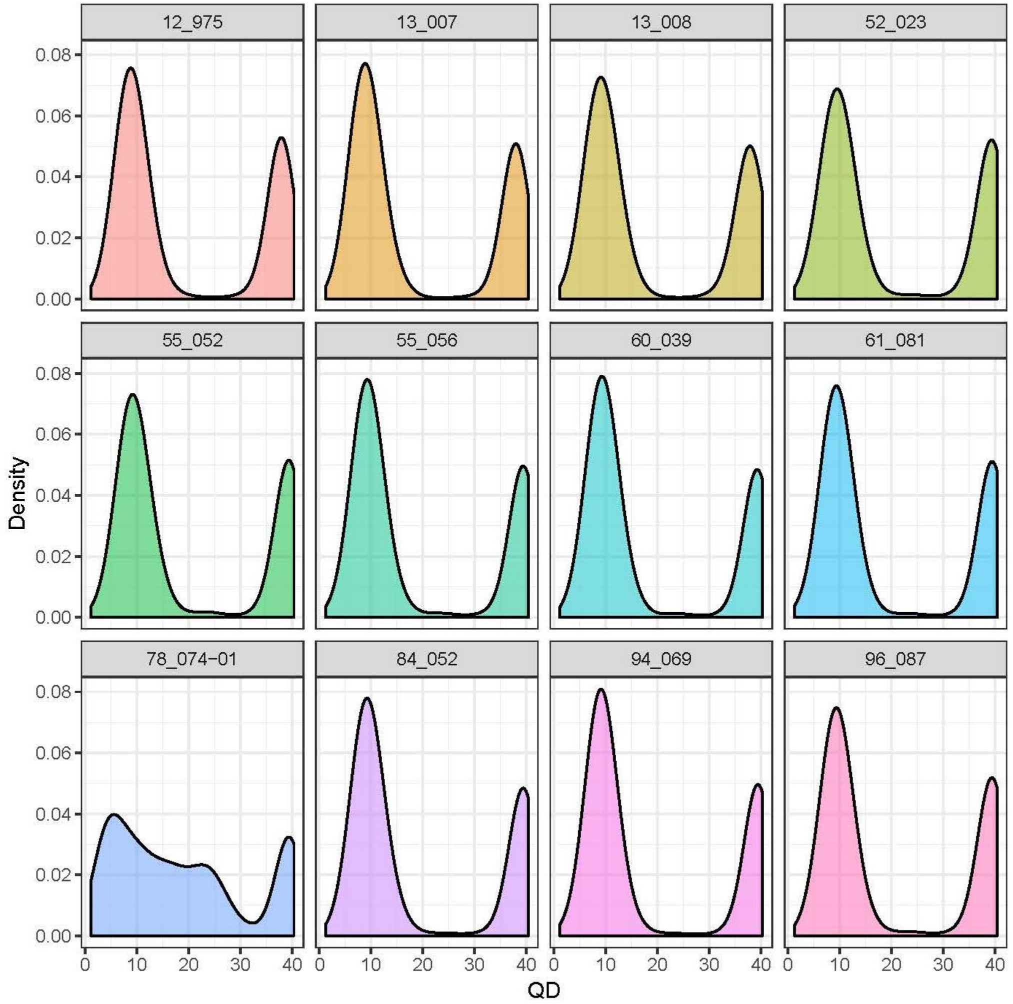

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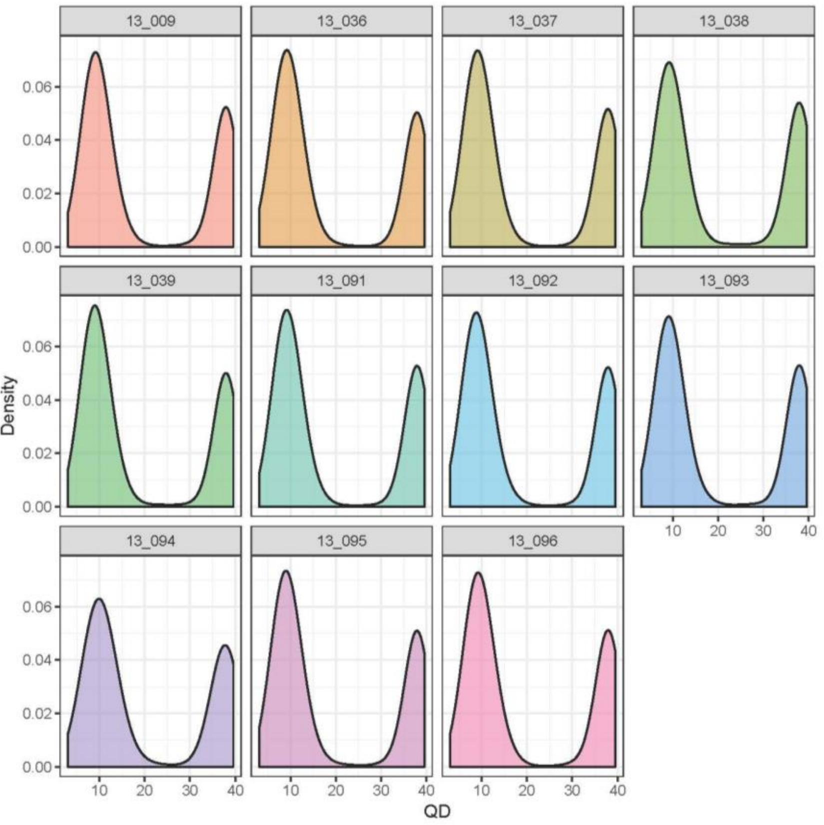

d.

Figure S4.2: Graphs representing quality–density. (QD) scores of all variants identified in each sample in this study. Sample code is shown above each graph. The graphs were generated by ggplot2 facet_grid. QD represents the variant quality score by the unfiltered heterozygous reference depth. QD annotation normalizes the variant quality in order to make it possible to compare variants with different alternative allele read depths based on quality. The plots show two peaks where the majority of variants were found.

FIGURE S4.3: Coverage depth of known PD genes. The coverage of six known PD genes ATP13A2, LRRK2, PINK1, PRKN, and SNC using the Ion AmpliSeq™ Neurological Diseases Panel and Ion Torrent sequencing platform is presented. The y-axis scale is 50, 100, 150, 200 and 25

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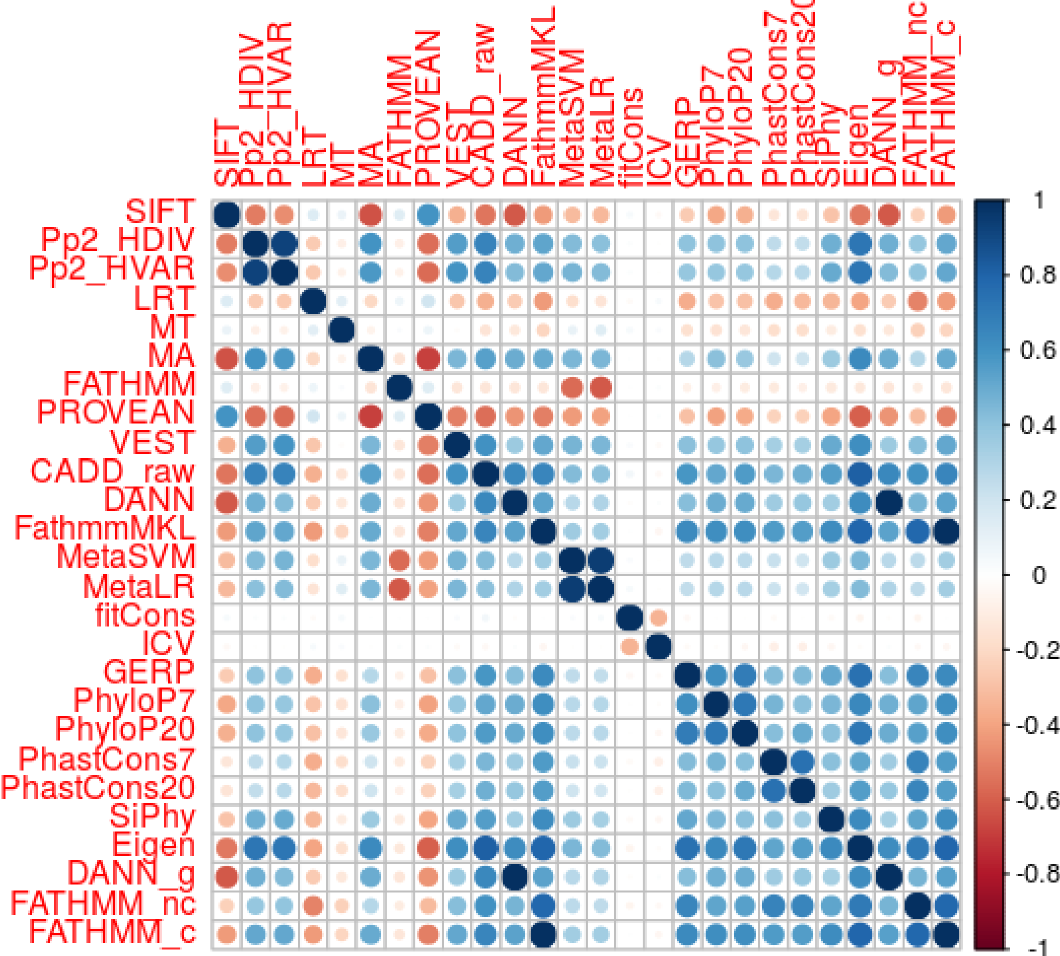

Figure S4.4: Correlation between prediction and conservation scores. We plotted the correlation matrix of 26 mutation scoring algorithm outputs for the 60 variants predicted to be deleterious identified in this study using variant scores obtained from tNGS data. **MetaLR** and **MetaSVM** were well-correlated and were used to predict the deleteriousness of sequence variants. See Figure 3 for comparison of 17 different scores for each of the 60 deleterious variants.

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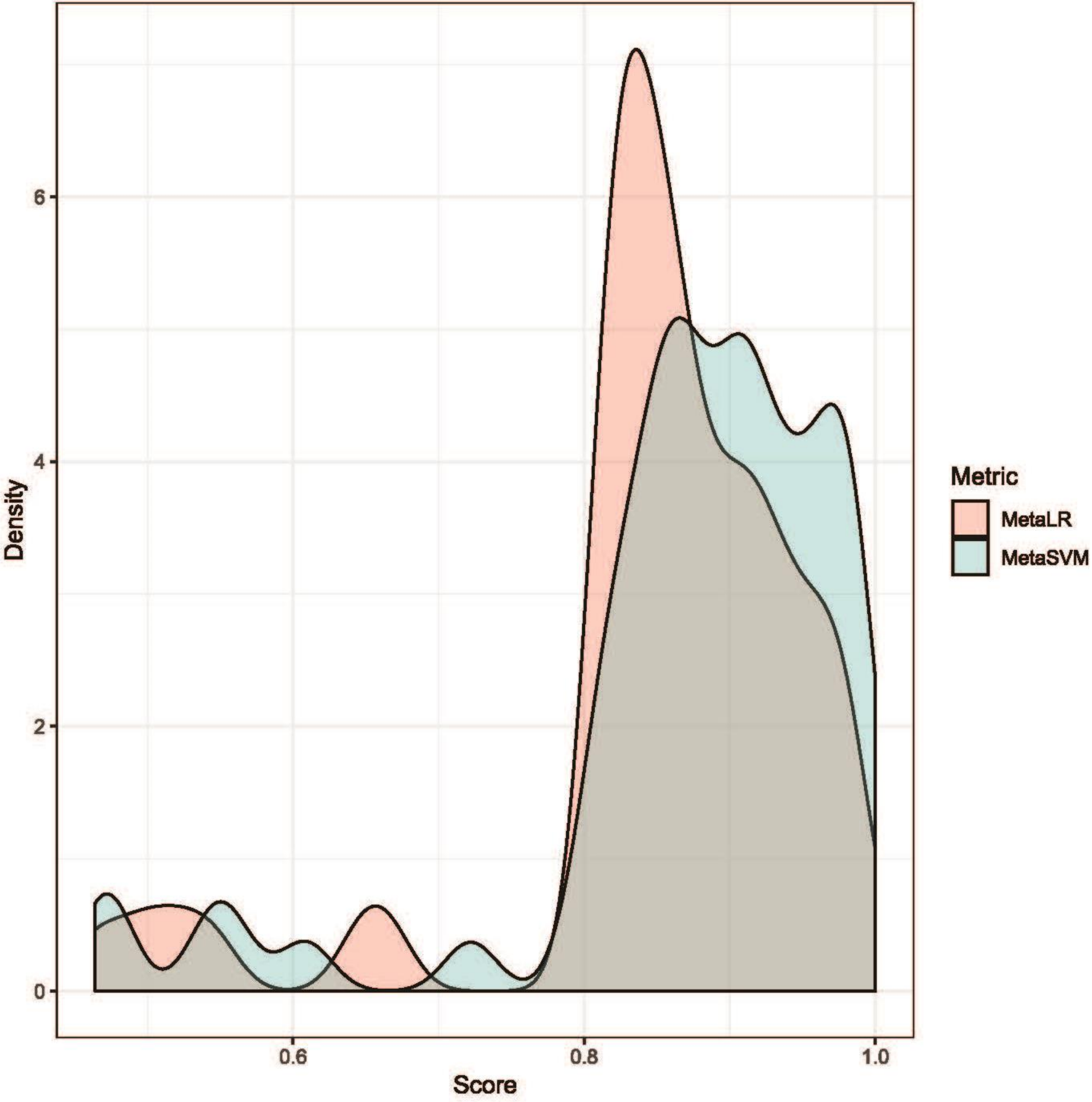
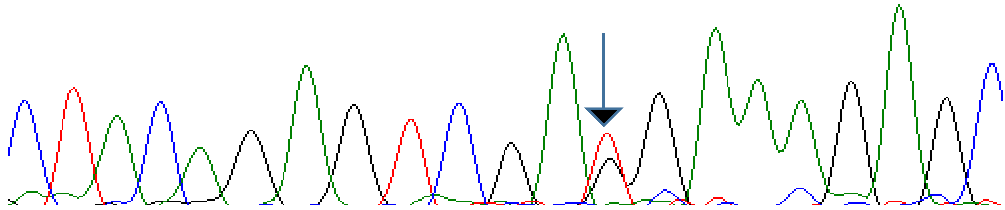
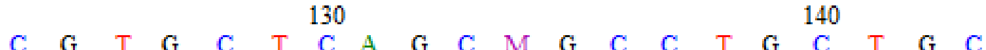
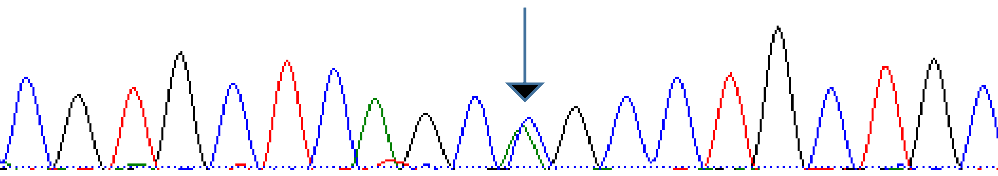


Figure S4.5: Density plot of the metaLR and metaSVM scores for the 60 selected variants. Variants based on score alone contributed to the peak above 0.8, whereas variants in known PD genes had scores > 0.45.



a. PRKN c.1182T>G (heterozygous missense D245E (NM_103988)) in a male Black South African PD patient. AAO of PD was 35 years.





b. ATP13A2 c.3010A>C (heterozygous missense S960R (NM_001141974)) in a male Black South African PD patient. AAO of PD was 39 years.

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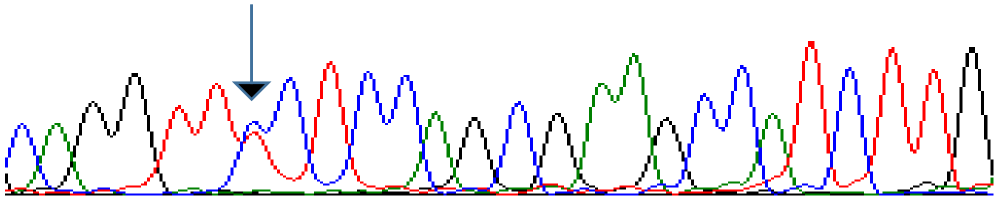
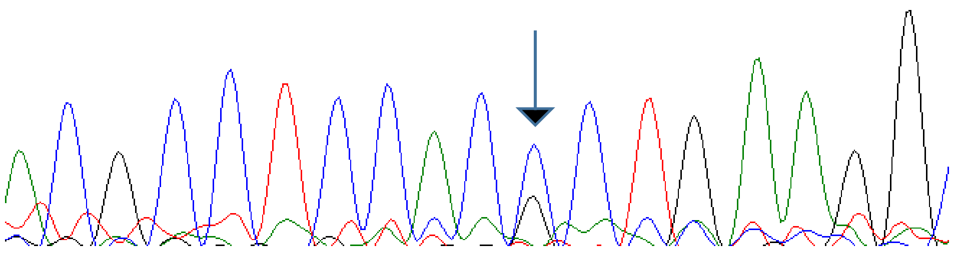


c. PINK1 c.683C>T (heterozygous missense S228F (NM_032409)) in a male Nigerian PD patient. AAO of PD was 53 years.



d. PINK1 c.913C>G (heterozygous missense P305A (NM_032409)) in a female Black South African PD patient. AAO of PD was 30 years.

Figure S4.6: Sanger sequencing results for selected variants. Chromatograms (a-d) demonstrating the variants identified in the tNGS. Blue arrows, locations of sequence variants.

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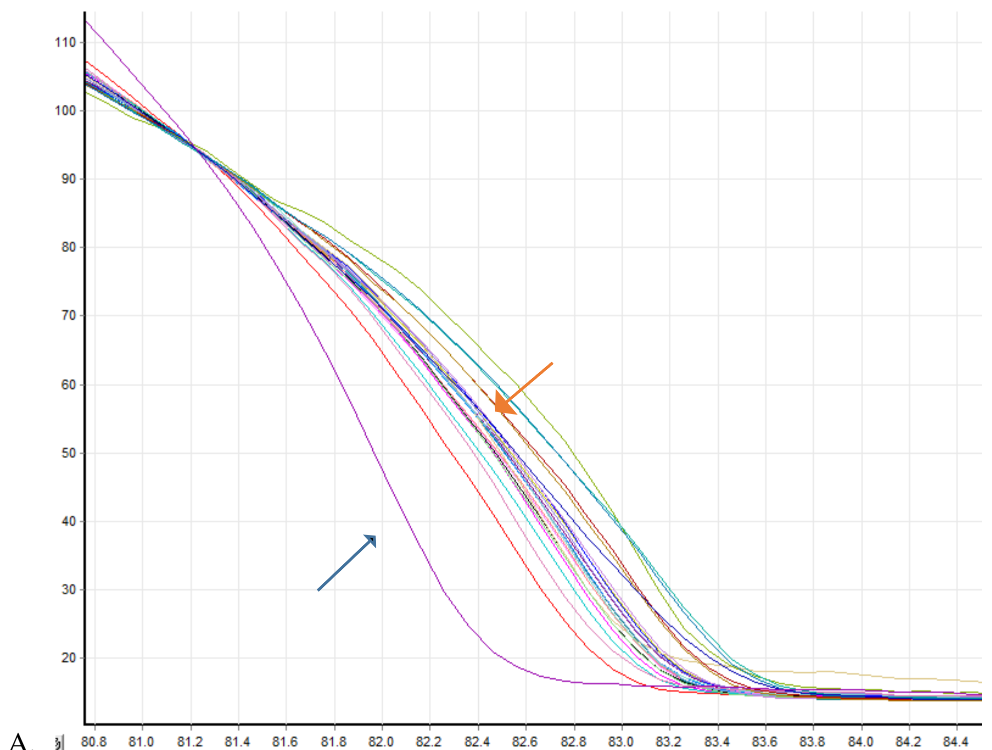
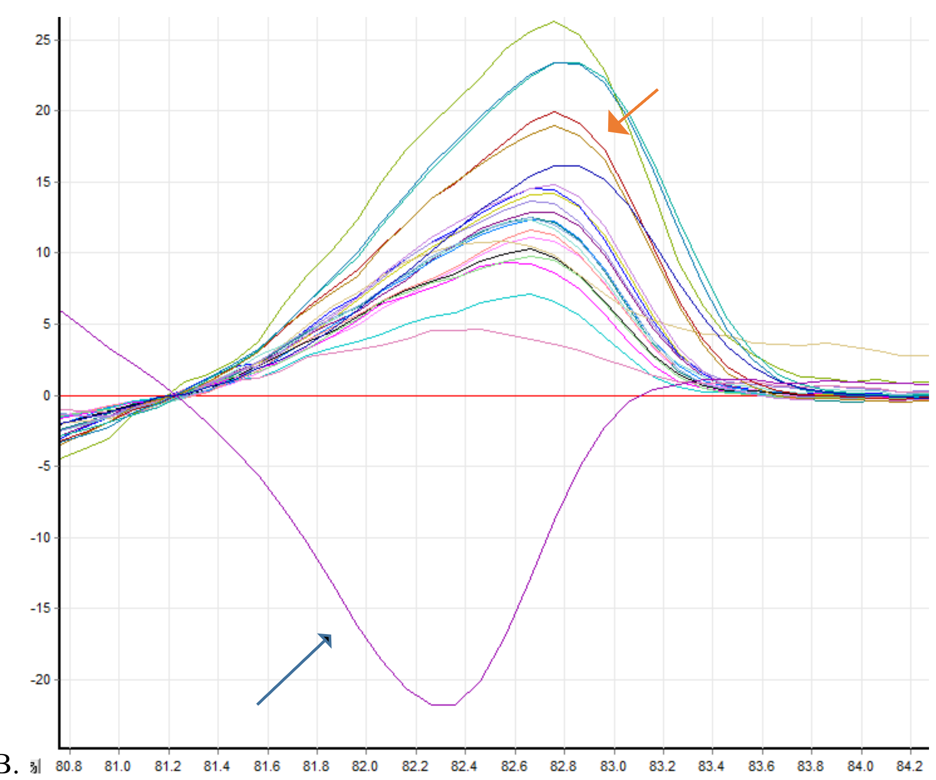
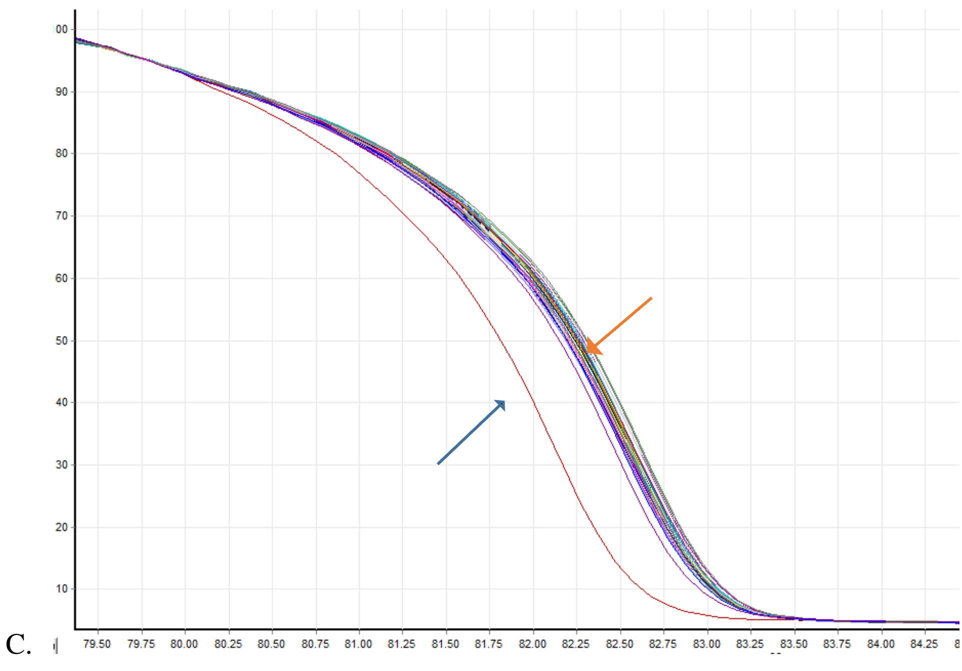
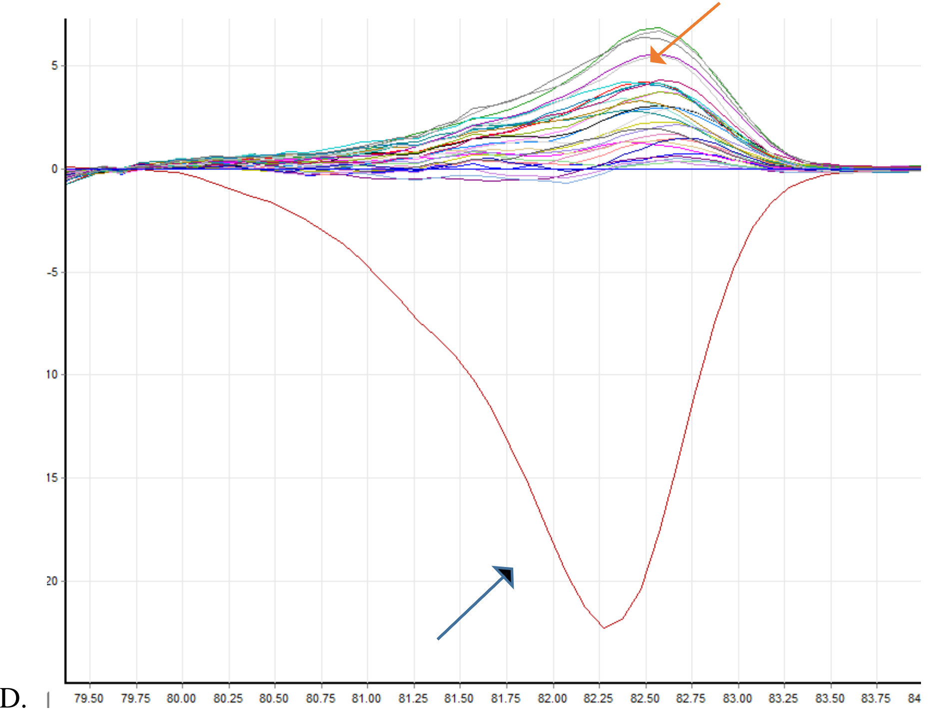






C.



D.

Figure S4.7: HRM screening for two variants in150 Black South African controls. Symbols: dark blue arrow, the mutant allele; red arrow, a group of control samples. Screening for PRKN D245E (A, B), and ATP13A2 S960R (C, D). Normalised graphs (A, C) and difference graphs (B, D) for the genotypes. The HRM melting temperature was 80-95ºC for PRKN D245E and 75-95ºC for ATP13A2 S960R.

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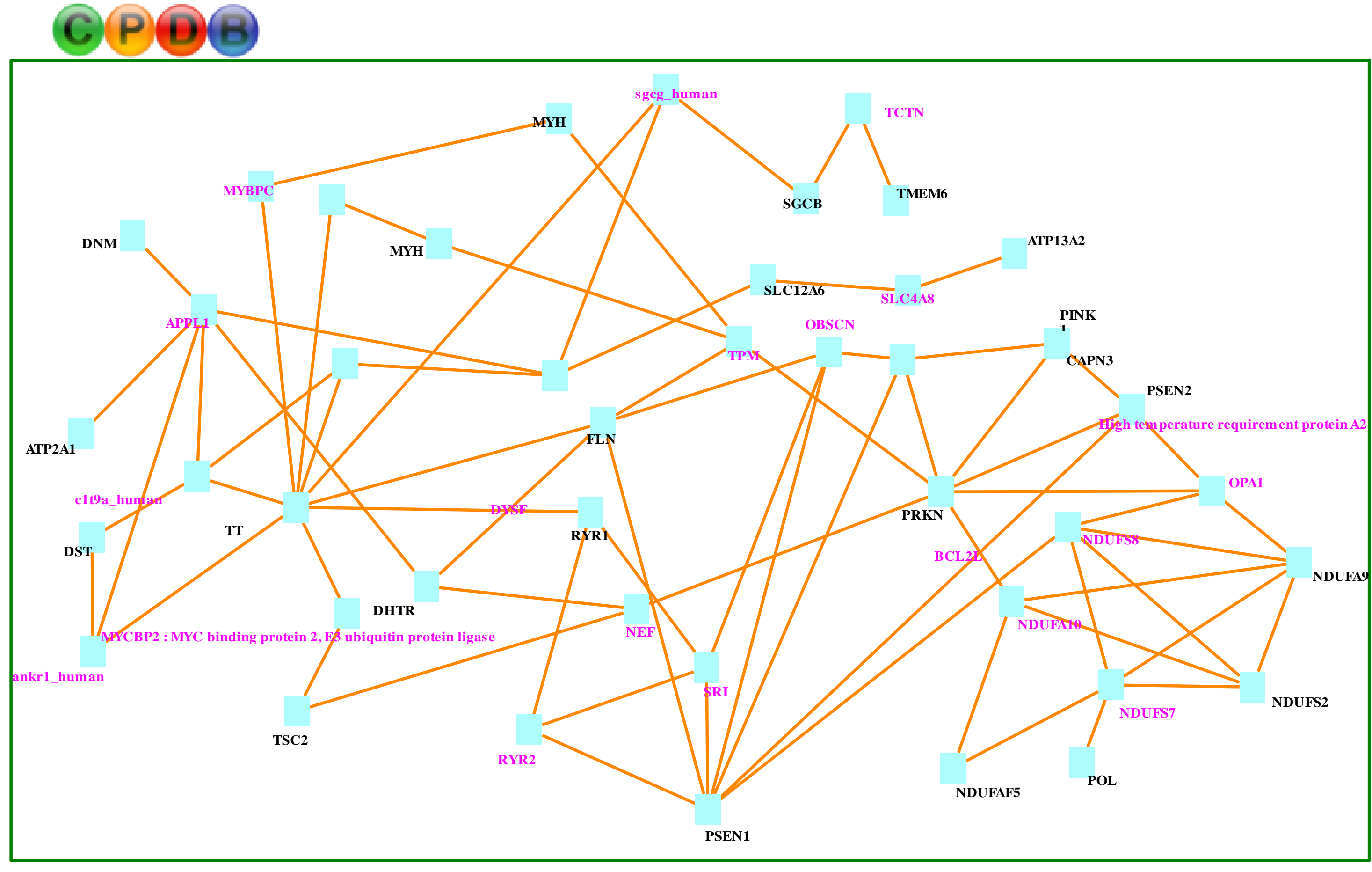

FIGURE S4.8: ConsensusPathDB network analysis of the 44 genes with variants predicted to be deleterious. We observed protein-protein interactions, on which 20 proteins were intermediate nodes and 24 proteins were seed nodes of biological processes. Produced using ConsensusPathDB http://cpdb.molgen.mpg.de/CPDB/cySVis

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Physical entity color

gene
protein
protein complex
RNA
compound
family
unknown

Interaction color

protein interaction
genetic interaction
biochemical reaction
gene regulatory interaction
drug-target interaction

Node label color

Black node labels denote seed nodes;

Magenta node labels denote intermediate nodes.

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CHAPTER FIVE

GENERAL DISCUSSION

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CHAPTER FIVE

GENERAL DISCUSSION

In the Chapters one and two, the rationale for studying the genetics of PD in the Black South African and Nigerian PD patients were described. Important issues were highlighted, such as the notable differences that exist in the haplotypes of the people who are from SSA. The differences have important ramifications for the disease pathophysiology and responses to treatments. Universally, the signs and symptoms of PD are the same in different parts of the world, including the majors signs observed in the PD patients in SSA. However, misdiagnosis, and the lack of neurologists in the rural communities to diagnose PD are some of the major challenges facing the investigators performing prevalence and incidence studies of PD in SSA.

Furthermore, these Chapters explained that genetic and environmental factors are profoundly involved in PD pathophysiology. Similarly, the reasons for implementing a tNGS technique for screening large numbers of genes involved in neurological diseases were stated. tNGS allows for a custom-design panel to screen genes of interest accurately and fast. SSA populations are greatly admixed and could harbour unique mutations in PD, hence by screening for pathogenic mutations in the PD patients in the unique SSA patients novel pathogenic mutations could be identified.

The present study lays the foundation for future studies on genetics of PD in SSA. There is not yet an ideal platform to screen for pathogenic mutations in patients, because each approach has advantages and disadvantages. A number of known PD genes that have been previously studied and associated with PD were highlighted and the mechanisms of PD pathology were discussed. Overall, a total of 15 Black SA and 42 Nigerian were studied for CNV in PD genes using the MLPA technique.

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Similarly, 33 Black SA and 14 Nigerian PD patients were studied for putative pathogenic mutations in candidate genes using a tNGS technique. Mutations found in PD patients from other geographic regions were not identified in this study, supporting the hypothesis that SSA PD patients may harbour novel mutations.

5.1 The Collaboration to Study the Genetics of PD in South Africa and Nigeria

The rationale for screening as many PD patients as possible for pathogenic mutations is strongly supported by literature (Lin and Farrer, 2014). Screening for disease mutations in a particular population, also provides an opportunity to identify novel mutations that might be unique to the people affected with the disease.

One of the challenges of PD studies in SSA is that sample sizes are relatively small. It therefore, requires a joint effort to recruit a large number of patients and ethnically matched controls in SSA. To address this matter, a research collaboration was established with the PD research group at Stellenbosch University located in Cape Town, South Africa, the Neurology Units of the Obafemi Awolowo University, Ile-Ife, in Nigeria, and the Mayo Clinic at Jacksonville, Florida, United States. The goal was to carry out cutting-edge research on the genetics of PD in SSA. This was the first time a research collaboration was established between South Africa and Nigeria to study the genetics of PD in Black PD patients.

The collaboration received funding from the Fogarty International Centre, National Institutes of Health (NIH) Bethesda, MD, USA. Bringing together the potentially large numbers of Black PD patients recruited from Nigeria, the most populous country in SSA, and in South Africa, where studies on the genetics of PD are in progress, provided an excellent starting point for this collaboration.

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The Mayo Clinic's Udall Center of Excellence in PD, in Florida, USA has been continuously funded by the NIH since 1999 attesting to their research excellence. This provided the ideal basis for innovative and multidisciplinary research on PD for this study. The funding covered two years. Ethics applications were submitted to both the Nigerian and the South African Ethics Committees. Patients were recruited, blood samples were collected for genomic DNA isolation and tNGS for studying pathogenic mutations in the PD patients was implemented.

The knowledge of genetic involvement in PD is still very limited in SSA. A previous study conducted to assess the knowledge, attitude and interest in genetic testing for mutations in PD in the Caucasians showed that the majority of participants thought it was likely they inherited a PD gene and showed that they were interested in genetic screening for PD (Falcone et al 2011).

Also, a study that provided insights into how people in SSA population perceive PD showed that the knowledge on PD was relatively high. However, many people opted not to provide a blood sample for genetic screening. The participants thought that PD was caused mostly by stress, certain foods or drinks, mental illness or other disorders. Some participants identified witchcraft as a cause of PD (Mokaya et al 2017). These beliefs are the major reasons why genetic studies on PD are not common in SSA. There is need for more awareness on PD genetics in these populations to ascertain the contributions of genetic mutations as one of the contributing factors of PD.

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5.1.1 Sample shipment

PD patients were recruited from Nigeria between the time period of 10 March 2017 to 20 July 2018. Material transfer agreement certificate issued by the Department of Health Republic of South Africa allowed the transfer of biological samples between the two countries. The recruiters informed the shipping company and completed the paper documentation needed to arrange for the sample pick-up and the shipping of the samples to South Africa.

Due to the sensitivity of the shipment, DHL Medical Express was contracted to take care of the shipment. They handle ambient, controlled at room temperature, and chilled or frozen shipments, with capabilities in place for monitoring these during transit. Ambient boxes were delivered to the Genetic Laboratory at Stellenbosch University and they included the packaging instructions inside the boxes. These boxes keep biological substances at a steady temperature during transit.

It usually took an approximately two weeks to transfer blood samples from the Nigerian recruitment sites to the Stellenbosch University Campus where the genetic studies were carried out. We recorded damage to blood sample tubes, which occurred in only one occasion and led to poor quality genomic DNA.

The ethics approvals to commence this study took three months. Participant information and informed consent forms used for research involving genetic studies, and the environmental exposure to hazard questionnaire form were completed by the patients. This information was catalogued and used for the patients' demographics in this study.

The tNGS was carried out at the DNA Sequencing Unit, Central Analytical Facilities of Stellenbosch University in Stellenbosch, South Africa. Analyses were performed in collaboration with the Bioinformatics Unit, Division of Molecular Biology and Human Genetics, Stellenbosch University. Patients and controls were also recruited in South Africa where there is an on-going recruitment for genetic studies on PD.

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5.1.2 Pilot Study to Check Feasibility

To check the performance of the Ampliseq 751genes Neurological Diseases panel, a pilot study involving the screening of eight PD patients including Caucasians, and Black South Africans was done. The study revealed that the Ion Ampliseq Neurological panel is a timeous and cost-effective method for screening of sequence variants in DNA extracted from both blood and saliva samples. Also, the preliminary analysis showed that the Black individuals seemed to have more sequence variations than the White South Africans in the genes studied. The pilot study also served as an internal control, as one sample was included in every run to determine the technical variability between different runs (unpublished data).

The patients' demographic and clinical information such as the AAO, AAR, family history of PD, sex, other disorders, prominent signs and symptoms and history of exposure to environmental toxins were collected. This information was entered on a study-specific REDCap database for easy accessibility.

5.2 Overall Study Outcomes

The review of previous genetic studies on PD patients in SSA suggested that genetic screening for pathogenic mutations in PD in these populations is still relatively new as limited information exists. It is therefore difficult to actually quantify the genetic risks for PD in SSA PD patients. Herein, the present study screened for mutations in the genes that are not previously linked to PD, and also included 17 of the known PD genes. The aim was to identify putative genes related to PD pathogenesis in SSA.

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The patients' demographic data showed that they have lived in their individual environments for a long period. Almost all the South African patients were diagnosed with early-onset PD. Early onset PD has implication in PD genetics. Nigerian patients, however, were late-onset PD, with some of them having a long history of exposure to different environmental toxins, such as herbicides. Environmental toxins could influence the development of PD (Nicolopoulou-Stamati et al., 2016). Similarly, people that are exposed to pesticides and other toxic chemicals could develop PD (Nicolopoulou-Stamati et al., 2016). Increasing age is the greatest risk factor for the development of PD. The number of neurons reduces in old people (Dickson, 2012). The reduction in the dopaminergic neurons activities often lead to the onset of PD symptoms (Chu and Kordower, 2007).

This study found four putative pathogenic CNVs in sporadic PD patients and concentrated on the 60 missense variants predicted to be deleterious identified in 44 genes including *ATP13A2*, *PRKN* and *PINK1* genes in the patients (**Figure 5.1**). Deleterious missense variant is known to change protein structure, solubility and dynamics and may affect the overall function of a gene. I*n silico* pathogenicity prediction tools mostly based their predictions on these principles by calculating the degree of conservation within species of the mutated site and its effect on RNA splicing and protein structure.

It is noteworthy to stress that sporadic PD cases were focused on in this study because sporadic forms of PD are seen predominantly, showing non-Mendelian inheritance pattern and lack of a definite family history (Kalinderi et al. 2016; Verstraeten et al. 2015).

Studies have described how different heterozygous missense, insertion, compound heterozygous, frame shift and duplication mutations in *PINK1* and *PRKN* in the monogenic PD patients, occurred virtually at every site on these genes (Pickrell and Youle, 2015). It was intriguing however that previously reported mutations in PD genes were not identified in the current study.

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These findings corroborate the previous genetic studies in SSA PD patients and indicated that common mutations like *LRRK2* G2019S are a rare cause of PD in these populations (Blanckenberg et al., 2013c). Previous genetic studies in SSA individuals with PD identified a duplication of exon 2 and a deletion of exon 9; and an heterozygous G430D mutation in the *PRKN* gene in Black South African patients, while an heterozygous deletions of exon 2 and exon 4 in *PRKN* gene were identified in Zambians with PD (Keyser et al., 2010c; Yonova-Doing et al., 2012). The above-mentioned variants were not identified in this study. The variants predicted to be deleterious identified in this study in the 44 genes have not been reported in PD patients before. Although, *PRKN*, *PINK1* and *ATP13A2* genes have been studied in the European, North American and North African population and found to harbour other pathogenic mutations (Singleton et al., 2013). Hence, new genes are to be studied in order to uncover the genetic architecture that impacts disease susceptibility in PD sporadic cases.

The availability of high-throughput genetic analysis techniques is increasing the information available for PD genetics. NGS technique has been effective to discover new candidate genes. The NGS requires selecting variants with high-quality calls to identify variants predicted to be deleterious which can be used for functional studies to verify the roles of the genes in PD (Jansen et al., 2017). The known PD genes belong to biological pathways involved in autophagy, apoptosis or mitochondrial function (Poewe et al., 2017).

tNGS and other methods used in the present study to screen for mutations in the Black South African and Nigerian PD patients are established and reliable (Quail et al., 2012). A recent study that used the Ion torrent tNGS sequencing platform to screen for mutations in PD genes in the Spanish population, confirmed this platform to be effective (Gorostidi et al., 2016).

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For variant identifications and annotation, certain metrics are critical for variant interpretation. Recently, a joint consensus recommendation by the Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists was published, and provided standards and guidelines for variant identification and annotation (Li et al., 2017). The criteria includes supporting reads (depth of coverage) and variant allele frequency, and using up-to-date bioinformatics pipelines. It also includes identifying the genomic locations of the variants, to determine if it is coding or non-coding (Li et al., 2017; Li and Wang, 2017). These criteria were considered in the variant evaluation processes in our study.

The quality of the BAM files collected in this study was good and was important for the additional information retrieved for each variant presented for annotations. Variant annotation is crucial to accurate interpretation of somatic sequence variants. The most applicable QC criteria applied to any NGS data are not restricted (Utturkar et al., 2015), usually, a minimum coverage required to call a base accurately at any position is (≥ 20X read depth) (Craig et al., 2008). Because NGS platforms are likely to make errors, it is therefore, important to use stringent criteria for variant annotations, to have confidence in the sequencing data and the variants selected. In our study, we used stringent threshold to discover high confidence variants and assessed the pathogenicity with 26 different scoring algorithms.

Identified variants might not be sufficient to produce PD symptoms in some people, but could increase their risk for developing PD. The interactions between genetic variants, in conjunction with environmental factors could lead to the development of PD. The identification of these factors provides information on pathogenic pathways that may be therapeutic targets (Cannon and Greenamyre, 2013). Determination of the pathogenicity or clinical impact of variants in PD remains a monumental task.

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tNGS analyses identified variants predicted to be deleterious in three known PD genes and 41 other genes in the SSA PD patients' part of the current study. Also, the MLPA assay identified four putative pathogenic CNVs in three known PD genes (*PARK 7*, *PRKN* and *PINK1*).

Interestingly, the 41 non-PD genes with variants predicted to be deleterious found in the Black South African and Nigerian PD patients encode important proteins. The proteins share biological pathways that have connections to the pathophysiology of neurological disorders including PD. In the present study, pathogenicity of the variants identified were determined by their deleteriousness scores.

Variants identified in *PINK1* and *PRKN* genes were further studied using protein modelling and the protein structures indicated that (*PRKN D234E,, PINK1 S228F and PINK1 P305A*) variants have strong implications for PD pathogenesis because their positions are in a domain that regulate autophagy, supporting the fact that these genes are prominent disease-causing genes.

Studies have shown that *PINK1/PARKIN* pathway facilitates mitophagy by altering mitochondrial trafficking (Wang et al. 2011). The *PINK1/PARKIN* pathway affects mitochondrial functions and trafficking by proteasomal degradation of specific mitochondrial outer membrane proteins. It also targets the entire mitochondria for autophagic degradation by selective recruitment of adaptor proteins to other mitochondrial outer membrane substrates (Narendra et al. 2012).

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Parkin at serine 65 and/or PINK1 at threonine 257 have been used for examining activity of PINK1-Parkin signalling pathway *in vivo* (Kondapalli et al., 2012). Although, *PINK1* directly phosphorylates *Parkin* at S65 domain to stimulate *Parkin* E3 ligase activity and recruitment to mitochondria (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012), mutation at S65 *Parkin* prevents phosphorylation by *PINK1*. The *Parkin* mutation S65A did not influence Parkin translocation to mitochondria, despite a lack of phosphorylation in S65A, Parkin still translocates to mitochondria in a PINK1 kinase-dependent process, indicating that other cytosolic *PINK1* substrates are involved in *Parkin* activation (Kane et al., 2014), suggesting that, there are other mutations in *PINK1* and *Parkin* mitochondrial quality control processes in PD pathogenesis.

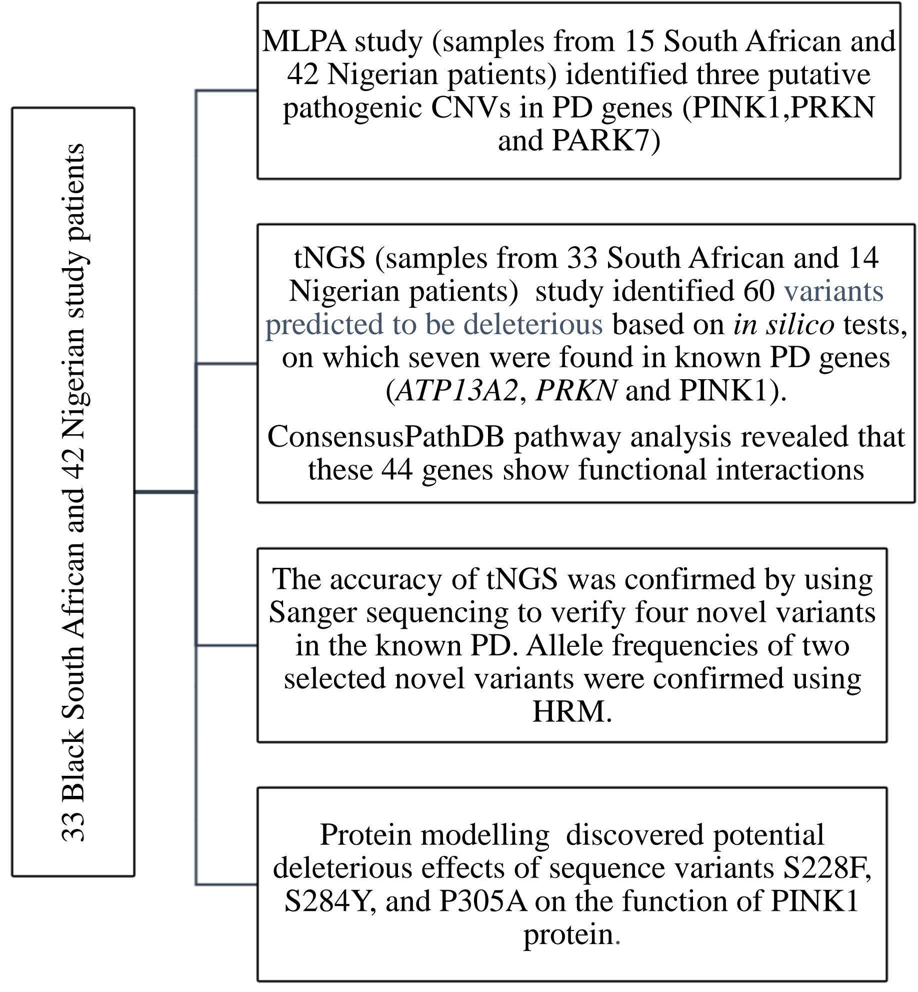

Figure 5.1: Overview of the study outcomes

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Growing evidence is supporting mutations in *ATP13A2* gene to be deleterious and disease-causing in PD patients. The previously reported mutations in *ATP13A2* gene in PD are described in **Chapter 1**. *ATP13A2* is located inside the lysosomal membrane and its proper function is essential to the lysosomal membrane stability. Variant predicted to be deleterious in this gene could impair lysosomal membrane stability which in turns affects the process of mitophagy degradation. In the present study, the deleterious variant *S960R* identified in the *ATP13A2* gene in one of the PD patients suggests that SSA patients with PD may be susceptible to mutations in this gene, which may increase their risk for developing the disease.

As hypothesized in this study, novel variants predicted to be deleterious were identified in PD genes and other genes that have not been previously associated with PD. Especially the frequently identified mutations in PD in Europe, North America, North Africa and Asia were not found in these patients. The Consensus PathDB analysis revealed biological networks that exist between the 44 genes that share mechanisms of pathology like autophagy, mitochondrial dysfunction, neuroinflammation, oxidative stress and cellular/receptor damage.

No variant predicted to be deleterious however was identified in *SNCA* gene. SCNA has a crucial role in PD pathology, *SNCA* gene mutations produced deleterious effects in dopaminergic neurons by reducing the affinity of α-synuclein for lipids, thus increasing the tendency of the protein to form oligomers through a concentration-dependent pattern and continuous accumulations of toxic α-synuclein fibrils (the major component of Lewy bodies) (Winner et al. 2011). Lewy body exist in Nigerian PD patients as it described in **Chapter 2** of this study. Previous studies on PD genetics in SSA also showed that mutations in this gene are rare. Nonetheless, more patients are to be screened for mutations including CNVs in *SNCA* gene before drawing final conclusion.

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5.3 Study Limitations and Future Follow-up Studies

Major limitations to this study were discussed extensively in the **Chapters 3,** and **4**. In addition to those limitations, a total of 18 genes with associations with PD and reported in literature, were not on the Ampliseq 751-gene panel. Some genes like the *APOE* gene which are often studied in PD-dementia patients were not on the panel used. Similarly, the *coiled-coil-helix-coiled-coil-helix domain 2 (CHCHD2)* gene. Whole genome analysis in a Japanese family with AD PD (Funayama et al. 2015) found a heterozygous mutation. *CHCHD2* should, therefore, also be considered a candidate gene for PD.

Therefore, using a whole exome sequencing to screen for mutation in the SSA PD patients in the future will allow to screen all genes in the human genome. Whole genome sequencing will provide the opportunity to identify copy number variations which are difficult to identify in a targeted gene panel. Also, putative pathogenic non-coding variants could be revealed with a whole genome sequencing approach.

Mutations in *vacuolar protein sorting 13C (VPS13C)* have association with the development of autosomal recessive early-onset forms of PD. It has been proven that *VPS13C* encodes a member of a family of *VPS13* (Velayos-Baeza et al. 2004) and *VPS13C* is located on the outer mitochondrial membrane.

The *Ubiquitin C-terminal hydrolase L1* (*UCHL1*) gene is among the genes first implicated in PD. The mutations in this gene have not yet been fully proven to cause PD. The presence of Lewy bodies and the function of *UCHL1* in the proteasome pathway suggest that *UCHL1* could be a compelling PD candidate.

Sequence analyses also confirmed *GIGYF2* gene as a PD gene in 12 unrelated familial PD patients from Italy and France, the study revealed seven different heterozygous mutations in the *GIGYF2* gene.

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The CAG repeat expansions within the coding region of *Ataxin-2 (ATXN2)* were gene identified in a dominantly inherited familial forms of PD (Gwinn–Hardy et al. 2000; Payami et al. 2003), and the clinical examinations suggest that cerebellar ataxia is a predominant symptom among PD patients.

Mutations in the *DNAJC6* gene in juvenile-onset atypical parkinsonism with autosomal recessive inheritance have been reported. (Edvardson et al. 2012; Koroglu et al. 2013). Similarly, mutations in *DNAJC13* gene have been reported in patients with a dominant form of PD characterized by α-synuclein positive Lewy bodies.

The *SYNJ1* gene is among the newly identified PD genes. The *SYNJ1* gene encodes synaptojanin-1, a presynaptic phosphoinositide phosphatase protein which has a role in the regulation of synaptic vesicle endocytosis, important in the recycling of proteins. It has a similar role to synaptojanin-1in endocytosis and *parkin* (Quadri et al. 2013; Krebs et al. 2013; Olgiati et al. 2014).

Mutations identified in the aforementioned genes require further studies to understand their true significance in PD. Likewise in this study the other 41 novel genes with variants predicted to be deleterious in PD will require extensive further studies as discussed in **Chapter 4**.

5.4 Conclusions

In conclusion, the genetic analyses described here, which included MLPA and the advanced bioinformatics tools led to the identification of novel pathogenic mutations in the Black South African and Nigerian PD patients. Mutations were found in known PD genes and in genes not previously known to harbour mutations in PD patients. Further studies are required to ascertain the biological functions of these mutations. Moreover, their role in PD pathobiology needs to be validated. The results could advance the discovery and the development of improved treatment approaches and drug interventions for PD.

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APPENDIX I: List of variants or mutations in genes identified in PD

Gene	Mutation	Family member with PD	Reference
PINK1	Gly32Arg	1Asian	Choi JM, *Neurogenetics* 9: 263-9, 2008
PINK1	Lys24fs and Cys549fs	4 Caucasians	Ibáñez P, *Brain* 129: 686-94, 2006
PINK1	Leu67Phe	1 Caucasian	Marongiu R, *Hum Mutat* 29: 565, 2008 Ferraris A, *Mov Disord* 24: 2350-7, 2009
PINK1	Arg68Pro	1 Caucasian	Valente EM, *Ann Neurol* 56: 336-41, 2004 Marongiu R, *Hum Mutat* 29: 565, 2008
PINK1	Gln129Stop	5 Caucasians and 4 Tunisians	Ishihara-Paul L, *Neurology* 71: 896-902, 2008
PINK1	Thr145Met	2 Caucasians and 1 Tunisian	Ishihara-Paul L, *Neurology* 71: 896-902, 2008
PINK1	Arg152Trp	3 Tunisians	Ishihara-Paul L, *Neurology* 71: 896-902, 2008
PINK1	Glu240Lys	2 Caucasians and 1 Asian	Rogaeva E, *Arch Neurol* 61: 1898-904, 2004. Ibáñez P, *Brain* 129: 686-94, 2006
PINK1	Arg246Stop	4 Asians and 2 Jewish	Hatano Y, *Ann Neurol* 56: 424-7, 2004 Tan EK, *Mov Disord* 21: 789-93, 2006 Ephraty L, *Mov Disord* 22: 566-9, 2007
PINK1	Tyr258Stop	3 Indian South Africa and I Asian	Tan EK, *Mov Disord* 21: 789-93, 2006 Keyser RJ, *Neurogenetics* Epub: , 2009
PINK1	Leu268Val	2 Asians	Tan EK, *Clin Genet* 68: 468-70, 2005 Marongiu R, *Hum Mutat* 29: 565, 2008
PINK1	Arg276Gln	5 Asians	Marongiu R, *Hum Mutat* 29: 565, 2008 Biswas A, *Parkinsonism Relat Disord* Epub: , 2009
PINK1	Asp297fs	2 Asians	Kumazawa R, *Arch Neurol* 65: 802-8, 2008 Savettieri G, *Parkinsonism Relat Disord* 14: 509-12, 2008
PINK1	Ala383Thr	5 Tunisia and 2 Caucasians	Ibáñez P, *Brain* 129: 686-94, 2006 Abou-Sleiman PM, *Ann Neurol* 60: 414-9, 2006 Marongiu R, *Hum Mutat* 29: 565, 2008
PINK1	Gly411Ser	2 Tunisians and 5 Caucasians	Zadikoff C, *Mov Disord* 21: 875-9, 2006 Abou-Sleiman PM, *Ann Neurol* 60: 414-9, 2006 Toft M, *J Neurol Neurosurg Psychiatry* 78: 82-4, 2007 Mellick GD, *Parkinsonism Relat Disord* 15: 105-9, 2009 Brooks J, *J Med Genet* 46: 375-81, 2009
PINK1	Trp437Stop	5 Caucasians	Valente EM, *Science* 304: 1158-60, 2004 Bonifati V, *Neurology* 65: 87-95, 2005 Criscuolo C, *Mov Disord* 21: 1265-7, 2009
PINK1	Gln456Stop	22 (Caucasian and Tunisians)	Bonifati V, *Neurology* 65: 87-95, 2005 Ibáñez P, *Brain* 129: 686-94, 2006 Hedrich K, *Arch Neurol* 63: 833-8, 2006 Zadikoff C, *Mov Disord* 21: 875-9, 2006 Abou-Sleiman PM, *Ann Neurol* 60: 414-9,

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			2006 Marongiu R, *Hum Mutat* 29: 565, 2008
PINK1	Glu476Lys	12 (Tunisians, Jordanian and Caucasians	Valente EM, *Ann Neurol* 56: 336-41, 2004 Rogaeva E, *Arch Neurol* 61: 1898-904, 2004 Bonifati V, *Neurology* 65: 87-95, 2005 Abou-Sleiman PM, *Ann Neurol* 60: 414-9, 2006 Marongiu R, *Hum Mutat* 29: 565, 2008 Myhre R, *BMC Neurol* 8: , 2008
LRRK2	Ala211Val	3 Caucasian	Xiromerisiou G, *Eur J Neurol* 14: 7-11, 2007 Nuytemans K, *Hum Mutat* Epub: , 2009
LRRK2	Glu334Lys	2 Caucasians	Nichols WC, *Neurology* 69: 1737-44, 2007 Lesage S, *J Med Genet* Epub: , 2009
LRRK2	Arg793Met	6 Caucasians	Zimprich A, *Neuron* 44: 601-7, 2004 Farrer M, *Neurology* 65: 738-40, 2005 Berg D, *Brain* 128: 3000-11, 2005 Toft M, *Acta Neurol Scand Suppl* 187: 72-5, 2007 Chen-Plotkin AS, *Neurology* 70: 521-7, 2008 Covy JP, *Mov Disord* 24: 32-9, 2009
LRRK2	Leu1114	4 Caucasians	Zimprich A, *Neuron* 44: 601-7, 2004 Farrer M, *Neurology* 65: 738-40, 2005 Berg D, *Brain* 128: 3000-11, 2005
LRRK2	Arg1325Gln	3 Caucasians	Nuytemans K, *Eur J Hum Genet* 16: 471-9, 2008 Lesage S, *J Med Genet* Epub: , 2009
LRRK2	Thr1410Met	7 Caucasians	Lesage S, *J Med Genet* Epub: , 2009 Abdalla-Carvalho CB, *Eur J Neurol* Epub: , 2010
LRRK2	Arg1441Gly	126 Caucasians, Latin Americans, Jewish and Asian	Paisán-Ruíz C, *Neuron* 44: 595-600, 2004 Mata IF, *Neurosci Lett* 382: 309-11, 2005 Mata IF, *Neurogenetics* 6: 171-7, 2005 Gaig C, *Arch Neurol* 63: 377-82, 2006 Simón-Sánchez J, *Mov Disord* 21: 1954-9, 2006 Dächsel JC, *Neurosci Lett* 410: 80-4, 2006 Deng H, *J Neurol Sci* 251: 102-6., 2006 González-Fernández MC, *Parkinsonism Relat Disord* 13: 509-15, 2007 Gómez-Esteban JC, *Neurosurgery* 62: 857-62, 2008 Gao L, *Eur J Neurol* Epub: , 2009 Gorostidi A, *Neurogenetics* 10: 157-9, 2009 Mata IF, *Parkinsonism Relat Disord* 15: 370-3, 2009 Martí-Massó JF, *Mov Disord* 24: 1998-2001, 2009
LRRK2	Arg1441Cys	28 Caucasians, Americans, Asianss	Zimprich A, *Neuron* 44: 601-7, 2004 Zabetian CP, *Neurology* 65: 741-4, 2005 Goldwurm S, *J Med Genet* 42: e65, 2005 Mata IF, *Neurogenetics* 6: 171-7, 2005 Di Fonzo A, *Eur J Hum Genet* 14: 322-31, 2006 Gaig C, *Arch Neurol* 63: 377-82, 2006 Tan EK, *Mov Disord* 21: 997-1001, 2006 Hedrich K, *Mov Disord* 21: 1506-10, 2006

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			Pankratz N, *Mov Disord* 21: 2257-60, 2006 Gosal D, *Mov Disord* 22: 291-2, 2007 Nuytemans K, *Eur J Hum Genet* 16: 471-9, 2008 Haugarvoll K, *Neurology* 70: 1456-60, 2008 Pchelina SN, *Eur J Neurol* 15: 692-6, 2008 Latourelle JC, *BMC Med* 6: , 2008 Floris G, *Parkinsonism Relat Disord* 15: 277-80, 2009 Shojaee S, *Mov Disord* 24: 1023-7, 2009
LRRK2	Arg1441His	8 Caucasians	Zabetian CP, *Neurology* 65: 741-4, 2005 Mata IF, *Neurogenetics* 6: 171-7, 2005 Spanaki C, *Neurology* 67: 1518-9, 2006 Huang Y, *Mov Disord* 22: 982-9, 2007 Ferreira JJ, *Mov Disord* 22: 1194-201, 2007 Lin CH, *J Biomed Sci* 15: 661-7, 2008 Lesage S, *J Med Genet* Epub: , 2009
LRRK2	Gly2019Ser	772 (Ashkenazi Jewish, Arab Berber (Tunisia), Caucasian and Latin Americans)	Numerous references, strongly known
LRRK2	Ile2020Thr	3 Caucasians	Zimprich A, *Neuron* 44: 601-7, 2004 Berg D, *Brain* 128: 3000-11, 2005 Ohta E, *Neurosci Lett* 417: 21-3, 2007
PRKN	Gln34Arg	8 Asians and Indian South Africa	Chaudhary S, *Parkinsonism Relat Disord* 12: 239-45, 2006 Biswas A, *Parkinsonism Relat Disord* 12: 420-6, 2006 Myhre R, *BMC Neurol* 8: , 2008 Bardien S, *Parkinsonism Relat Disord* 15: 116-21, 2009
PRKN	Arg42Pro	11 Caucasian	Terreni L, *Neurology* 56: 463-6, 2001 Hedrich K, *Neurology* 58: 1239-46, 2002 Bertoli-Avella AM, *Mov Disord* 20: 424-31, 2005 Clark LN, *Arch Neurol* 63: 548-52, 2006 Pellecchia MT, *Mov Disord* 22: 559-63, 2007 Wang Y, *Arch Neurol* 65: 467-74, 2008 Sironi F, *Parkinsonism Relat Disord* 14: 326-33, 2008 Macedo MG, *Mov Disord* 24: 196-203, 2009
PRKN	Asn52fs	31 Caucasians	Abbas N, *Hum Mol Genet* 8: 567-74, 1999 Hedrich K, *Neurology* 58: 1239-46, 2002 Hoenicka J, *Arch Neurol* 59: 966-70, 2002 Nichols WC, *J Med Genet* 39: 489-92, 2002 Muñoz E, *J Neurol Neurosurg Psychiatry* 73: 582-4, 2002 Rawal N, *Neurology* 60: 1378-81, 2003 Hedrich K, *Mov Disord* 19: 1146-57, 2004 Sun M, *Arch Neurol* 63: 826-32, 2006 Dächsel JC, *Neurosci Lett* 410: 80-4, 2006 Lesage S, *J Med Genet* 45: 43-6, 2008 Bras J, *BMC Neurol* 8: , 2008

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			Lohmann E, *Neurology* 72: 110-6, 2009 Nuytemans K, *Hum Mutat* Epub: , 2009 Camargos ST, *Mov Disord* 24: 662-6, 2009 Brooks J, *J Med Genet* 46: 375-81, 2009 Pankratz N, *Neurology* 73: 279-86, 2009
PRKN	Ala82Glu	8 Caucasians	Hedrich K, *Hum Mol Genet* 10: 1649-56, 2001 Varrone A, *Neurology* 63: 2097-103, 2004 Kay DM, *Ann Neurol* 61: 47-54, 2007 Lesage S, *J Med Genet* 45: 43-6, 2008 Håkansson A, *Parkinsonism Relat Disord* 14: 520-2, 2008 Sironi F, *Parkinsonism Relat Disord* 14: 326-33, 2008 Nuytemans K, *Hum Mutat* Epub: , 2009 Brooks J, *J Med Genet* 46: 375-81, 2009 Pankratz N, *Neurology* 73: 279-86, 2009
PRKN	Pro113fs	25 Caucasians	Farrer M, *Ann Neurol* 50: 293-300, 2001 Hedrich K, *Neurology* 58: 1239-46, 2002 Nichols WC, *J Med Genet* 39: 489-92, 2002 Foroud T, *Neurology* 60: 796-801, 2003 Chen R, *Parkinsonism Relat Disord* 9: 309-12, 2003 Scherfler C, *Brain* 127: 1332-42, 2004 Wiley J, *Mov Disord* 19: 677-81, 2004 Poorkaj P, *Am J Med Genet A* 129A: 44-50, 2004 Hedrich K, *Mov Disord* 19: 1146-57, 2004 Poorkaj P, *BMC Neurol* 5: , 2005 Clark LN, *Arch Neurol* 63: 548-52, 2006 Sun M, *Arch Neurol* 63: 826-32, 2006 Wang Y, *Arch Neurol* 65: 467-74, 2008 Brooks J, *J Med Genet* 46: 375-81, 2009 Pankratz N, *Neurology* 73: 279-86, 2009 Sammler EM, *Mov Disord* 24: 2442-3, 2009
PRKN	Lys211Asn	8 Caucasians	van de Warrenburg BP, *Neurology* 56: 555-7, 2001 Kann M, *Ann Neurol* 51: 621-5, 2002 Nichols WC, *J Med Genet* 39: 489-92, 2002 Foroud T, *Neurology* 60: 796-801, 2003 Klein C, *Eur J Hum Genet* 13: 1086-93, 2005 Hertz JM, *Eur J Neurol* 13: 385-90, 2006 Aguiar P de C, *Mov Disord* 23: 1228-33, 2008 Barsottini OG, *Arq Neuropsiquiatr* 67: 7-11, 2009 Pankratz N, *Neurology* 73: 279-86, 2009
PRKN	Cys441Arg	10 Asians	Shyu WC, *Parkinsonism Relat Disord* 11: 173-80, 2005 Chan DK, *J Neural Transm* 115: 715-9, 2008 Guo JF, *Mov Disord* 23: 2074-9, 2008 Lohmann E, *Neurology* 72: 110-6, 2009 Lee MJ, *Mov Disord* 24: 104-8, 2009

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PRKN	Pro437Leu	27 Caucasians	Hedrich K, *Neurology* 58: 1239-46, 2002 Nichols WC, *J Med Genet* 39: 489-92, 2002 Foroud T, *Neurology* 60: 796-801, 2003 Rawal N, *Neurology* 60: 1378-81, 2003 Djarmati A, *Hum Mutat* 23: , 2004 Poorkaj P, *Am J Med Genet A* 129A: 44-50, 2004 Hertz JM, *Eur J Neurol* 13: 385-90, 2006 Sun M, *Arch Neurol* 63: 826-32, 2006 Kay DM, *Ann Neurol* 61: 47-54, 2007 Nuytemans K, *Hum Mutat* Epub: , 2009 Brooks J, *J Med Genet* 46: 375-81, 2009 Pankratz N, *Neurology* 73: 279-86, 2009 Brooks JA, *Neurobiol Aging* Epub: , 2009
PRKN	Gly430Asp	8 Asians and Caucasians)	Hedrich K, *Neurology* 58: 1239-46, 2002 Scherfler C, *Brain* 127: 1332-42, 2004 Shyu WC, *Parkinsonism Relat Disord* 11: 173-80, 2005 Sun M, *Arch Neurol* 63: 826-32, 2006 Mellick GD, *Parkinsonism Relat Disord* 15: 105-9, 2009 Pankratz N, *Neurology* 73: 279-86, 2009
PRKN	Cys431Phe	13 (Asians and Caucasians)	Maruyama M, *Ann Neurol* 48: 245-50, 2000 Brooks J, *J Med Genet* 46: 375-81, 2009
PARK7	ex1-5del	3 Caucasians	Bonifati V, *Science* 299: 256-9, 2003
PARK7	Ala179Thr	2 Caucasians	Macedo MG, *Mov Disord* 24: 196-203, 2009 Nuytemans K, *Hum Mutat* Epub: , 2009
SNCA	tripSNCA [F-I]	12 Caucasians	Farrer M, *Ann Neurol* 55: 174-9, 2004 Fuchs J, *Neurology* 68: 916-22, 2007 Puschmann A, *Parkinsonism Relat Disord* 15: 390-2, 2009 Puschmann A, *Parkinsonism Relat Disord* 15: 390-2, 2009
SNCA	tripSNCA [S]	13 Caucasians	Waters CH, *Ann Neurol* 35: 59-64, 1994 Muenter MD, *Ann Neurol* 43: 768-81, 1998 Gwinn-Hardy K, *Acta Neuropathol* 99: 663-72, 2000 Singleton AB, *Science* 302: 841, 2003 Farrer M, *Ann Neurol* 55: 174-9, 2004
SNCA	dupSNCA [A]	8	Nishioka K, *Ann Neurol* 59: 298-309, 2006 Nishioka K, *Mov Disord* Epub: , 2009
SNCA	Ala53Thr	22 families	Polymeropoulos MH, *Science* 276: 2045-7, 1997 Papadimitriou A, *Neurology* 52: 651-4, 1999 Athanassiadou A, *Am J Hum Genet* 65: 555-8, 1999 Scott WK, *Neurogenetics* 2: 191-2, 1999 Spira PJ, *Ann Neurol* 49: 313-9, 2001 Michell AW, *J Neurol Neurosurg Psychiatry* 76: 596-7, 2005 Ki CS, *Clin Genet* 71: 471-3, 2007 Choi JM, *Neurogenetics* 9: 263-9, 2008 Puschmann A, *Parkinsonism Relat Disord* Epub: , 2009

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SNCA	Ala30Pro	3 Caucasians	Krüger R, *Nat Genet* 18: 106-8, 1998 Seidel K, *Ann Neurol* 67: 684-9, 2010
ATP13A2	Leu1059Arg and L1085WfsX1088	2 Caucasians, many cases in Asians	Park et al, *Human mutation* 2011.

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APPENDIX II: List of medications that have been approved to treat Parkinson's diseases as of May 2018.

Mechanism of action	Generic name	Trade name®	Common side effects
DOPA Decarboxylase inhibitor/DA precursor	Carbidopa/Levodopa	Sinemet	Nausea, dizziness, orthostatic hypotension, anxiety, dyskinesia, confusion, hallucinations, somnolence
	Carbidopa/Levodopa (controlled release)	Sinemet CR	
	Carbidopa/Levodopa (orally disintegrating)	Parcopa	
	Carbidopa/Levodopa (extended release capsules)	Rytary	
	Carbidopa/Levodopa (enteral suspension)	Duopa	
COMT inhibitor, inhibits breakdown of levodopa	Entacapone	Comtan	Same as carbidopa/ levodopa. In addition: diarrhea, discoloration of body fluids.
	Tolcapone	Tasmar	Tasmar can cause elevated liver function enzymes.
DOPA decarboxylase inhibitor/DA	Carbidopa/Levodopa Entacopone	Stalevo	Same as carbidopa/ levodopa and COMT inhibitor
DA agonist	Pramipexole	Mirapex	Nausea, dizziness, orthostatic hypotension, swelling of ankles, dyskinesia, hallucinations, confusion, somnolence, sleep attacks, impulse control disorders
	Pramipexole (extended release)	Mirapex ER	Neupro can lead to a skin reaction at the patch site
	Ropinirole	Requip	

	Ropinirole (extended release)	Requip XL	
	Apomorphine (injection)	Apokyn	
	Rotigotine (transdermal patch)	Neupro	

Mechanism of action	Generic name	Trade name®	Common side effects
MAO-B inhibitor, inhibits breakdown of dopamine	Selegiline	Eldepryl	Selegiline can insomnia
	Selegiline (orally disintegrating)	Zelapar	Dizziness, gastrointestinal dyskinesia, hallucinations, confusion, headache
	Rasagiline	Azilect	Note possible interactions
	Safinamide	Xadago	Safinamide effects through mechanisms of well
Mixed mechanisms, including NMDA antagonism	Amantadine	Symmetrel	Hallucinations, leg swelling, dizziness, mottled skin (livedo reticularis),

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	Amantadine (extended release)	Gocovri	confusion, dry mouth and eyes, constipation, dizziness, orthostatic hypotension,
Anticholinergic	Trihexyphenidyl Benztropine	Artane Cogentin	Dry mouth and eyes, constipation, urinary retention,

This table contained information from the American Parkinson Disease Association (www.apdaparkinson.org).

APPENDIX III: Description of tools used in variant characterization in this study.

Annotations Database	Prediction Algorithm/Conservation Score	Description	Method	Categorical Prediction	Author(s)
SIFT_pred SIFT_score	SIFT	Sort intolerated from tolerated	P (An amino acid at a position is tolerated | The most frequent amino acid being tolerated)	D: Deleterious (SIFT<=0.05); T: tolerated (SIFT>0.05)	Pauline Ng, Fred Hutchinson Cancer Research Center, Seattle, Washington
Polyphen2_HDIV_pred Polyphen2_HDIV_score	Polyphen v2	Polymorphism phenotyping v2	Probabilistic Classifier Training sets: HumDiv	D: Probably damaging: >=0.957, P: possibly damaging 0.453<=pp2_hdiv<=0.956 B: benign pp2_hdiv<=0.452	Ivan Adzhubei Havard Medical School
Polyphen2_HVAR_pred Polyphen2_HVAR_score	Polyphen v2	Polymorphism phenotyping v2	Machine learning Training sets: HumVar	D: Probably damaging >=0.957 P: possibly damaging 0.453<=pp2_hdiv<=0.956 B: benign: pp2_hdiv<=0.452	Shamil Sunyaev Havard Medical School
LRT_pred LRT_score	LRT	Likelihood Ratio Test	LRT of H0: each codon evolves neutrally vs H1: the codon evolves under negative selection	D: Deleterious N: Neutral U: Unknown Lower scores are more deleterious	Sung Chung & Justin Fay, Washington University

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Annotations Database	Prediction Algorithm/Conservation Score	Description	Method	Categorical Prediction	Author(s)
MutationTaster_pred MutationTaster_score	MutationTaster	Bayes Classifier	A: disease_causing_automatic D:disease_causing N:polymorphism [probably harmless]; P:polymorphism_automatic[known to be harmless higher values are more deleterious		Markus Schuelke, the Charité –Universitätsmedizin, Berlin
MutationAssessor_pred MutationAssessor_score	MutationAssessor	Entropy of multiple sequence alignments	H/M means functional and L/N means non-functional higher values are more deleterious	H: high; M: medium; L: low; N: neutral	Reva Boris Computation Biology Center Memorial Sloan Kettering Cancer Center, New York
FATHMM_pred FATHMM_score	FATHMM	Hidden Markov Model (HMM)	Functional analysis through HMM	D: Deleterious T: Tolerated <lower values are more deleterious	Shihab Hashem, University of Bristol, UK
PROVEAN_pred PROVEAN_score		Protein Variation Effect Analyzer	Clustering of homologus sequences	D: Deleterious N: Neutral Higher values are more deleterious	Choi Y J. Craig Venter Institute
VEST3_score	VEST V3	Variant effect scoring tool	Random forest classifier	Higher values are more deleterious	Rachel Karchin, John Hopkins University

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Annotations Database	Prediction Algorithm/Conservation Score	Description	Method	Categorical Prediction	Author(s)
CADD_raw CADD_phred	Combined annotation dependent depletion	Linear kernel Support Vector Machine	Higher values are more deleterious		Jay Shendure, Xiaohui Xie, University of California – Irvine
DANN_score	DANN	Deleterious Annotation of genetic variants using Neural Networks	Neural network	Higher values are more deleterious	Jay Shendure & Xiaohui Xie University of California – Irvine
fathmm-MKL_coding_pred	FATHMM-MKL	predicting the effects of both coding and non-coding variants using nucleotide-based HMMs	Classifier based on multiple kernel learning	D: Deleterious Score >= 0.5 T: Tolerated Score < 0.5	Shihab Hashem University of Bristol, UK
MetaSVM_pred MetaSVM_score	MetaSVM	Support vector machine		D: Deleterious; T: Tolerated; Higher scores are more deleterious	Coco Dong University of South Carolina Biostatistics Department, South Carolina
MetaLR_pred MetaLR_score	MetaLR	Logistic regression		D: Deleterious; T: Tolerated; Higher scores are more deleterious	Coco Dong University of South Carolina Biostatistics Department, South Carolina

Annotations Database	Prediction Algorithm/Conservation Score	Description	Method	Categorical Prediction	Author(s)
integrated_fitCons_score integrated_confidence_value	FitCons	Fitness consequences of functional annotation	Integrate functional assays like ChIP-Seq with conservation measure of transcription factor binding sites	Higher scores are more deleterious	Christodoulidou A Briza Cold Spring Harbor Laboratory
GERP++_RS GERP++_NR	Genome Evolutionary Rate Profiling ++	Maximum likelihood estimation procedure	Higher scores are more deleterious		Eugne Davydov Stanford University, CS Department
phyloP7way_vertebrate	PhyloP	Phylogenetic p-values	Phylogentic p-values calculated from a LRT, score-based test, GERP test uses data on 7 species	Higher scores are more deleterious	Adam Siepel, University of California Santa Cruz (UCSC), Santa Cruz, California
phyloP20way_mammalian	PhyloP	Phylogentic p-values	Phylogenetic (phylo-HMM). Uses data on 20 species	Higher scores are more deleterious	Adam Siepel, University of California Santa Cruz (UCSC), Santa Cruz, California
phastCons7way_vertebrate	phastCons	A phylogenetic hidden Markov model (phylo-HMM) Use 7 species	Higher scores are more deleterious		Adam Siepel, University of California Santa Cruz (UCSC), Santa Cruz, California
phastCons20way_mammalian	phastCons	Phylogenetic (phylo-HMM). Uses data on 20 species	Higher scores are more deleterious		Adam Siepel, University of California Santa Cruz (UCSC), Santa Cruz, California

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Annotations Database	Prediction Algorithm/Conservation Score	Description	Method	Categorical Prediction	Author(s)
SiPhy_29_way	SiPhy	Probablistic framework, HMM. Uses data on 29 species.	Higher scores are more deleterious		Manual Garber Broad Institute of MIT & Harvard, Boston

dbNSFP Information assessed on: https://brb.nci.nih.gov/seqtools/colexpanno.html#annotableANNOVAR.

APPENDIX IV: List of articles published articles identified and used in the review on PD studies in Nigeria.

Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
1	Owolabi	Gastrointestinal complications in newly diagnosed Parkinson's disease: A case-control study.	2014	35	227-231	Trop Gastroenterol	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	2.6E+07	
2	Ojagbemi	Relationship between cognitive dysfunction and behavioural symptoms in Nigerian patients with Parkinson's disease no dementia.	2013	3	293-300	J Parkinsons Dis	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	2.4E+07	
3	Ojagbemi	Neuropsychiatric symptoms in Nigerian patients with Parkinson's disease.	2013	128	9 to 16	Acta Neurol Scand	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	2.3E+07	
4	Femi	Clinical profile of parkinsonian disorders in tropics: Experience at Kano, northwestern Nigeria	2012	3	237-241	J Neurosci Rural Pract	Research article	Prevalence and Symptoms and signs	Yes	Yes	Text and Tables 1,2	3505306	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
5	Ojo	Plasma homocysteine level and its relationship to clinical profile in Parkinson's disease patients at the Lagos University Teaching Hospital.	2011	30	319-324	West Afr J Med	Research article	Biochemical/pathology	Yes	Yes	Text and Table 2	2.3E+07	
6	Okubadejo	Clinical profile of parkinsonism and Parkinson's disease in Lagos, Southwestern Nigeria.	2010	10	1	BMC Neurol	Research article	Prevalence	Yes	Yes	Text and Table 1	2806862	
7	Okubadejo	Analysis of Nigerians with apparently sporadic Parkinson disease for mutations in LRRK2, PRKN and ATXN3.	2008	3	e3421	PLoS One	Research article	Genetics	Yes	Yes	Text and Table 3	2559870	
8	Akinyemi	Cognitive dysfunction in Nigerians with Parkinson's disease.	2008	23	1378-1383	Mov Disord	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	1.9E+07	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
9	Okubadejo	Parkinson's disease in Africa: A systematic review of epidemiologic and genetic studies.	2006	21	2150-2156	Mov Disord	Review	Review	Yes	Yes	Text	1.7E+07	
10	Okubadejo	Longitudinal study of mortality predictors in Parkinson's disease in Nigerians.	2005	34	365-369	Afr J Med Med Sci	Research article	Symptoms and signs	Yes	Yes	Text	1.7E+07	
11	Muthane	Melanized nigral neuronal numbers in Nigerian and British individuals.	2006	21	1239-1241	Mov Disord	Research article	Biochemical/pathology findings	Yes	Yes	Text	1.7E+07	
12	Okubadejo	Frequency and predictors of autonomic dysfunction in Parkinson's disease: a study of African patients in Lagos, Nigeria.	2004	11	45-49	Niger Postgrad Med J.	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	1.5E+07	
13	Igbokwe	Xenobiotic metabolism in	1993	70	807-809	East Afr Med J	Research article	Environmental risk factors	Yes	Yes	Text	8026358	

Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
		idiopathic Parkinson's disease in Nigerian Africans.											
14	Schoenberg	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.	1988	38	645-646	Neurology	Research article	Prevalence	Yes	Yes	Text and Table 1	3352927	
15	Schoenberg	Environmental risk factors for Parkinson's disease: the epidemiologic evidence.	1987	14	407-413	Can J Neurol Sci	Review	Review	Yes	Yes	Text	3315146	
16	Osuntokun	Parkinsonism in the Nigerian African: a prospective study of 217 patients.	1979	56	597-607	East Afr Med J	Research article	Symptoms and signs	Yes	Yes	Text and Table 1	520239	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
17	Osuntokun	The pattern of neurological illness in tropical Africa. Experience at Ibadan, Nigeria.	1971	12	417-442	J Neurol Sci	Research article	Prevalence	Yes	Yes	Text and Table 1	4324654	
18	Schoenberg	Descriptive epidemiology of Parkinson's disease: disease distribution and hypothesis formulation	1987	45	277-83	Adv Neurol	Review	Review	Yes	No	No	3493626	No data on Nigerian PD.
19	Osuntokun	Neurological disorder in Nigerian Africans: a community-based study	1987	75	13-21	Acta Neurol Scand	Research article	Prevalence	Yes	Yes	Text and Table 1	3033973	
20	Owolabi	Progressive supranuclear palsy misdiagnosed as Parkinson's disease: A case report and review of literature	2016	3	S44-S47	Annals of Medical and Health Sciences Research	Research article	Other disease with similar symptoms	Yes	Yes	Text	3853608	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
21	Okubadejo	An analysis of genetic studies of Parkinson's disease in Africa	2008	14	177-182	Parkinsonism and Related Disorder	Review	Review	Yes	Yes	Text	1.8E+07	
22	Akinyemi	Epidemiology of parkinsonism and Parkinson's disease in Sub-Saharan Africa: Nigerian profile	2012	3	233-234	Journal of Neurosciences in Rural Practice	Review	Review	Yes	Yes	Text	2.3E+07	
23	Owolabi	Pattern of neurological admissions in tropics: Experience at Kano, Northwestern Nigeria	2010	13	167-170	Annals of Indian Academy of Neurology	Research article	Prevalence	Yes	Yes	Text and Table 1	2.1E+07	
24	Ojo	Frequency of cognitive impairment and depression in Parkinson's disease: A preliminary case-control study	2012	53	65-70	Nigerian Medical Journal	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	3530250	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
25	Owolabi	Pulmonary function tests in patients with Parkinson's disease: A case-control study	2016	19	66-70	Nigerian Journal of Clinical Practice	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	2.7E+07	
26	Ekenze	Profile of neurological admissions at the University of Nigeria Teaching Hospital Enugu	2010	19	419-422	Nigerian J Med	Research article	Prevalence	Yes	Yes	Text and Table 1	2.2E+07	
27	Ogunrin	Trace metals in patients with Parkinson's disease: a multi-center case-control study of nigerian patients	2013	1	31-38	J Neurol Epi	Research article	Environmental risk factors	Yes	Yes	Text	N/A	
28	Olaitan	Depression among patients with Parkinson's disease in Nigerian tertiary hospital	2013	13	96-103	Nigerian Health J	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	N/A	

Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
29	Olaitan	Non-motor features in Parkinson's disease patients attending neurology clinic at a tertiaryinstitution in Nigeria: A preliminary report	2014	14	114-118	Nigerian Health J	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	N/A	
30	Temitope	Falls and their associated risks in Parkinson's disease patients in Nigeria	2016	9	160-165	J Mov Disord	Research article	Symptoms and signs	Yes	Yes	Text and Table 2	5035939	
31	Ben-Shlomo	Incidental Lewy body disease.	1994	344	1503	Lancet	Letter to editor	No study	Yes	No	No	N/A	No data.
32	Jendroska	Incidental Lewy body disease in Black Africans.	1994	344	882-883	Lancet	Research article	Biochemical/pathology	Yes	Yes	Text	N/A	
33	Odeku	Cranial meningiomas in the Nigerian African.	1973	4	275-287	Afr J Med Sci	Reserch article	Other disease with similar symptoms	Yes	Yes	Text	4749181	
34	Osuntokun	Neuropsychiatric manifestations of typhoid fever in 959 patients.	1972	27	7 to 13	Arch Neurol	Research article	Other disease with similar symptoms	Yes	Yes	Text	4340378	

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Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
35	Dada	The Nigerian neurological profile.	1970	31	746-755	Dis Nerv Syst	Research article	Prevalence	Yes	Yes	Text and Table 1	4992399	
36	Osuntokun	Non-embolic ischaemic cerebrovascular disease in Nigerians.	1969	9	361-388	J Neurol Sci	Reserch article	Other disease with similar symptoms	Yes	Yes	Text	5345116	
37	Oguniyi	Treatment of parkinsonian syndromes in developing countries.	1997	26	1 to 2	Afr J Med Med Sc	Review	Review	Yes	Yes	Text	1.1E+07	
38	Adebayo	Neuropsychiatric and Parkinsonian manifestations of dementia: A case report in a Nigerian woman	2013	12	46-48	Annal of African medicine	Research article	Other disease with similar symptoms	Yes	Yes	Text	2.3E+07	
39	Solomon	Parkinsonism caused by adverse drug reactions: a case series	2011	5	105	Journal of medical case reports	Research article	Other disease with similar symptoms	Yes	Yes	Text	N/A	

Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
40	Talabi	A 3-year review of neurologic admissions in University College Hospital Ibadan, Nigeria.	2003	22	150-1	West Afr J Med	Research article	Prevalence	Yes	Yes	Text and Table 1	1.5E+07	
41	Chapp-Jumbo	Neurologic admissions in the Niger Delta area of Nigeria - a ten year review	2004	23	14-20	African Journal of Neurol sci	Research article	Prevalence	Yes	Yes	Text and Table 1	N/A	
42	Tucci	Study of the genetic variability in a Parkinson's Disease gene: EIF4G*1*	2012	518	19-22	Neuroscience letters	Research article	Genetics	Yes	Yes	Text and Table 3	3769807	
43	Salawu	Current management of Parkinson's disease	2010	9	55-61	Annal of African medicine	Review	Review	Yes	Yes	Text	2.1E+07	
44	Lekoubou	Epidemiology of neurodegeneration diseases in sub-Saharan Africa: a systematic review	2014	14	Not in print	BMC Public health	Review	Review	Yes	Yes	Text	4094534	

Count	First Author	Title	Year	Vol	Pages	Journal	Article type	Area of study on PD	Full text obtained (Yes/No)	Used for Review (Yes/No)	Used in	PMID	Reason for exclusion
45	Okubadejo	Prevalence of essential tremor in urban Lagos, Nigeria: a door-to-door community-based study	2012	12	Not in print	BMC Neurol	Research article	Other disease with similar symptoms	Yes	Yes	Text	2.3E+07	

APPENDIX V: Information on the Ampliseq 751 genes neurological diseases panel

The link to this table is provided below:
https://www.ampliseq.com/tmpl/view.action?tmplDesignId=62969292#/?listAction=tmplCoverageSummaryList&tmplDesignId=62969292&wrapperId=ajaxTableWrapper

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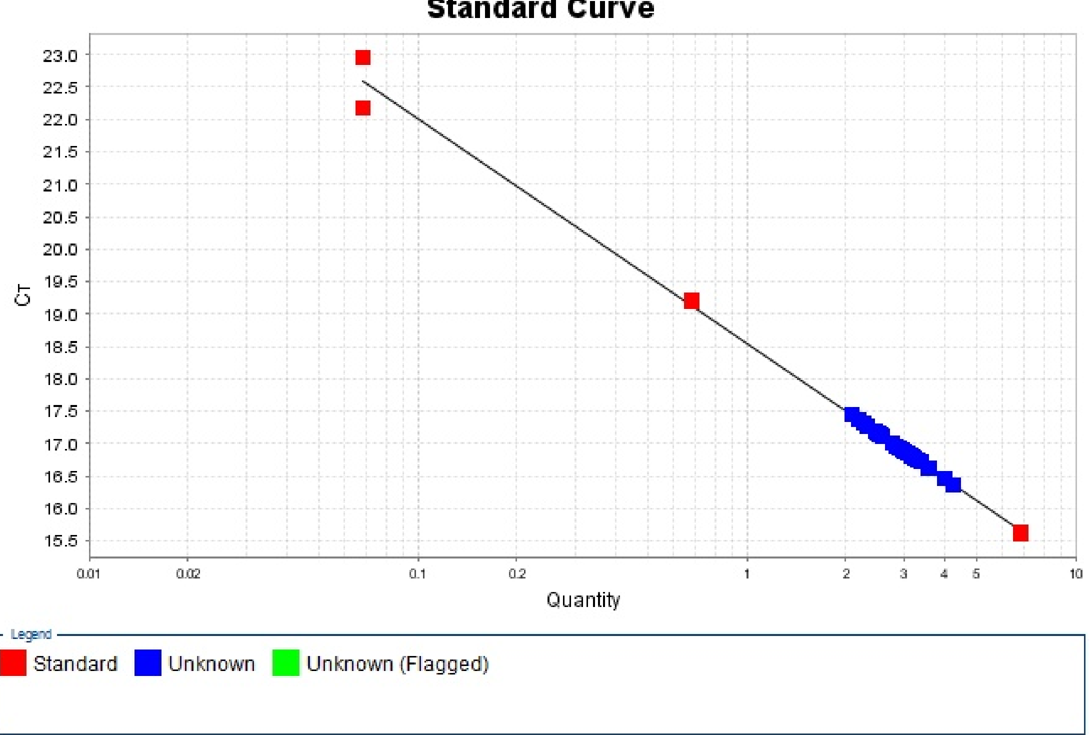

A.

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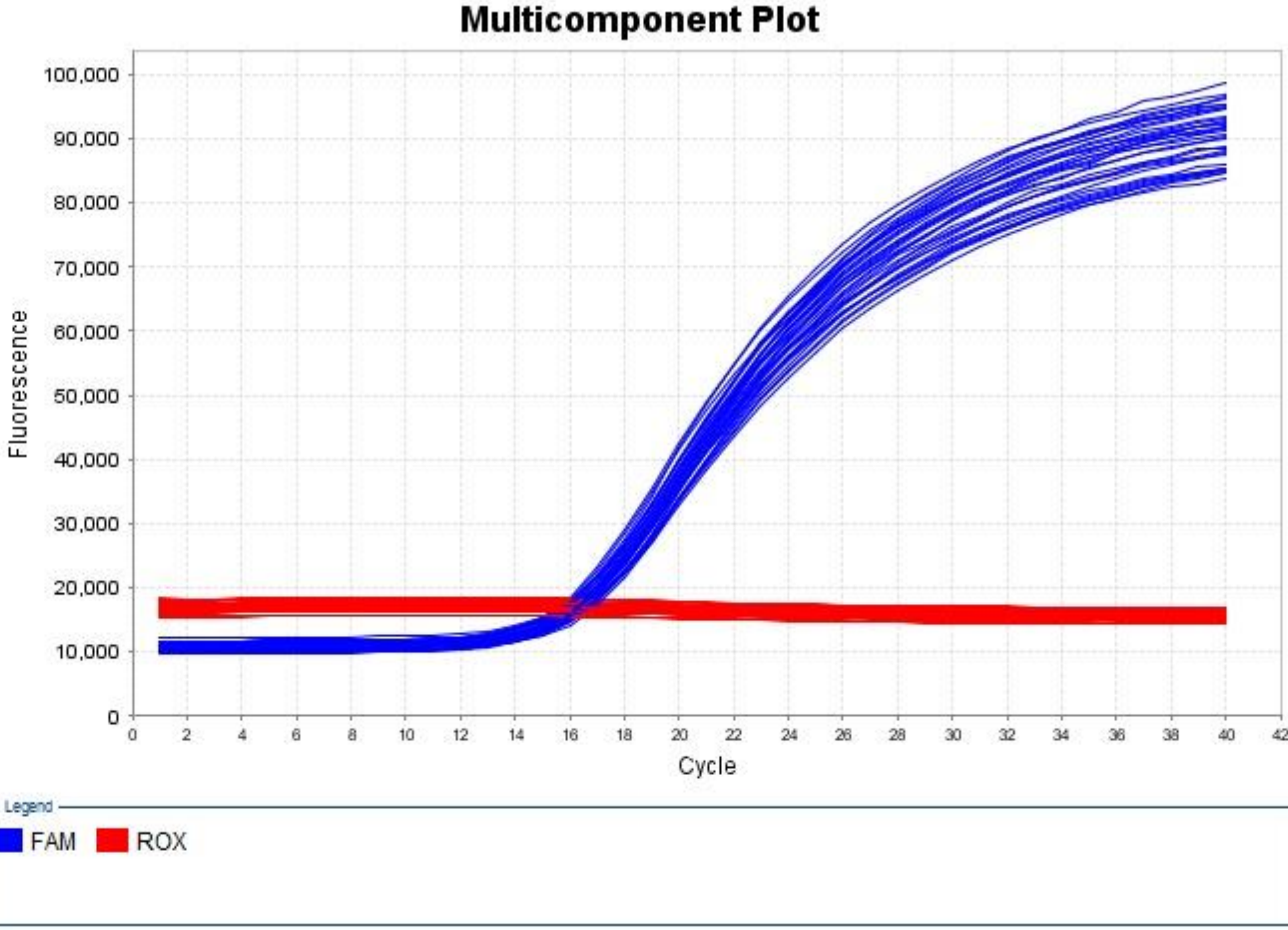

B.

APPENDIX VI: Plotted graph representing library quantification during amplification using RT-PCR. Standard curve (A) shows the standardized graph normalized with internal controls; and (B) multicomponent plot showing library enrichments during amplification.