CELEBRATING THE DIVERSITY OF GENETIC RESEARCH TO DISSECT THE PATHOGENESIS OF PARKINSON'S DISEASE

EDITED BY: Soraya Bardien, George Damion Mellick, Nobutaka Hattori, Owen A. Ross and Suzanne Lesage

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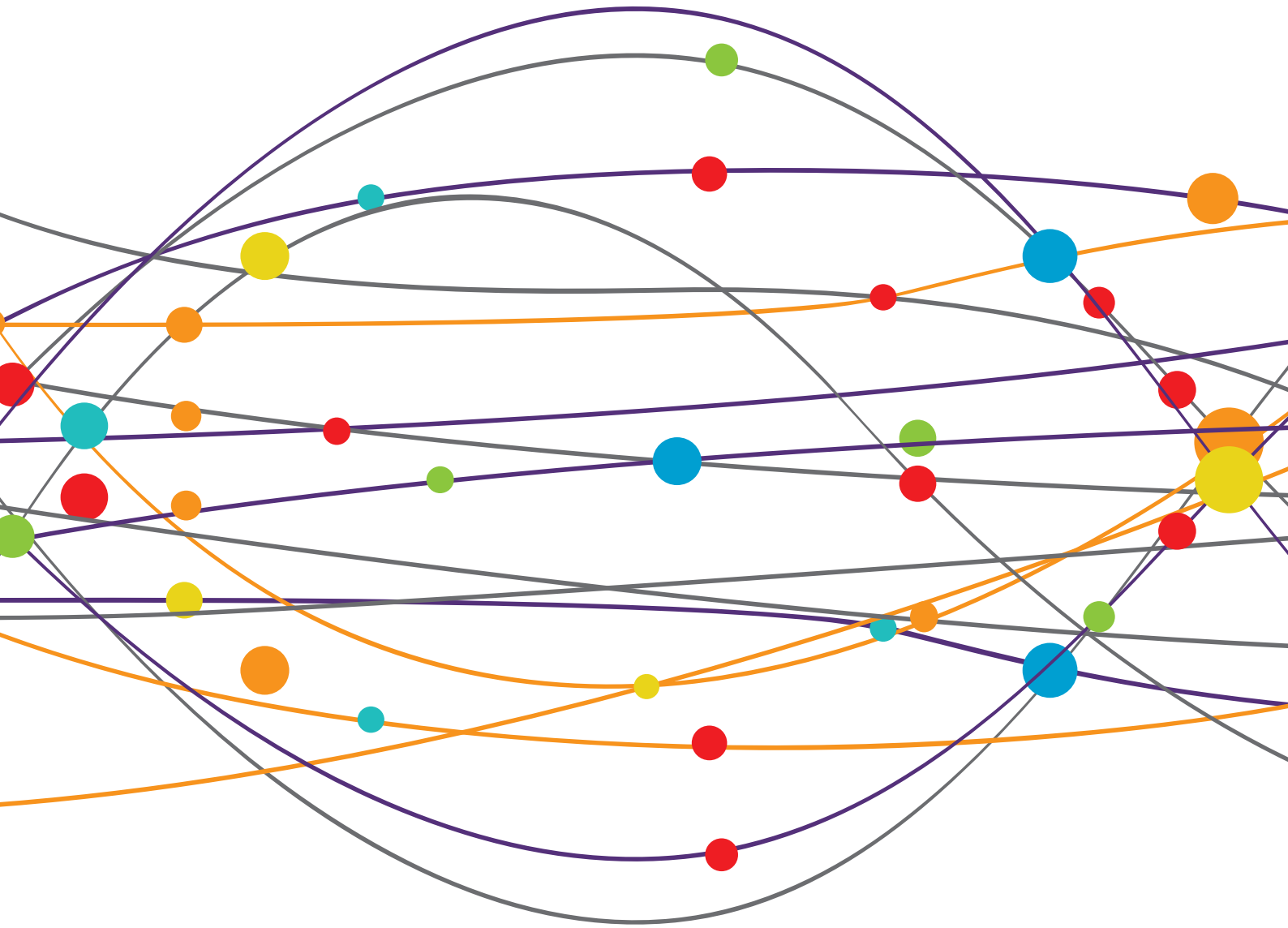

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CELEBRATING THE DIVERSITY OF GENETIC RESEARCH TO DISSECT THE PATHOGENESIS OF PARKINSON'S DISEASE

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EDITORIAL
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Editorial: Celebrating the Diversity of Genetic Research to Dissect the Pathogenesis of Parkinson's Disease

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Editorial on the Research Topic

Celebrating the Diversity of Genetic Research to Dissect the Pathogenesis of Parkinson's Disease

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INTRODUCTION

Parkinson's disease (PD) is the fastest growing neurological disorder worldwide, taking into account age-standardized rates for prevalence, disability and deaths (1). PD is characterized by a clinical symptomatology involving both motor and non-motor symptoms. According to the Global Burden of Disease study (2018), the global burden of this disorder has more than doubled over the past two decades from 2.5 million patients in 1990 to 6.1 million patients in 2016 (2).

In this editorial and eBook, we highlight the research done on PD by members of a global consortium known as the Genetic Epidemiology of Parkinson's disease (GEoPD) Consortium. We begin the editorial by providing a brief history of how GEoPD was started and how it has subsequently developed into an international endeavor. We then briefly summarize the completed and ongoing projects, and conclude with the future vision of this unique consortium.

FROM FRIENDS ON A ROAD TRIP TO AN INTERNATIONAL ROADMAP FOR SOLVING THE PUZZLES OF PD

GEoPD is a group of researchers dedicated to promoting education, scientific research, and translational development in PD. It is the longest running worldwide Consortium on PD, operating since 2004, and initially funded by a Michael J. Fox Foundation award to form "global genetic consortia." GEoPD enables unfettered access to a "family" of multi-disciplinary expertise, including specialty neurologists, geneticists, biologists, epidemiologists or statisticians. From its inception, GEoPD has always maintained its tradition of diversity and inclusion with an active and growing membership from more than 60 sites and 30 countries on six continents.

The democratization of data, resources, projects and funding are long-established principles. To ensure open, honest collaboration and transparency without politics and control, GEoPD has always maintained an elected leadership. The GEoPD President is elected from and by a

Steering Committee, for which each member serves a 2-year term that is renewable. Election to the Steering Committee is based on past contributions to the Consortium including: (1) directing a collaborative project, and/or; (2) directing a Core service (as specified below), and/or; (3) hosting the annual meeting.

Participation and projects are funded by and collectively for the members, generally through grants and sponsorships from different funding agencies and national societies. Projects include whole exome and genome sequencing, custom array genotyping, and studies that range from longitudinal assessment of idiopathic PD to monogenetic parkinsonism in families, to analyses of genetic and environmental variables using a Mendelian randomization approach.

The first GEoPD meeting was organized by Demetrius ("Jim") Maraganore in 2004 in Greece and was attended by six investigators that included Alexis Elbaz, Matt Farrer, and Rejko Kruger, who remain active members of the Consortium as Core leaders, and as past and current GEoPD Presidents. The six of them drove in a minibus from Athens, in the southeast, to Ioannina in the northwest, *via* Patras (**Figure 1**). Their tour guides on this fateful journey were Jim Maraganore and John Ioannidis. In part, the mission was to visit the ancestral origin of the alpha-synuclein p.A53T mutation, the first genetic mutation to be associated with familial late-onset parkinsonism. Focused on PD and genetic epidemiology, it was a remarkable journey of scientific discovery. They jointly elected to share that journey with likeminded colleagues around the world and Rejko Kruger coined the name "GEoPD" on the minibus. Ever since, one member of the Consortium has elected to host the meeting in a different part of the globe.

The aptly described GEoPD "World Tour" is CME-accredited and provides a forum where colleagues catch up, in person and/or virtually, and discuss their work and the latest developments in PD research. It is an educational opportunity that openly shares unpublished data and insights, that engagingly debates controversy in the field, and in a convivial setting. A central part of the meeting is reserved for a "data blitz" session that gives members ∼10 min to highlight unpublished data and seek the help and collaboration of the entire membership. As a first exemplary project, GEoPD assessed the role of alpha-synuclein beyond autosomal dominantly inherited PD and established common regulatory polymorphisms in the *SNCA* gene as a risk factor for sporadic PD worldwide (3). This first global confirmation of *SNCA* as a risk factor in sporadic PD was subsequently confirmed by an unbiased approach in the era of genome-wide association studies (GWAS) (4).

DEVELOPING A TRULY INTERNATIONAL COLLABORATION

GEoPD is currently organized into five cores, each having a specific mandate about one of the following areas: bioinformatics, biology, clinical, communications, epidemiology, and statistics. The consortium's main mission is to promote multi-investigator research projects. Annual Meetings, held since 2005, offer a valuable forum for consortium members to discuss unpublished data and ideas, highlight research questions or needs, and identify global opportunities for partnership. These in-person meetings are organized and hosted each year in a different country by one of the members; the first "official" meeting was organized in Paris, France in 2005, and subsequent meetings took place in Santorini (Greece, 2006), Trondheim (Norway, 2008), Tübingen (Germany, 2009), Toronto (Canada, 2010), Evanston (USA, 2011), Seoul (South Korea, 2012), Lübeck (Germany, 2013), Vancouver (Canada, 2014), Tokyo (Japan, 2015), Luxembourg (Luxembourg, 2016), Cairns (Australia, 2017), Paris (France, 2018), Cape Town (South Africa, 2019), and in 2020 the first virtual meeting (due to the Coronavirus-2019 pandemic) hosted by the group in Milan, Italy.

Using the large multi-ethnic clinical and genetic datasets (currently including over 40,000 PD patients and 40,000 controls, mainly of European and Asian origins), multiple advanced analyses are performed to assess emerging mutations or variants associated with PD, and several studies have been published. We have examined the role of ∼120 *LRRK2* coding variants in ∼15,000 individuals, to implicate frequent substitutions in idiopathic PD and neuroprotection (5). Additionally, interactions between *LRRK2* and *PARK16 (RAB7L1; RAB29)* variants were not replicated in GEoPD efforts (6). Similarly, we have not been able to provide any evidence of an interaction of LRRK2 p.R1398H, which has a protective effect, with *MAPT* or *SNCA* variants (7). We have also questioned the role of intermediate size repeat expansions in *SCA2*, *SCA3*, *SCA6*, and *SCA17* (8), or *C9orf72* expansions (9), as risk factors for idiopathic PD and our findings excluded a major role of any of these intermediate/expanded repeats in PD pathogenesis. Overall, the GEoPD consortium has contributed more than 20 original, globally collaborative articles to advance our understanding of the genetic architecture of PD [(3, 5–23), Markopoulou et al.; Rajan et al.]. Recently, a unique global initiative from our consortium aims to identify all patients and relatives with *SNCA* multiplications to inform alpha-synuclein targeted therapeutic development (22). Longitudinal clinical assessments, genealogic information, genotyping data, and *SNCA* locus breakpoints from 59 families with *SNCA* multiplications are publicly available via a website that has been created as a forum for data exchange.

ONGOING PROJECTS

Details about the ongoing collaborative projects of the consortium can be found at the GEoPD website (https://www.geopd.net/projects). These include: **Monogenic PD** (a project to collect clinical and genetic information on mutation-positive monogenic PD individuals to inform genotype-phenotype correlations (21); **LONG-PD** a prospective study to assess disease progression, treatment response and outcomes in a longitudinal manner over >10 years in different ethnic cohorts of PD patients; **Courage PD** (COmprehensive Unbiased Risk factor Assessment for Genetics and Environment in Parkinson's Disease); **RVCD** (a study identifying rare sequence variants segregating in Mendelian forms of PD); and the **Trios** project, which aims to study PD-affected individuals and both of their biological parents using whole exome sequencing.

FIGURE 1 | Founding members of GEoPD in Greece in 2004. Permission for publishing this figure has been obtained from the five individuals shown.

THE FUTURE VISION OF GEoPD

Over the past two decades, GEoPD has significantly contributed to the genetic dissection of PD, and established genetics as an entry point to decipher molecular mechanisms underlying neurodegeneration in this increasingly common age-related disorder. The mission of GEoPD is to inspire and unite researchers, at a global scale, to advance multidisciplinary research on genetic and environmental causes of PD, and to share insights and resources to enable translational neuroscience and clinical applications. The democratically elected structure of GEoPD allows direct and equal participation of all member sites worldwide; intellectual scientific contributions to the GEoPD enterprise, publicly voiced, discussed and ratified by the membership, are then supported by the efforts of the collective. Hence, the science is neither convened nor limited by the desires and constraints of funding agencies, nor political influence. This concept provides assurance that each site can jointly own and participate in federated data and sample infrastructure. Furthermore, this vision continues to draw new members from countries whose populations remain underrepresented in worldwide research, to promote their research in a global effort.

The advance of large-scale genotyping technologies and high-throughput sequencing has not yet directly translated into corresponding advances in elucidating the missing heritability of PD. New strategies are required to disentangle the complex genetic architecture of PD, to establish the molecular pathogenesis of this disorder, and to inform therapeutic development. To date, most genetic discoveries have been made in Caucasians of European ancestry, but these populations do not include the genetic diversity of different ethnicities worldwide. Identifying new genes, consolidating candidate genes and defining the impact of genetic variability in diverse populations has been a priority for GEoPD since its inception. This is underscored by the outreach to underrepresented populations at the first African GEoPD meeting in 2019 which served as the basis for this Frontiers eBook.

To fully capitalize on opportunities for genotype-phenotype correlations, GEoPD has addressed the emerging need for deep clinical phenotyping in PD and control cohorts (i.e., to improve stratification of patient heterogeneity, inform prognosis and enhance clinical trials). This is reflected by our efforts to harmonize clinical data-capture across GEoPD sites worldwide [e.g., in the LONG-PD study (led by Katerina Markopoulou) or Minimal Dataset initiatives available to all sites *via* the Elixir node for translational medicine data in Luxembourg].

Our understanding of genotype-phenotype correlations is currently limited due to a lack of systematic assessment of the functional role of novel gene regulatory variants and splicing defects (e.g., *via* differential regulation of gene expression). The increasing number of novel PD genes and risk variants being identified further underscores the need for functional validation of novel mutations using models to define disease-relevant molecular pathways. "Mechanism-based" stratification of molecular heterogeneity will inform genetically-stratified patient participation in clinical trials, and the first targeted

treatment options (i.e., precision medicine). In this context, patient-based induced pluripotent stem cells (iPSC) including isogenic controls, provide an essential tool to study different disease-related variants in defined genetic backgrounds.

While rare mutations in monogenic forms of PD pave the way for precision medicine, the biological pathways impacted can reveal generic mechanisms that apply to larger groups of idiopathic patients (24). Many more genes have been revealed by genome-wide association meta-analyses that assess frequent, polymorphic variants of minor effect, although that polygenic risk is not predictive (25). However, recent innovations in whole genome sequencing, distributed cloud computing and artificial intelligence promise far greater breakthroughs in medical research discovery. We now have the opportunity to define the joint contribution of all genetic variants, including those of intermediate frequency and modest effect, provided that sample sizes are sufficiently large. The collaborative spirit of the GEoPD consortium strongly supports these larger scale international initiatives. Hence, we have embraced the Global Parkinson's Genetics Program (GP2) effort, led by Andrew Singleton, supported by the US National Institutes of Health and the recent Aligning Science Across Parkinson's (ASAP) initiative, that provides the opportunity to synergize and maximize the precious research contribution of people worldwide, with and without PD.

The 21 articles appearing in our first eBook in "Frontiers in Neurology: Neurogenetics Research Topic" highlights the breadth and depth of our scientific inquiry, and come from researchers working in Africa, Asia, Australia, Europe and North America. This reflects our expanding international collaborative effort, including the elected host site for the next GEoPD Annual Meeting in Omsk in southwestern Siberia in October 2021.

We welcome new members to join us by visiting our website at https://www.geopd.net/component/users/?view=registration.

AUTHOR CONTRIBUTIONS

GDM, SB, and OAR conceptualized the Editorial. All authors participated in drafting, writing, and reviewing the text.

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Evidence of a Recessively Inherited CCN3 Mutation as a Rare Cause of Early-Onset Parkinsonism

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The study of consanguineous families has provided novel insights into genetic causes of monogenic parkinsonism. Here, we present a family from the rural Khyber Pakhtunkhwa province, Pakistan, where three siblings were diagnosed with early-onset parkinsonism. Homozygosity mapping of two affected siblings and three unaffected family members identified two candidate autozygous loci segregating with disease, 8q24.12-8q24.13 and 9q31.2-q33.1. Whole-exome sequence analysis identified a single rare homozygous missense sequence variant within this region, *CCN3* p.D82G. Although unaffected family members were heterozygous for this putative causal mutation, it was absent in 3,222 non-Parkinson's disease (PD) subjects of Pakistani heritage. Screening of 353 Australian PD cases, including 104 early-onset cases and 57 probands from multi-incident families, also did not identify additional carriers. Overexpression of wild-type and the variant CCN3 constructs in HEK293T cells identified an impaired section of the variant protein, alluding to potential mechanisms for disease. Further, qPCR analysis complemented previous microarray data suggesting mRNA expression of *CCN3* was downregulated in unrelated sporadic PD cases when compared to unaffected subjects. These data indicate a role for CCN3 in parkinsonism, both in this family as well as sporadic PD cases; however, the specific mechanisms require further investigation. Additionally, further screening of the rural community where the family resided is warranted to assess the local frequency of the variant. Overall, this study highlights the value of investigating underrepresented and isolated affected families for novel putative parkinsonism genes.

Keywords: parkinson, genetic, CCN3, NOV, early-onset, extracellular matrix

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INTRODUCTION

In most cases, the cause of Parkinson's disease (PD) is unknown, and although the risk may be influenced by a number of common genetic (1) and environmental factors (2), only a minority of cases can be ascribed to known genetic causes. These rare monogenic causes of parkinsonism often arise in and are detected through the study of consanguineous families from remote regions. Notably, mutations in *DJ-1* (3), *PINK1* (4, 5), *DNAJC6* (6), *SYNJ1* (7), *PLA2G6* (8), and the putative genes *PODXL* (9) and *ADORA1* (10) were identified in consanguineous parkinsonism families. While most PD cases do not possess these rare genetic variants, their discovery provides

insight into the cellular mechanisms involved in the development of the disease, and ongoing screening of affected families is a powerful platform to identify further candidates. To this end, a consanguineous family from a rural district in the Khyber Pakhtunkhwa province, Pakistan, who presented with early-onset parkinsonism but had screened negative for known causes of recessive parkinsonism, underwent further genetic analysis. This study reports the finding of a rare, putatively pathogenic, p.D82G mutation in *CCN3* (also known as *NOV*) in this family. The gene encodes a secreted matricellular protein, which may have a role in adhesion, cellular signaling, cell migration, angiogenesis, and calcium homeostasis (11).

MATERIALS AND METHODS

Genetic Analysis

Due to the remote location of the family, only the proband (IV:3) was available for diagnosis by an expert movement disorder specialist. Details of the family were acquired through interviews with the proband. DNA was extracted from venous blood donated by patients from the Khyber Pakhtunkhwa province, Pakistan (Ethics: Ref. 65/IRBEB/PGMI/LRH), using the phenol chloroform method, and from Queensland, Australia (Ethics: Ref. ESK/04/11/HREC), using a salting-out method described previously (12).

Approximately 300,000 single-nucleotide polymorphisms (SNPs) were genotyped using the HumanCytoSNP-12 BeadChip and the iScan system (Illumina) in the two patients IV:3 and IV:5, as well as three unaffected members, III:2, III:5, and IV:4. All samples had SNP call rates >95%. Homozygosity mapping was performed using GenomeStudio (Illumina) and Homozygosity Mapper (13). Copy number variation (CNV) detection was performed using the cnvPartition plugin within GenomeStudio (Illumina).

Whole-exome sequencing (WES) was performed in patient IV:3 using the Nextera Rapid Capture Exome Enrichment chemistry and sequenced under the 2× 75-bp pair-end configuration on the MiSeq sequencer (Illumina) at the Griffith University DNA sequencing facility. The sample produced an average read depth of 30× over the 45-Mb target region, with ∼93.5% of calls above Q30. The data were prepared as recommended by the Genome Analysis Toolkit (GATK) developers (14). Sequence variants differing to the human consensus sequence hg19/GRCh37 were identified by the HaplotypeCaller algorithm (GATK) and annotated by ANNOVAR (15). Variants were filtered by the following parameters: (1) missense variants; (2) gnomAD minor allele frequency (MAF) <0.001; (3) homozygous.

The mutation was confirmed by Sanger sequencing, which was performed by amplifying the region surrounding the mutation using the following primers: 5′-GGTTTCTCCTTGTCTCGCCT-3′ (forward) and 5′-GCTGCAGGAGAAGAGGTCAA-3′ (reverse). Amplification products then underwent BigDye Terminating v3.1 reaction which were analyzed on the genetic analyzer 3130 × l (Applied Biosystems) at the Griffith University DNA sequencing facility.

High-Resolution Melt Analysis

High-resolution melt (HRM) analysis was used in 320 Australian PD samples to assess genetic variants in the 135-bp region surrounding the putative mutation, encompassing 57% of exon 2. HRM primers were 5'-GCTCATGCTGTCTGGTGTGT-3′ (forward) and 5′-GATTACCGTGCAGATGCCA-3′ (reverse). Briefly, products were amplified using the GoTaq kit (Promega) with 1.5 mM $MgCl_2$, 200 μM dNTPs (Bioline), 200 nM primers (Sigma-Aldrich), and 1 μM dsDNA binding Syto9 dye, with the following PCR profile on a RotorGene 6000 (QIAGEN Inc.): 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Fluorescence was acquired at the 72°C step, and the product underwent high-resolution melt between 83° and 93°C.

Quantitative PCR

Quantitative PCR (qPCR) was used to verify observations from microarray data of human olfactory neurosphere-derived (hONS) cells (16), available at www.ebi.ac.uk/arrayexpress (Accession: E-TABM-724). Total RNA was extracted from hONS cells donated by nine unrelated idiopathic Queensland PD cases and eight unaffected controls using TRIzol (Thermo Fisher Scientific) at 60–80% confluence. RNA (200 ng) was converted to cDNA using the SuperscriptIII First-Strand Synthesis SuperMix kit (Thermo Fisher Scientific). qPCR analysis amplified *CCN3* as well as endogenous controls *RPL13* and *TBP*, using the following primers: *CCN3* forward 5′-CGGCGGTAGAGGGAGATAAC-3′, *CCN3* reverse 5′-GCCTGTAAGCTGCAAGGGTA-3′, *RPL13* forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, *RPL13* reverse 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′, *TBP* forward 5′-CCACTCACAGACTCTCACAAC-3′, and *TBP* reverse 5′-CTGCGGTACAATCCCAGAACT-3′. Products were amplified using the PowerUp SYBR green kit (Applied Biosystems), the thermal cycling conditions were UDG activation 50°C for 2 min, polymerase activation 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min. A relative standard curve was used to determine the expression of *CCN3* to the geometric mean of *RPL13* and *TBP*. Statistical analysis was conducted in R (v3.5.1). Briefly, two outliers (controls) were removed for high values [relative expression (log2): 2.3 and 1.7]. Normality and Levene's test of equal variance were assessed using the ggplot2 (v3.0) and car (v3.0-5) packages. A Student's *t*-test was conducted under the assumptions of normal distribution and equal variance using the stats (v3.5.1) package. The Bonferroni correction was used to control for multiple comparisons.

V5-Tagged Expression Construct Design

Briefly, cDNA was prepared from a control hONS cell line and the *CCN3* coding sequence, lacking the stop codon, was amplified using the Pfusion HF polymerase (New England Biolabs). Next, the *CCN3* amplicon was inserted into pDONR201 and then into the pEF-DEST51 expression vector using the Gateway BP and LR Clonase II Enzyme mix, respectively (Thermo Fisher Scientific). The c.A245G (NM_002514) point mutation was introduced by the QuikChange Lightning Multi Site-Directed Mutagenesis

Kit (Agilent Technologies) using the following primers: 5′-CTGGAGCCATGCGGCGAGAGCAGTGGC-3′ (forward) and 5′-GCCACTGCTCTCGCCGCATGGCTCCAG-3′ (reverse). Correct sequence identity was verified by Sanger sequencing.

Cell Culture and Transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. For immunoblot experiments, 75,000 cells were plated into a 24-well plate overnight (Nunc™) and transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific). For immunostaining experiments, cells were plated at a density of 37,500 cells overnight on poly-ornithine (Sigma-Aldrich)-coated glass coverslips. The cells were either fixed or lysed 48 h post-transfection, and supernatants were also collected by centrifugation for 10 min at 300 × g to remove cell debris and analyzed. Lysis was performed using 10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% Triton-X100, incubated for 15 min on ice followed by centrifugation for 5 min at 4°C. Fixation was achieved by incubating coverslips in 4% paraformaldehyde (PFA) for 10 min.

Immunoblotting

Immunoblots were performed using Tris-Glycine gels and standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) protocols. Nitrocellulose membranes were probed with rabbit-anti-V5 (1:3,000; Cell Signaling Technology) and mouse-anti-α-tubulin (1:18,000; Sigma-Aldrich) antibodies overnight at 4°C, followed by goat-anti-mouse-680RD and goat-anti-rabbit-800CW secondary antibodies (both 1:24,000 LI-COR) for 60 min at room temperature. Membranes were imaged on an Odyssey-Fc imaging system (LI-COR).

Immunostaining

Coverslips were permeabilized and blocked in PBS containing 10% horse serum and 0.3% Triton-X100 for 60 min. CCN3 was detected using rabbit-anti-V5 antibody (1:1,000) for 90 min at room temperature and detected using donkey-anti-rabbit-555 secondary antibody (1:1,000, Thermo Fisher Scientific). Coverslips were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and imaged on an Olympus FV1000 confocal microscope.

RESULTS

Patient IV:3 was diagnosed with levodopa-responsive parkinsonism at the age of 31 after developing rigidity and gait abnormalities. The symptomatology progressed, and at the age of 36 presented with severe rigidity, frequent falling events, mild tremor, hypomimia, difficulty swallowing, and a stooped posture. Magnetic resonance imaging (MRI) scans were unremarkable (data not shown). The proband had reported patient's IV:5 and IV:6 also presented with similar symptomatologies, while the parents were not affected (**Figure 1A**). CNV analysis and WES in IV:3 excluded known causes of genetic parkinsonism, including *PARK2* and *SNCA* dosage. Homozygosity mapping of the two affected siblings suggested that two autozygous loci, 8q24.12-8q24.13 and 9q31.2-q33.1, segregated with disease. Collectively, these encompassed 97 protein-encoding genes.

WES identified one missense variant in these genes with a MAF <0.001, *CCN3* p.D82G (exon 2, c.A245G, NM_002514) on chromosome 8 using the parameters described. Subsequent Sanger sequencing showed that the affected sibling (IV:5) was also homozygous for this mutation, while the unaffected brother (IV:4), mother (III:5), second-degree relative (III:2) were found to be heterozygous for this mutation (**Figure 1A**). By inference, the father (III:6) would also be heterozygous for this mutation. The variant resided in the insulin-like growth factor binding domain of the CCN3 protein (**Figure 1B**).

The variant was rare, with a MAF of 4.012×10^{-6} in the gnomAD dataset, and was not identified in the exomes of 168 Pakistani subjects from the Greater Middle East (GME) Variome Project (17); 3,222 subjects of Pakistani heritage (18); or 3,044 subjects from the AnnEx database, which contained a mixture of ethnic groups and movement disorders, including those with parkinsonism as the predominant phenotype (https://annex.can.ubc.ca). Further, HRM analysis and previous WES data did not identify the mutation in 353 Australian PD samples, including 104 early-onset cases (age at onset <50 years) and 57 probands from multi-incident families. The variant had a CADD score of 24.7 (19), which was suggestive of a deleterious variant.

To assess if the p.D82G mutation had any effect on the CCN3 protein, both $CCN3^{WT}$ (wild-type) and $CCN3^{D82G}$ were expressed in HEK293T cells. Due to the fact that CCN3 is a secreted protein, $CCN3^{WT}$ was primarily detected in the cell culture medium as expected (**Figure 2A**), with barely any detectable protein within the cell (**Figure 2B**). Furthermore, $CCN3^{D82G}$ was also detected at a similar level in the cell culture medium (**Figure 2A**). However, when the $CCN3^{D82G}$ cell lysate was analyzed, we detected a significant increase in cellular CCN3 protein (27.65 ± 13.01 fold increase, $p < 0.05$; **Figure 2B**). We next investigated the subcellular localization of $CCN3^{D82G.}$ by immunofluorescence staining of HEK293 cells transfected with either $CCN3^{WT}$ or $CCN3^{D82G}$. Subcellular localization confirmed $CCN3^{WT}$ was detectable in less transfected cells than the $CCN3^{D82G}$ (**Figure 2C**), suggesting the $CCN3^{D82G}$ had impaired secretion when compared to $CCN3^{WT}$.

We next investigated the expression of *CCN3* mRNA in hONS cells derived from unrelated sporadic cases and controls to validate observations from microarray data (16), which suggested that *CCN3* was decreased by 7.3% in PD patients ($p < 0.01$; **Figure 3**). Interestingly, the qPCR analysis confirmed *CCN3* expression was downregulated in PD cases by 52.8% ($p < 0.001$) when compared to *RPL13* and *TBP* (**Figure 3**).

DISCUSSION

Here we present evidence that a rare homozygous mutation in *CCN3* found in a family in rural Pakistan may be a novel cause of parkinsonism. After excluding other causes of early-onset

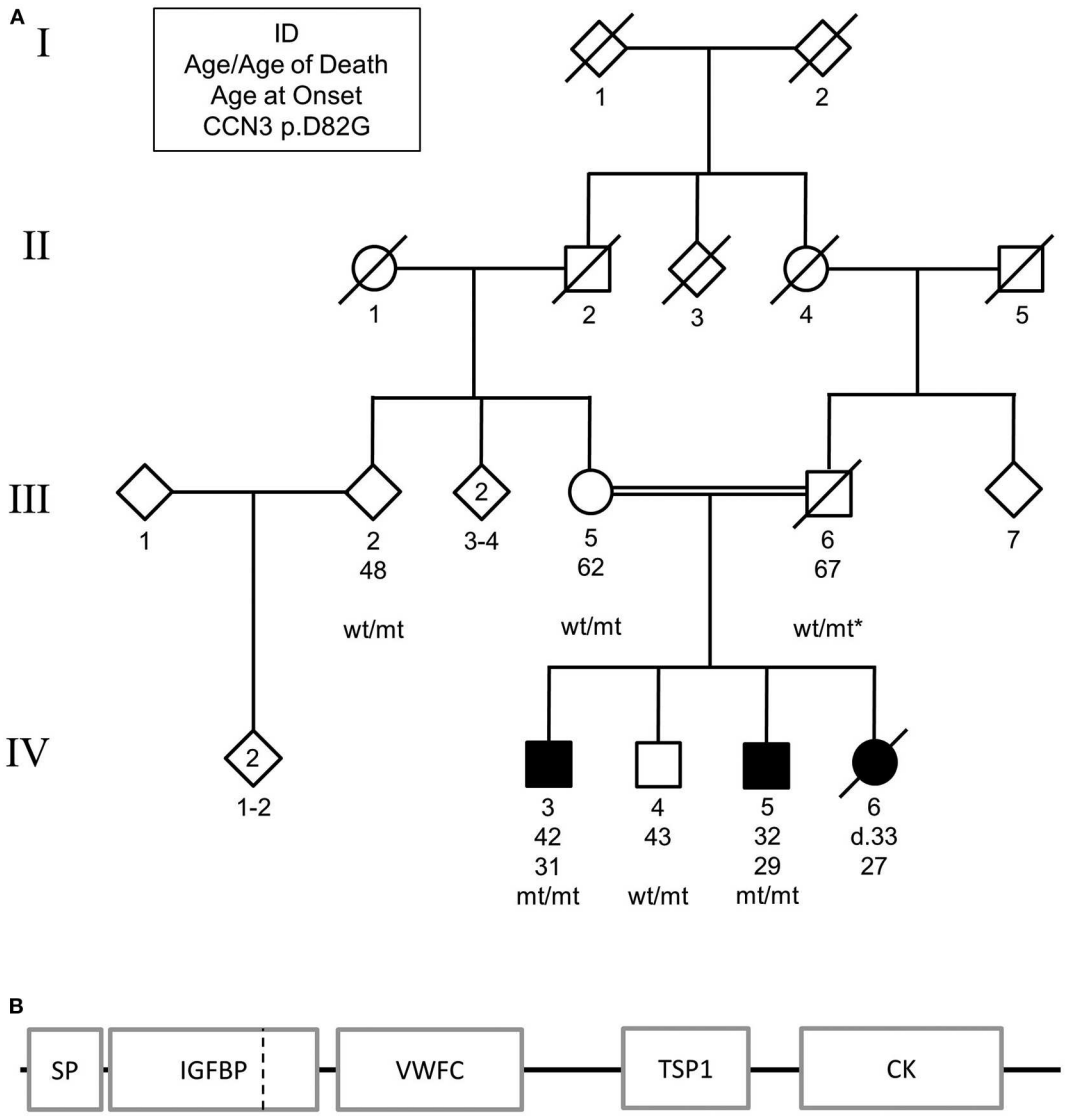

FIGURE 1 | (A) Pedigree of family carrying *CCN3* p.D82G homozygous mutation. Solid shapes indicate those affected by parkinsonism. Squares represent males, circles represent females, and diamonds represent undefined. * indicates an inferred genotype. Non-essential pedigree information has been omitted or modified to protect the privacy of the family. **(B)** Schematic of mutation location in protein structure. SP, signal peptide; IGFBP, insulin-like growth factor binding protein; VWFC, von Willebrand factor type C; TSP1, thrombospondin type-1 repeat; CK, cysteine-knot, C-terminus. Dashed vertical line represents mutation location.

parkinsonism within affected family members, homozygosity mapping and WES identified only one suitable candidate for disease: *CCN3* c.A245G (p.D82G). Prior to this study, the mutation had only been reported once as a heterozygous variant in a European subject above 80 years of age in the gnomAD dataset (20). We did not detect the variant in 353 Australian samples, which included 57 multi-incident families and 104 early-onset cases. Further, the mutation was not detected in three other WES datasets with a combined total of 6,434 samples of which 3,390 were of Pakistani heritage. These data suggest the mutation is very rare across multiple populations; however, screening the community from the same rural district as the family may provide further insight into the local frequency of the sequence variant. Although the CCN3 aspartic acid residue at position 82 is multi-allelic, the reported rate of the asparagine and glutamic acid amino acid changes were still rare (20), have lower CADD scores compared to the glycine substitution, 24.4 and 18.2, respectively (19), and may have different effects on protein function. Notably, glycine is achiral and has been noted to affect flexibility in protein conformation (21, 22). Hence, we still consider this mutation as a good candidate for disease in affected members of this family.

Notably, we observed that a portion of the $CCN3^{D82G}$ protein is consistently retained within the cell, while still being able to be secreted. This suggests that the aspartic acid residue in the insulin-like growth factor (IGF)-like binding domain is

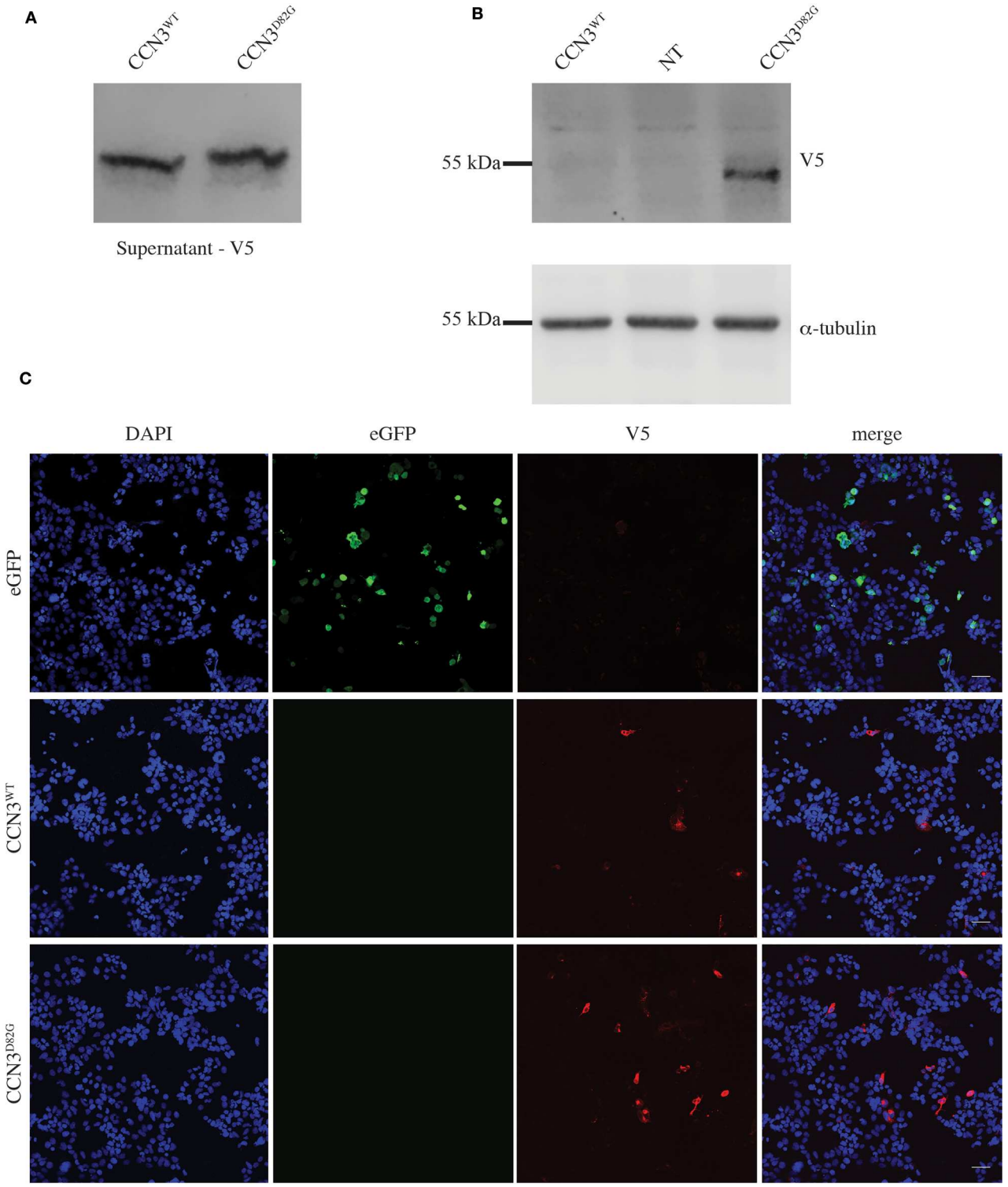

FIGURE 2 | CCN3^{D82G} has impaired secretion. **(A)** V5 Immunoblot of cultured supernatant from non-transfected (NT) and CCN3^{WT-} and CCN3^{D82G}-expressing cells. **(B)** Immunoblots of lysates from non- NT and CCN3^{WT-} and CCN3^{D82G}-expressing cells probed with either V5 or α-tubulin. **(C)** Immunofluorescence imaging of eGFP (enhanced green fluorescent protein) and CCN3^{WT-} and CCN3^{D82G}-expressing cells.

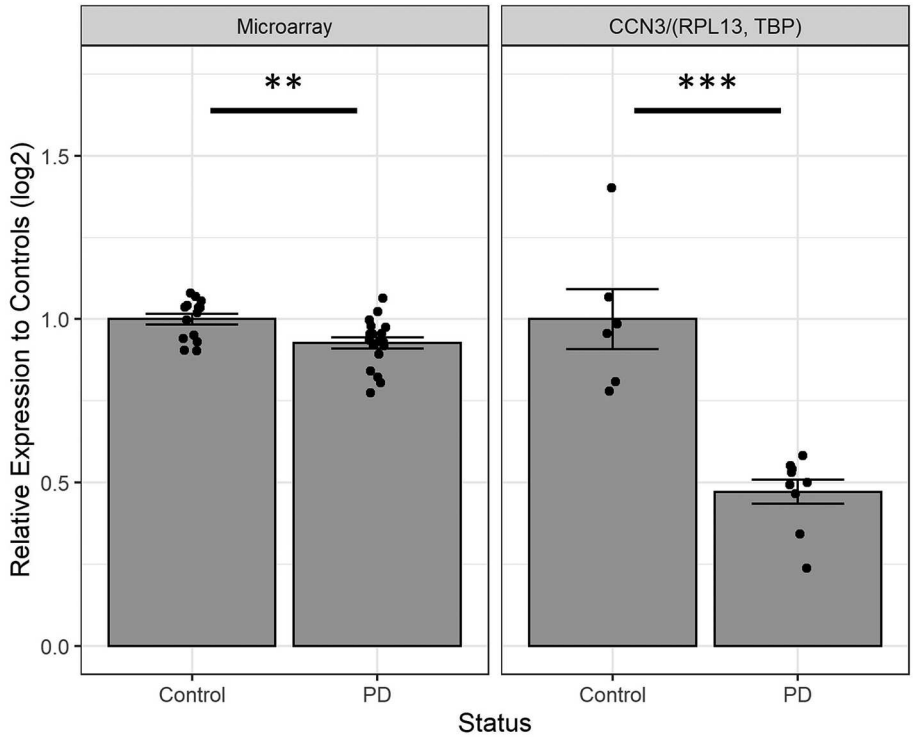

FIGURE 3 | Comparing *CCN3* mRNA expression between cases and controls from microarray and qPCR data. Expression data were normalized to control-derived hONS samples and displayed on a log scale. qPCR data were normalized to geometric mean of *RPL13* and *TBP*. Error bars represent standard error. $^{**}p < 0.01$, $^{***}p < 0.001$.

important for normal secretion. This finding is interesting as the CCN3 IGF-like binding domain has little to no IGF binding affinity (23), thus supporting the importance of this domain for another function.

Mechanisms in which this aberrant CCN3 protein may lead to disease are unclear. It is noteworthy that *CCN3* mRNA is expressed in developing human brains and has been detected in the substantia nigra, pontine abducens, thalamic nuclei, and striatum at 32 weeks gestation (24). Further, the CCN3 protein was observed in motor neurons of the ventral horn (25). Thus, CCN3 may have a role in the development of the nervous system. Alternatively, chemokines CCL2 and CXCL1 in rat astrocytes were found to increase upon recombinant CCN3 protein exposure (26), suggesting a possible neuroinflammatory role. Further investigation is strongly warranted to characterize potential mechanisms affected by the aberrant CCN3 protein.

It is also noteworthy that hONS cells from sporadic PD cases had a lower expression of *CCN3*. These primary human cells were previously shown to encapsulate aspects of disease, such as metabolic, oxidative (16, 27), and mitochondrial phenotypes (28). Consistently, the disease-specific decreased expression was also observed through microarray analysis (16). These data suggest *CCN3* expression may have a role in sporadic disease, or conversely, disease status may have an effect on *CCN3* expression. This observation also warrants further investigation.

CONCLUSION

While we cannot exclude mutations residing outside the exome, the evidence presented in this study indicates that the best candidate for disease in this family was the rare homozygous *CCN3* p.D82G mutation. Notably, the mutation impaired secretion of the CCN3 extracellular matrix protein; however, the pathway affected by the aberrant protein within the affected family requires further investigation. We propose that other consanguineous families with early-onset parkinsonism from this region should also be examined for this and other rare variants. Interestingly, this study also presents evidence to suggest that *CCN3* expression is downregulated in idiopathic PD; however, the molecular causes of this downregulation and the role CCN3 has in idiopathic PD also require further elucidation. Nevertheless, this study has identified a strong interesting parkinsonism candidate, CCN3, highlighting the utility of screening isolated affected families for identification of putative disease-causing genes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Postgraduate Medical Institute (PGMI), Lady Reading Hospital (LRH), Peshawar (Ref. 65/IRBEB/PGMI/LRH) and the Human Research Ethics Committee (HREC) at Griffith University (Ref: ESK/04/11/HREC). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SK, ZA, and MN contributed to the collection of patient information and sample. SB, SK, and JF conducted the genetic analysis. SB and MÖ conducted and analyzed the genotyping. SB and SP conducted and analyzed the expression analysis. JI designed the expression construct. AS conducted and analyzed cell culture, immunoblotting, and immunostaining. GM led and supervised the overall project. SB, AS, SK, GM, SW, JF, and CL contributed to study design and conception. SB and AS prepared the manuscript. All authors contributed to the manuscript revision.

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Olfactory Dysfunction in Familial and Sporadic Parkinson's Disease

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This minireview discusses our current understanding of the olfactory dysfunction that is frequently observed in sporadic and familial forms of Parkinson's disease and parkinsonian syndromes. We review the salient characteristics of olfactory dysfunction in these conditions, discussing its prevalence and characteristics, how neuronal processes and circuits are altered in Parkinson's disease, and what is assessed by clinically used measures of olfactory function. We highlight how studies of monogenic Parkinson's disease and investigations in ethnically diverse populations have contributed to understanding the mechanisms underlying olfactory dysfunction. Furthermore, we discuss how imaging and system-level approaches have been used to understand the pathogenesis of olfactory dysfunction. We discuss the challenging, remaining gaps in understanding the basis of olfactory dysfunction in neurodegeneration. We propose that insights could be obtained by following longitudinal cohorts with familial forms of Parkinson's disease using a combination of approaches: a multifaceted longitudinal assessment of olfactory function during disease progression is essential to identify not only how dysfunction arises, but also to address its relationship to motor and non-motor Parkinson's disease symptoms. An assessment of cohorts having monogenic forms of Parkinson's disease, available within the Genetic Epidemiology of Parkinson's Disease (GEoPD), as well as other international consortia, will have heuristic value in addressing the complexity of olfactory dysfunction in the context of the neurodegenerative process. This will inform our understanding of Parkinson's disease as a multisystem disorder and facilitate the more effective use of olfactory dysfunction assessment in identifying prodromal Parkinson's disease and understanding disease progression.

Keywords: olfactory dysfunction, genetics, idiopathic Parkinson's disease, longitudinal studies, biomarker, cognition, monogenic Parkinson's disease, neurodegeneration

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INTRODUCTION

Since Ansari and Johnson (1) first reported that olfactory dysfunction (OD) occurs in Parkinson's disease (PD), OD has been evaluated using tests of odor identification, odor discrimination, odor-threshold detection and electrophysiology (2–4). OD is not PD-specific and is prevalent in aging and other diseases, particularly in neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, and rapid-eye-movement sleep-behavior disorder (5–12). OD can severely impact the quality of life, affecting interpersonal and eating habits, patient safety, and nutritional intake (13–15). Because OD is prominent in PD (16, 17) and its onset may signal prodromal PD,

it is important to understand how and when OD arises, the mechanisms underlying its association with PD progression, and identify interventions for OD.

OD PREVALENCE IN PD

Cross-sectional studies revealed that OD occurs in sporadic PD prior to the initiation of dopaminergic therapy [reviews: (3, 4, 18–20)]. The reported prevalence of OD in sporadic PD varies substantially: 45–50% (1, 21, 22), 70–80% (2, 23), and 90–97% (24, 25). This may reflect challenges in PD diagnosis, OD measurement, sample size, normative group selection, and age. Prevalence of OD generally decreases when adjusted for age-related norms, as the prevalence of OD is over 50% past age 65 and 62–80% past age 80 (26, 27).

Interestingly, OD in monogenic PD exhibits variable penetrance and expressivity. In manifesting carriers with *GBA* (β-glucosylceramidase), *SNCA* (α-synuclein, point or gene-multiplication), *LRRK2* (leucine-rich repeat kinase 2), *PINK1* (PTEN-induced kinase 1), or *DJ1* (PARK7: Parkinsonism-associated deglycase) mutations, and in *MAPT* (microtubule-associated protein tau)-associated frontotemporal dementia and parkinsonism, OD-penetrance overlaps with that in sporadic PD [(28–64); reviews: (3, 65–68)]. While different studies report varying, sex- or allele-differential OD prevalence in mutation carriers relative to sporadic PD controls [tabulated in Doty (3)], two key OD features seen in sporadic PD persist in many monogenic forms. First, while many carriers are hyposmic when they phenoconvert to show motor symptoms, some carriers have mostly preserved olfaction (28, 40). Second, the distribution of OD in monogenic PD cohorts is similar to sporadic PD (**Figure 1**). The striking exception is *PRKN* (parkin RBR E3-ubiquitin protein ligase) and *VPS35* (VPS35 retromer-complex component) manifesting carriers, who have normal olfaction or only mild OD (70–75). As discussed below, the preserved olfaction in *PRKN* carriers and possibly some subsets of *LRRK2* carriers appears related to an absence of Lewy bodies (LBs) in the olfactory bulb and/or the olfactory system (76–78).

Mutations in *LRRK2*, *PINK1*, *GBA*, *SNCA*, and *PRKN* have similar effects on OD across ethnically and geographically diverse populations. Hence, if a mutation causes OD, its effect-size on OD-related neurodegenerative processes is large relative to genetic background and environmental exposure. Since these mutations increase substantially PD risk, targeted investigations of non-manifesting mutation carriers of *LRRK2*, *PINK1*, *GBA*, and *SNCA* provide a unique opportunity to understand OD in PD.

OD CHARACTERISTICS IN PD

Though many fundamental questions about OD in PD have been raised for some time (24, 79, 80) and studied in diverse patient cohorts and contexts, consensus answers are not always available, as described below. Sometimes, conflicting findings reflect the tests used or their interpretation. As discussed more fully by

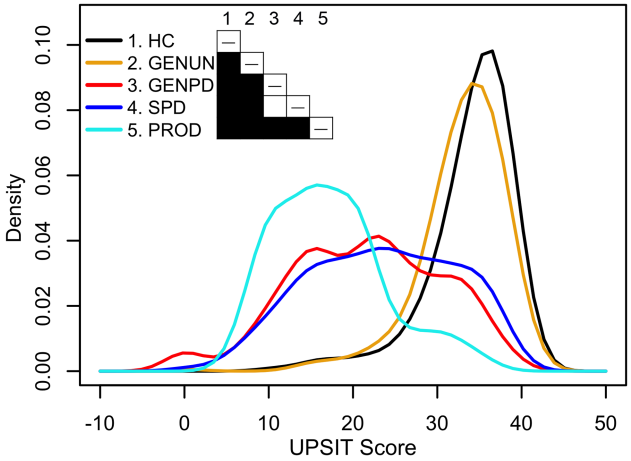

FIGURE 1 | Univariate density estimates of scores on the University of Pennsylvania Smell Identification Test (UPSIT) in five PPMI cohorts (69). Cohorts: 198 healthy controls (HC, black) age-matched with 491 sporadic Parkinson's disease patients (SPD, blue, ≥2 of resting tremor, bradykinesia, or rigidity, with resting tremor or bradykinesia required, or either asymmetric resting tremor or asymmetric bradykinesia; PD diagnosis ≤2 years; Hoehn and Yahr stage I–II; scan-confirmed dopaminergic deficit; ≥30 years at diagnosis; no dopaminergic medications ≥6 months after baseline assessment), 310 asymptomatic genetic Parkinson's disease patients who have a mutation, or are a first-degree relative of an individual having a mutation, in LRRK2, SNCA, or GBA (GENUN, gold), 220 symptomatic genetic Parkinson's disease patients who have a mutation in LRRK2, SNCA, or GBA (GENPD, red), and 61 individuals selected for REM-behavior sleep disorder and/or hyposmia (PROD, cyan). Shading in the table cells indicates the *P*-value (white: $P \geq 0.05$, black: $P < 0.001$) obtained from pairwise non-parametric bootstrap tests of equal densities using 1,000 permutations.

Doty (81), while the results on psychophysical tests of OD (tests of odor identification, odor discrimination, or odor-threshold detection) are strongly correlated, they vary in reliability and sensitivity and assess different neurophysiological, neurological, and/or psychological aspects. Most often, OD is evaluated using tests of odor identification. Using those tests, variable OD is seen in all studies of sporadic PD and those monogenic PD forms resembling sporadic PD (*SNCA*, *GBA*, *LRRK2*), including at motor-symptom onset. **Figure 1** (69) illustrates this using univariate density estimates of odor-identification-test scores obtained from the Parkinson's Progression Marker Initiative (PPMI). While the score distributions of early-stage, dopamine-transporter-scan positive, dopaminergic-treatment naïve sporadic PD (blue), and age-matched healthy controls (black) are distinct, both groups have normosmic, hyposmic, and anosmic membership. This is also observed in manifesting *SNCA*, *GBA*, and *LRRK2* carriers (red line), which here have a score distribution like sporadic PD. Indeed, anosmia is not always seen in manifesting carriers in nuclear families with monogenic PD (28, 52). Hence, like PD motor symptoms, OD has variable penetrance in sporadic and some monogenic PD. Unlike them, OD is frequently seen in otherwise healthy aging and other neurodegenerative diseases, suggesting that OD can result from a confluence of processes.

Though OD in PD presents non-uniformly, community-based prospective studies demonstrated that it can appear up to 4 years before motor-symptom onset (79, 80); in MAPT carriers it can appear 2 years before symptom onset (52). Consequently, OD has been used in biomarker panels for predicting risk and/or progression of PD [(59, 82–88); reviews: (18, 19, 89–91)]. For this purpose, it is important to elucidate: (1) whether OD in PD is distinguishable from OD in other diseases and aging; (2) how its onset and progression relates to motor-symptom onset and progression; (3) whether OD severity is associated with disease stage, duration, or predicts disease progression; and (4) what clinical tests of OD measure in the context of the disease process. Well-designed studies of OD in monogenic PD can address each issue.

DISTINGUISHING FEATURES OF OD IN PD

Central to understanding whether the etiology of OD in PD is shared with that in the elderly or other neurodegenerative diseases is identifying whether OD has PD-specific characteristics. PD does affect supra-threshold estimates of perceived odor intensity, which appears spared in Alzheimer's disease, schizophrenia, and the elderly (92), but does not affect the trigeminal system (93). Combined with imaging, it can help distinguish disorders whose initial presentation overlaps with PD, such as progressive supranuclear palsy, cortico-basal degeneration, or multiple system atrophy [(94–96); reviews: (20, 97)].

Many studies have identified a set of odors or pattern of OD that best evaluates OD in their cohort (98–107). Most often however, the odor sets are dissimilar in different PD populations (108). This likely reflects odor identification being influenced strongly by prior exposure and population variation in odorant-receptor alleles. Multiple analyses have indicated that there is not odor-selective hyposmia in PD. Highly compelling is an odor-item analysis indicating that the discriminatory power of odor subsets is not shared across independently selected groups (109). Additional support comes from longitudinally evaluating hyposmia in subjects with sporadic PD, subjects without neurodegenerative disease, and in *MAPT*-mutation carriers. They reveal odor-identification irreproducibility as a general feature of OD: subjects do not misidentify the same odors on replicate odor-identification tests (52). In a longitudinal study of sporadic or monogenic PD subjects recruited from ethnically diverse populations, comparison of results across populations would be facilitated by using a universal olfactory test that is independent of odor-specific insensitivity or prior experience (110).

HOW IS OD RELATED TO DISEASE ONSET AND PROGRESSION?

The etiological mechanisms underlying the variable presentation of both PD motor symptoms and OD remain unclear. The olfactory epithelium in PD appears normal (111), but it is unknown whether PD impacts its neurogenic niche (112), the functional integration of axons from differentiating olfactory-receptor neurons into the olfactory bulb, and how either process impacts OD. α-Synuclein deposits are found in the olfactory bulb and anterior olfactory nucleus at Braak stage I (113–115), and glomerular volume is reduced by half in PD (116). Since the olfactory bulb plays a critical role in the spatiotemporal coding of smell, OD early in disease might reflect the incomplete inhibition of olfactory inputs at the level of the olfactory bulb (117) and the reported increase in dopaminergic neurons (118, 119). Studies of OD in monogenic PD offer a compelling hypothesis for the variable expressivity of OD: early OD reflects LB development in the olfactory bulb. LBs are prominent neuropathological features in monogenic PD forms with OD (*SNCA*, *GBA*, *PINK1*, and *DJ1*), but not in *PRKN*-related PD where olfaction is preserved (28–64, 70–77). Progressive OD is also seen in mice expressing forms of human α-synuclein exhibiting olfactory-bulb Lewy pathology (78, 120, 121). *ATP13A2* (ATPase cation transporting 13A2) carriers exhibit OD (65) but not LB (122), but show atypical PD. Since LB and olfactory dysfunction are not always seen in *LRRK2* carriers, and some *LRRK2* alleles have fewer LB (40–49, 76, 77, 123), additional support for this hypothesis would come if the relatively preserved olfaction in a subgroup of *LRRK2* carriers were also associated with fewer olfactory-bulb LB. If this hypothesis is correct, screening hyposmic individuals using PET ligands under development to image LB in the olfactory bulb (124)[1] would help identify those having increased risk of developing PD-motor symptoms.

Early olfactory deficits are consistent with the olfactory vector hypothesis for PD pathogenesis and the caudo-rostral spread of LB pathology (113–115). It is interesting however, that some individuals with normal olfaction lack olfactory bulbs (125). This suggests that the establishment and maintenance of olfactory circuits has considerable functional plasticity. The projections of the olfactory tract form circuits spanning multiple cortical areas, including the entorhinal and orbitofrontal cortices and utilize multiple neurotransmitter systems (**Figure 2**). Therefore, olfactory-bulb pathology may not be the sole determinant of OD. As discussed below, early OD associated with olfactory bulb LB can be followed by later cholinergic denervation (126, 127). It will be important to address the extent to which OD in PD is associated with a loss of functional plasticity, whether it reflects the differential progression of the neurodegenerative process in one or multiple anatomical regions, the contributions of degenerative or compensatory changes in dopaminergic, or other neurotransmitter systems, including substance P and acetylcholine (128, 129), and how these associations relate to later motor-symptom onset and progression.

OD has significant, moderate to strong associations with nigrostriatal degeneration (105, 130, 131). In one study, 98.7% of PD subjects with imaging evidence of nigrostriatal dopaminergic denervation had OD (130). There, however, most still retained some olfactory function: 24.6% were anosmic and 73.2% were hyposmic [$N = 183$, motor-disease duration $= 6.4 \pm 4.3$ year, Hoehn and Yahr (H&Y) stage 1–5].

[1] https://www.michaeljfox.org/grant/18f-labeled-alpha-synuclein-ligands-pet-imaging-lewy-bodies

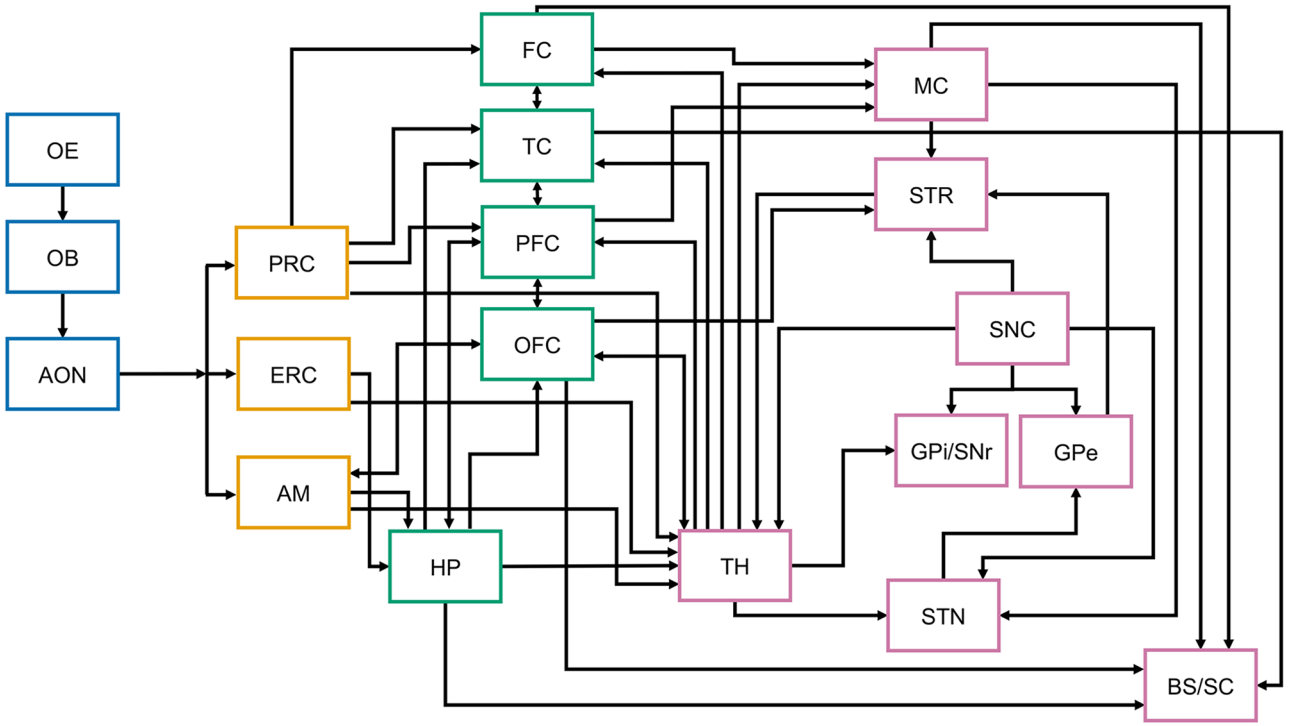

FIGURE 2 | Simplified schematic representation of central nervous system structures and connections involved in olfaction, memory, and motor control. The figure aims to illustrate the complexity of the connections of the olfactory system, associative cortices, thalamus, and the basal ganglia that may be differentially affected at different stages of Parkinson's disease. While the arrows represent anatomical and functional connectivity, not all known interconnections are included in this schematic representation. Differential neuronal loss and associated decrease in key neurotransmitter (acetylcholine, dopamine, etc.) levels at any of these structures has the potential to differentially affect their function and connectivity, thus directly and indirectly contributing to olfactory dysfunction. While in PD LB preferentially involve the brainstem at disease onset, their distribution in the olfactory and cortical areas depends on disease stage (113, 114). OE, olfactory epithelium; OB, olfactory bulb; AON, anterior olfactory nucleus; PRC, perirhinal cortex; ERC, entorhinal cortex; AM, amygdala; FC, frontal cortex; TC, temporal cortex; PFC, prefrontal cortex; OFC, orbitofrontal cortex; HP, hippocampus; TH, thalamus; MC, motor cortex; STR, striatum; SNC, substantia nigra pars compacta; GPi/SNr, globus pallidus interna/substantia nigra pars reticulata; GPe, globus pallidus externa; STN, subthalamic nucleus; BS, brainstem.

Consistent with these findings, screens for hyposmia increase the likelihood of identifying subjects with abnormal dopamine transporter binding (91). This may be a causal association or reflect coincident processes. Deficits in cholinergic transmission are a common element in OD in different diseases (132), and neurodegeneration affecting cholinergic circuits is found even early in PD [**Figure 2**, (133–136)]. Indeed, cholinergic denervation of the limbic archicortex in PD subjects at H&Y 2.5 ± 0.5 is a more robust determinant of poor odor-identification test scores than nigrostriatal dopaminergic denervation (126). When groups of PD patients having mild motor deficits and varying degrees of OD were compared, there was a more significant reduction of a putative cholinergic marker (i.e., short latency afferent inhibition of the motor cortex) when olfactory event-related potentials (a direct measure of the processing of olfactory information) were absent, than when only their latency and/or amplitude was altered (137). Curiously, a history of smoking (cholinergic stimulation) is also associated with better olfaction in PD (138).

The use of shared neural substrates in the premotor frontal and orbitofrontal cortex by olfaction and cognition (**Figure 2**), and the contribution of cholinergic deficits to OD provides a potential mechanism for why greater OD appears to identify the subset of sporadic and monogenic PD patients at greater risk of future cognitive impairment [(59, 107, 139–149); reviews: (27, 150)]. Thus, genome-wide screens in PD subjects for variants that influence risk of severe OD or protect from developing OD may identify genetic factors that increase risk of, or offer protection from, cognitive impairment in PD. PD-associated changes to central brain networks, brain-region specific structural integrity, and functional connectivity also are associated with OD (151–161). The importance of functional connectivity is highlighted by theta-specific phase coupling between the piriform cortex and hippocampus in the rapid differentiation of odor stimuli (162), and the ability of anosmic subjects having diminished functional connectivity to activate an olfaction-related functional network (163). A possible partial restoration of functional connectivity may explain why deep-brain stimulation of the subthalamic nucleus (DBS-STN) leads to modest odor-identification test score improvement (164–166). To obtain mechanistic insights into the variable presentation of OD, its relationship to motor symptom presentation and

later cognitive dysfunction, it would be fruitful to longitudinally study carriers of monogenic PD mutations utilizing functional imaging to evaluate how functional connectivity is altered during prodromal PD and disease progression.

Since olfactory system LB increase with advancing neuropathological PD stage (113–115) and many non-motor PD symptoms such as cognitive and autonomic dysfunction often increase in severity with disease progression, it is unclear why the severity of hyposmia is not consistently associated with motor signs, disease stage, or duration. This is especially striking because the density of synuclein-pathology in the olfactory bulb is positively correlated with motor scores (165). Some cross-sectional studies reveal that diminished scores on olfactory-function tests are associated with increased disease duration (167, 168), while others do not (22, 23, 25, 117, 148, 169, 170). Some studies have reported associations with more severe disease (22, 148, 168, 169, 171, 172) but others have not (24, 25, 117, 170, 173–175), even though hyposmia severity is associated with lower dopamine transporter activity (168). While OD does not always develop in parallel with other non-motor symptoms in either sporadic or monogenic PD (65), resolving whether it does develop in parallel with motor symptoms has implications for management. In one study of PD subjects with similar striatal dopamine transporter activities, normosmic individuals had lower levodopa-equivalent dose requirements than did hyposmic individuals at 2.5 years of follow-up (22), suggesting that a relative lack of OD may be associated with a clinically more benign disease course.

The conflicting results about whether OD relates to disease progression might be explained if OD does not appear gradually, but rather in a stepwise irreversible manner. Variability in the occurrence of LB within the olfactory bulb could be related to the degree of inhibition of olfactory inputs (117) and/or increase in dopaminergic neurons (118). This could contribute to variable expressivity in initial OD that remains relatively stable over time, possibly due to functional plasticity. Stepwise onset could arise from the convergence of multiple failing processes. While a primary early contributor is almost certainly the loss of functionality within the olfactory bulb, later contributions could derive from other olfactory-system regions. These could include the asynchronous stepwise failure of compensatory mechanisms and/or the onset of dysfunction in circuits involved in associative processing and interpretation of smell. Joining the gradual loss of functionality in the olfactory bulb to either of these processes would lead to a stepwise onset of OD in PD. In this scenario, different levels of OD would be observed upon breaching different functional lintels. A continuous scaled-test score distribution would be observed in a population, but longitudinally followed individuals would show stepwise score decline. Since many newly diagnosed cases are normosmic or hyposmic, whether or not an association is observed between OD and motor function in a cross-sectional study would depend strongly on the cohort's initial constitution.

Whether OD shows stepwise progression could be addressed by obtaining longitudinal data on OD in large PD cohorts. To date, most studies (e.g., PPMI) assess OD only at baseline. Hyposmia can be stable over periods of 2–6 years in sporadic PD (24, 117), *MAPT* mutation carriers (52), and *GBA* mutation carriers (50). Therefore, to assess the progression of OD accurately, follow-up longer than 5 years will be necessary. A more efficient approach is to assess the progression of OD in non-manifesting carriers from monogenic PD cohorts where disease risk is substantially increased, and the genetic cause is known. A longitudinal study using imaging methodologies able to evaluate when LB appear, the integrity of multiple neurotransmitter systems, and functional connectivity would help address the relative contribution of each to the onset and progression of OD and motor symptoms.

WHAT DO OLFACTORY-FUNCTION TESTS ASSESS ABOUT THE DISEASE PROCESS?

The stability of measurements of OD in PD suggests that it may be challenging to use them to directly assess the prodromal and symptomatic disease process outside of monogenic PD cohorts. Intriguingly, PD subjects often subjectively assess their olfactory ability as better than evaluated by validated clinical measures (13, 24, 176–178). One study (176) found 91% hyposmic subjects using the UPSIT, an objective odor-identification test, vs. 55% using a subject's subjective assessment. Lower scores on clinical tests have implications for a patient's quality of life. Patients unaware of their olfactory deficit may be at greater risk of harm because they may be unable to detect smoke or spoiled foods (178). However, this concern may be tempered if the perception of the patient is not fully captured by the objective assessment.

An explanation for the discrepancy between the objective and subjective assessments comes from finding that a loss of awareness of hyposmia is associated with mild cognitive impairment in PD (177). PD patients who overrate their sense of smell or are aware of their hyposmia have worse executive function than those who are objectively and subjectively normosmic (13). Memory is strongly related to olfaction, and deficits in olfaction and verbal learning/memory in PD are associated (107, 126, 179–182). Deficits in cognitive processes also indirectly contribute to lower scores on forced-choice odor-identification tests (69). Consequently, discrepancies in the metacognitive knowledge of hyposmic individuals—self-awareness of their olfactory ability—and objectively measured OD may reflect testing-related cognitive challenges in memory or decision making. This lack of metacognitive knowledge may be a sensitive biomarker of early cognitive decline (13). A lack of metacognitive knowledge may also identify individuals whose olfactory system can have functionality restored. If a subject's perceptual reality is better than their objectively assessed ability, some of the neural substrates used for processing olfactory information should be preserved. Assessing metacognitive knowledge within longitudinal studies of monogenic PD could help identify the neural substrates preserved when metacognitive knowledge does not match objective measurements, and which are lost when individuals self-perceive anosmia. This has pragmatic considerations for managing cognitive decline.

Identifying individuals whose olfactory system could have functionality restored also identifies candidates for potential

OD therapy. While motor symptom treatment is a primary concern in PD, improving non-motor symptoms like OD will improve patient quality of life (13, 183). Simple strategies to improve OD are lacking presently. While DBS-STN modestly improves OD (163–166), DBS-STN is currently used to treat motor complications of levodopa therapy in patients with an at-least 4 year disease duration. It will be informative to assess if other treatments currently under development, such as α-synuclein antibody therapy, gene-editing therapy or other molecular treatments specific to monogenic forms of PD, also have a beneficial effect on OD.

CONCLUSION

Elucidation of the mechanisms underlying OD in PD and their relationship to the onset and progression of motor and cognitive symptoms will contribute to comprehensive measures of OD being used to better understand, identify and manage PD. Well-characterized monogenic cohorts identified within the GEoPD and other international consortia (184) can serve as the ideal substrate for multifaceted longitudinal studies needed for this purpose.

AUTHOR CONTRIBUTIONS

KM and BC contributed to the conception of the review. BC wrote the first draft of the manuscript. BC and KM contributed to manuscript revision, read, and approved the submitted version.

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BRIEF RESEARCH REPORT
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Appendectomy, Tonsillectomy and Parkinson's Disease Risk: A Swedish Register-Based Study

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Introduction: The gut-brain hypothesis proposes that Parkinson's disease (PD) pathology may start in the gut and later spread to the brain in a prion-like manner. As PD pathology is redundant in the appendix and tonsils, which are important gut-associated lymphoid tissues, we examined whether appendectomy and tonsillectomy were associated with later PD risk.

Methods: The nested case-control study included 78,650 PD patients born in 1900–1980 and with a diagnosis of PD between 1964 and 2010. For each PD patient, we randomly selected 40 non-PD controls individually matched for sex and year of birth at the date of PD diagnosis. Appendectomy and tonsillectomy before PD diagnosis were ascertained from the Swedish Patient Register from 1964 onward. We calculated odds ratios (OR) with 95% confidence intervals (CI) using conditional logistic regression adjusting for country of birth, highest achieved education, COPD, comorbidity index, and number of hospital visits.

Results: Overall, we found 16% lower risk of PD linked to previous appendectomy (OR = 0.84, 95% CI: 0.80–0.88) and 8% lower risk of PD linked to previous tonsillectomy, although not statistically significant (OR = 0.92, 95% CI: 0.81–1.04). A 7 and 15% lower risk of PD was also noted ≥20 years after appendectomy and tonsillectomy, respectively. Similar associations were observed for men and women but were stronger for PD diagnosed after age 60.

Conclusion: Appendectomy and potentially also tonsillectomy were associated with a lower risk PD. A potential mechanism may involve surgical removal of alpha-synuclein redundancy in the appendix and tonsils.

Keywords: Parkinson's disease, appendectomy, tonsillectomy, nested case-control, register-based

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INTRODUCTION

The so-called dual-hit hypothesis about the pathogenesis of idiopathic Parkinson's disease (PD) states that neutrophic pathogens may enter the brain through two portals— the nasal cavity and the gut (1–3). Deposition of alpha-synuclein has been found throughout the entire gut with most dense expression in the appendix of both PD patients and healthy individuals (4). Although still controversial, there is evidence that alpha-synuclein pathology may be transported from cell to cell

and thereby spread from the nasal cavity or gut to the brain (2, 3). Mounting evidence has also linked neuroinflammation with PD development (5).

The tonsils and the appendix are important gut-associated lymphoid tissues in the mucosa-associated immune system (6). Surgical procedures to remove these organs often occur in early childhood or adolescence due to recurrent or acute infections, which may cause long-term alteration in immune function. Previous studies have linked these procedures to risk of multiple sclerosis (7) and inflammatory bowel diseases (8). Given the potential spread of alpha-synuclein from the nasal cavity and gut to the brain and the involvement of the tonsils and appendix in immune function, one might hypothesize that surgical removal of these organs may lower the risk of PD. However, previous results are inconclusive, two studies reported that appendectomy was linked to lower risk of PD or delayed PD onset (9, 10), whereas four other studies suggested either a slightly increased PD risk after appendectomy or no association (11–14). Evidence for tonsillectomy and PD risk is scarce; one study reported no association between tonsillectomy and PD risk (15).

We aimed to evaluate the association of appendectomy and tonsillectomy with PD risk in Swedish nationwide registers. In addition to examining appendectomy and tonsillectomy as binary exposures, we also explored the potential temporal relationship between these surgeries and PD risk and the impact of sex and age on the associations.

MATERIALS AND METHODS

Swedish Health and Population Registers

The Swedish *Patient Register* was established in 1964–1965 and collects information on dates of admission and discharge of hospitalizations, surgical procedures, and medical diagnoses (16, 17). Coverage of this register became complete in 1987 and was expanded to cover surgical day-care procedures in 1997 and outpatient visits in 2001 (18). The *Causes of Death Register* records nationwide information on deaths since 1952, and the *Total Population Register* contains information on dates of immigration and emigration (19). The Swedish *Population and Housing Censuses* were conducted every 5 years from 1960 to 1990 to collect detailed information on housing, civil status, and socioeconomic status (20).

Ascertainment of PD

PD cases were identified from the Patient Register using Swedish revisions of International Classification of Diseases (ICD) codes (i.e., ICD-7: 350 in 1964–1968; ICD-8: 342 in 1969–1986; ICD-9: 332.0 in 1987–1996; and ICD-10: G20 from 1997 onward). Both primary and secondary PD diagnoses were considered. PD is assigned as the primary diagnosis in the Patient Register when PD is considered the main reason for hospitalization, whereas it is assigned as a secondary diagnosis when another condition is considered the main reason. In a previous validation study, compared to clinical workup, positive predicted value (PPV) for inpatient PD diagnosis was 70.8% for primary or secondary PD diagnosis and increased to 80.3% when restricted to primary diagnosis (21).

Ascertainment of Appendectomy and Tonsillectomy

Information on appendectomy was obtained from the Patient Register according to the Swedish Classification of Operations and Major Procedures codes (4510, 4511, 4517, 0058 in 1964-1996, and JEA00, JEA01, JEA10 from 1997 onward). Information on tonsillectomy was obtained using the codes 2710 and 2720 in 1964-1996, and EMB10, EMB20, EMB30, EMB99 from 1997 onward.

Study Design

We conducted a nested case-control study based on individuals who were born between 1900 and 1980 and who participated in the Swedish Population and Housing Census in 1970 or 1980. PD cases were identified from the Patient Register between January 1, 1964 and December 31, 2010. For each PD case, we randomly selected 40 controls who were alive and living in Sweden without previous PD diagnosis, individually matched to the PD patient on sex and year of birth on the date of PD diagnosis. Date of PD diagnosis is hereafter referred to as index date. The final study population encompassed 78,650 PD cases and 3,146,000 non-PD controls.

Co-variates

We retrieved information on country of birth (Swedish vs. non-Swedish born) from the Total Population Register and data on educational attainment ($\leq$9, 10–12, $\geq$13 years, or unknown) from the Swedish Register of Education. Smoking has consistently been linked to lower risk of PD (22) and also to higher risk of appendectomy (23) and tonsillitis, which is the main indication for tonsillectomy (24). As self-reported information on smoking was not available, we used lifetime chronic obstructive pulmonary disease (COPD) as a proxy for smoking similar to a previous study (25). We obtained information on comorbidity from the Patient Register between 1964 and index date, and further weighted and categorized this information according to Deyo's modification of the Charlson's Comorbidity Index (0, 1–2, or $\geq$3 points) (26) ICD codes were presented in our previous study (25). As a measurement of surveillance bias related to comorbidity, including appendicitis or tonsillitis, such that these individuals may have more frequent hospital visits and therefore greater likelihood of receiving PD diagnosis compared to others, we obtained information on number of hospital visits before index date from the Patient Register (both inpatient and outpatient), categorized according to tertiles (0–1, 2, or $\geq$3 visits). Age at index date was categorized as $<$60, 60–69, 70–79, or $>$80 years.

Statistical Analysis

The associations between appendectomy, tonsillectomy, and PD risk were expressed as odds ratios (ORs) with 95% confidence intervals (CIs) estimated from conditional logistic regression. We first analyzed the combined effect of appendectomy and tonsillectomy in relation to PD risk. Individuals who underwent either appendectomy or tonsillectomy or both were defined as exposed and were compared with individuals who had neither surgery regarding PD risk. Second, we performed separate

regression analyses for appendectomy and tonsillectomy in relation to PD risk. We performed the above described analyses in two adjustment steps: first, conditional on sex and birth year matched sets; second, additionally adjusted for country of birth, educational attainment, COPD, comorbidity index, and number of hospital visits. We performed temporal relationship analyses exploring PD risk ≥5, ≥10, and ≥20 years after surgeries. We conducted sub-analyses including interaction terms between surgery and sex, as well as surgery and age at index date (<60, 60–69, 70–79, or ≥80). In addition, we performed a sensitivity analysis restricted to PD cases identified through primary diagnosis. We used Stata 15 and SAS 9.4 for statistical analyses with 2-sided alpha of 0.05.

RESULTS

The distributions of sex and year of birth were balanced between PD cases and controls due to matching (**Table 1**). The mean age (±SD) at PD diagnosis was 74.0 ± 9.19 years. Individuals who were born outside of Sweden, had ≤9 or 10–12 years of education, or had COPD showed lower PD risk. Individuals with unknown educational attainment, more comorbidities, or more frequent hospital visits had a higher risk of PD.

Appendectomy and PD Risk

We identified a total of 80,028 individuals who had an appendectomy. We observed a marginally significant 2% decreased PD risk after an appendectomy after adjusting for sex and birth year matching pairs (**Table 2**, model 1), and 16% decreased PD risk (HR = 0.84, 95% CI = 0.80–0.88) when additionally adjusted for country of birth, highest achieved education, COPD, comorbidity index, and number of hospital visits (**Table 2**, model 2). In the temporal relationship analyses, we noted 31, 24, and 22% lower risk of PD with in 5, 10, and 20 years after the surgery, respectively. The inverse associations remained statistically significant with 13, 12, and 7% decreased PD risk observed more than 5, 10, or 20 years post the surgery (**Table 2**, model 2). We found similar results for men and women and slightly stronger associations for PD diagnosed after age 60 (**Table 2**, model 2). Similar results were observed when restricted to PD defined through primary diagnosis (**Table S1**).

TABLE 1 | Characteristics of Parkinson's disease (PD) cases and controls from the Swedish total population 1,964–2,010, *N* = 3,224,650.

	PD cases, *N* (%)	Controls, *N* (%)	OR (95% CI)[a]	*P*-value[b]
Total	78,650 (100)	3,146,000 (100)		
Sex				
Male	43,533 (55.4)	1,741,320 (55.4)		
Female	35,117 (44.6)	1,404,680 (44.6)		
Age at index date, years				
<60	5,827 (7.4)	234,176 (7.4)		
60–69	16,041 (20.4)	641,304 (20.4)		
70–79	36,039 (45.8)	1,440,524 (45.8)		
≥80	20,743 (26.4)	829,996 (26.4)		
Born in Sweden				<0.01
Unknown	2 (0)	815 (0)		
No	4,464 (5.7)	186,688 (5.9)	0.95 (0.92–0.98)	
Yes	74,184 (94.3)	2,958,497 (94)	1	
Highest achieved education, years				<0.0001
Unknown	25,126 (31.9)	955,824 (30.4)	1.81 (1.72–1.90)	
≤9	30,247 (38.5)	1,273,971 (40.5)	0.89 (0.86–0.91)	
10–12	15,861 (20.2)	640,729 (20.4)	0.92 (0.89–0.95)	
>12	7,416 (9.4)	275,476 (8.8)	1	
Chronic obstructive pulmonary disease (COPD)				<0.0001
No	74,818 (95.1)	2,930,034 (93.1)	1	
Yes	3,832 (4.9)	215,966 (6.9)	0.69 (0.67–0.72)	
Comorbidity index				<0.0001
0	50,235 (63.9)	2,167,220 (68.9)	1	
1–2	21,768 (27.7)	749,584 (23.8)	1.29 (1.27–1.31)	
≥3	6,647 (8.5)	229,196 (7.3)	1.31 (1.27–1.34)	
Number of hospital visits				<0.0001
0–1	24,021 (30.5)	1,310,741 (41.7)	1	
2	20,913 (26.6)	755,964 (24.0)	1.72 (1.69–1.76)	
≥3	33,716 (42.9)	1,079,295 (34.3)	2.19 (2.15–2.24)	

[a] *Logistic regression conditional on birth year and sex;* [b] *Wald-test p-value for categorical variables.*

TABLE 2 | Appendectomy and risk of Parkinson's disease (PD), nationwide case-control analysis.

N	PD cases *N*	Controls OR (95% CI)	Model 1[aa] OR (95% CI)[b]	Model 2[b]
Appendectomy				
No	76,742 (97.6)	3,067,880 (97.5)	1	1
Yes	1,908 (2.4)	78,120 (2.5)	0.98 (0.93–1.02)	0.84 (0.80–0.88)
Years before index date				
<5	232 (0.3)	11,104 (0.4)	0.84 (0.73–0.95)	0.69 (0.60–0.79)
≥5	1,676 (2.1)	67,016 (2.1)	1.00 (0.95–1.05)	0.87 (0.83–0.92)
<10	511 (0.6)	22,371 (0.7)	0.91 (0.84–1.00)	0.76 (0.69–0.83)
≥10	1,397 (1.8)	55,749 (1.8)	1.00 (0.95–1.06)	0.88 (0.83–0.93)
<20	1,025 (1.3)	44,027 (1.4)	0.93 (0.87–0.99)	0.78 (0.73–0.83)
≥20	883 (1.1)	34,093 (1.1)	1.04 (0.97–1.11)	0.93 (0.87–0.99)
Stratified by Sex				
Male	874 (1.1)	36,133 (1.1)	0.97 (0.90–1.03)	0.84 (0.78–0.90)
Female	1,034 (1.3)	41,987 (1.3)	0.98 (0.92–1.05)	0.85 (0.80–0.90)
Stratified by age at index date, years				
<60	253 (0.3)	9,538 (0.3)	1.06 (0.94–1.21)	0.90 (0.79–1.02)
60–69	444 (0.6)	17,756 (0.6)	1.00 (0.91–1.10)	0.85 (0.77–0.93)
70–79	776 (1.0)	32,535 (1.0)	0.95 (0.89–1.02)	0.82 (0.76–0.88)
≥80	435 (0.6)	18,291 (0.6)	0.95 (0.86–1.05)	0.86 (0.78–0.94)

[a]Logistic regression conditional on birth year and sex; [b]Logistic regression conditional on birth year and sex, and additionally adjusted for country of birth, highest achieved education, COPD, comorbidity index, and number of hospital visits.

TABLE 3 | Tonsillectomy and risk of Parkinson's disease (PD), nationwide case-control analysis.

N	PD cases *N*	Controls OR (95% CI)	Model 1[aa] OR (95% CI)[b]	Model 2[b]
Tonsillectomy				
No	78,409 (99.7)	3,136,900 (99.7)	1	1
Yes	241 (0.3)	9,100 (0.3)	1.06 (0.93–1.21)	0.92 (0.81–1.04)
Years before index date				
<5	26 (0)	954 (0)	1.09 (0.74–1.61)	0.91 (0.62–1.34)
≥5	215 (0.3)	8,146 (0.3)	1.06 (0.92–1.21)	0.92 (0.80–1.05)
<10	59 (0.1)	2,014 (0.1)	1.17 (0.90–1.52)	0.98 (0.75–1.27)
≥10	182 (0.2)	7,086 (0.2)	1.03 (0.89–1.19)	0.90 (0.77–1.04)
<20	136 (0.2)	4,699 (0.1)	1.16 (0.98–1.37)	0.98 (0.83–1.16)
≥20	105 (0.1)	4,401 (0.1)	0.95 (0.79–1.16)	0.85 (0.70–1.03)
Stratified by Sex				
Male	141 (0.2)	5,247 (0.2)	1.08 (0.91–1.27)	0.92 (0.78–1.09)
Female	100 (0.1)	3,853 (0.1)	1.04 (0.85–1.27)	0.91 (0.75–1.11)
Stratified by age at index date, years				
<60	93 (0.1)	3,081 (0.1)	1.21 (0.98–1.50)	1.02 (0.83–1.26)
60–69	63 (0.1)	2,600 (0.1)	0.97 (0.75–1.25)	0.83 (0.64–1.07)
70–79	62 (0.1)	2,489 (0.1)	1.00 (0.77–1.28)	0.88 (0.68–1.13)
≥80	23 (0)	930 (0)	0.99 (0.65–1.50)	0.90 (0.60–1.36)

[a]Logistic regression conditional on birth year and sex; [b]Logistic regression conditional on birth year and sex, and additionally adjusted for country of birth, highest achieved education, COPD, comorbidity index, and number of hospital visits.

Tonsillectomy and PD Risk

We identified 9,341 individuals who underwent tonsillectomy. There was a trend that previous tonsillectomy was associated with a lower risk of PD in the fully adjusted model (**Table 3**, model 2), with a more prominent a risk reduction for tonsillectomy performed more than 20 years before PD diagnosis, but the associations were not statistically significant (**Table 3**, model 2). Similar results were observed when restricted to PD defined through primary diagnosis (**Table S2**).

DISCUSSION

In this nationwide nested case-control study, we found a lower PD risk in relation to appendectomy and a non-significant trend toward lower PD risk in relation to tonsillectomy. The inverse associations were generally stronger within 20 years after surgery but remained statistically significant more than 20 years post-surgery. The associations were similar in men and women, but stronger after age 60 compared to before.

According to Braak's hypothesis, alpha-synuclein, the hallmark for PD pathology, may originate in the gut and later migrate to the brain via the vagus nerve (1). In line with this, deposits of alpha-synuclein have been observed in the entire gastrointestinal tract more than 20 years before PD onset (27). Notably, however, out of the entire gastrointestinal system, mucosal alpha-synuclein was most abundant in the appendix in individuals without neurological disease (28). Alpha-synuclein aggregates were equally abundant in normal and inflamed appendiceal tissue (10). More intriguingly, we and others reported lower PD risk more than 20 years after vagotomy (i.e., a surgical procedure resecting the vagus nerve) (25, 29). This evidence collectively suggests that the appendix may act as a reservoir for alpha-synuclein, and, in line with our results, removing the appendix may be linked to lower risk for PD.

Braak's hypothesis was further extended to the dual-hit hypothesis stating that environmental neurotrophic pathogens may spread to the brain from the nasal gateway as well as from the gut (2, 3). Moreover, there is an increasing recognition of PD as a "prion-like" disease supported by the observations of cell-to-cell α-synuclein transmission in grafted neurons in PD patients (30, 31) and the spread of intragastrical injected α-synuclein from the enteric nerve system to the brain in mice (32). The tonsils and the appendix are important gut-associated lymphoid tissues that together with mesenteric lymph nodes, protect hosts against gastro-intestinal infections (6). In transmissible prion-disease, such as Creutzfeld Jakob disease, prions first accumulate within gut-associated lymphoid tissues such as tonsils and the appendix, and later spread to the brain via the enteric nervous system (6). A similar pattern may be hypothesized for the spreading of alpha-synuclein in PD.

One potential explanation for our observed decreased PD risk related to appendectomy is bias. Residual confounding is one potential bias that needs to be considered. Cigarette smoking has been consistently linked to lower risk of PD (22), and although evidence is scarce, it has also been associated with higher risk of appendicitis (23) and chronic or recurrent tonsillitis (24). As this

was a register-based study, we unfortunately lacked information on cigarette smoking, but we adjusted for COPD as a proxy for smoking. Another potential bias is reverse causation, which can be present if PD patients would be less likely to be diagnosed with or undergo appendectomy compared to the general population, for example due to very advanced PD. Further, PD has a long prodromal phase (33) and there is a delay between onset of PD symptoms to first inpatient register diagnosis of about 7 years (21). Taking advantage of the long follow-up time in our study, we performed temporal analyses that addressed this issue. Even though we found a stronger inverse association during the period shortly before appendectomy compared to the period longer before, we still observed a statistically significant inverse association more than 20 years before appendectomy. If the hypothesis that appendectomy or tonsillectomy protects against PD is true, a dose-response effect might be expected, such that the inverse association would be stronger with longer duration between surgery and PD. We may speculate that one reason that we did not observe such pattern is reverse causation, but we find it unlikely that our results are explained entirely by reverse causation. Another potential bias is incomplete coverage of the Patient Register before it became nationwide. Individuals who lived in counties covered by the register may be more likely to have both surgery and PD diagnoses captured and vice versa, but this would introduce an underestimation of an inverse association, meaning that the true association would be even stronger than the one observed.

Our results are in line with the previous studies that reported lower PD risk after appendectomy (9, 10). The Killinger et al. study (10) was also based on Swedish register data, but we used a different study design and different definitions of appendectomy and PD, resulting in that our study identified more than 3 times as many PD cases with appendectomy. We also adjusted for several co-variates, including COPD as a proxy for smoking, which they did not. Three previous studies reported that appendectomy was not related to PD risk (11, 13, 14) and one reported higher PD risk after appendectomy (12). Potential explanations include differential definitions of PD cases and controls, surveillance bias, which may result in an artificial positive association between PD and surgery, and inadequate length of follow-up precluding exploration of a potential long-term protective effect of appendectomy on PD. Our results for tonsillectomy were consistent with the Danish study that reported 5% non-significant lower risk of PD after tonsillectomy.

The main strengths of this study are the large population-based sample of more than 3.2 million individuals and the long study period between 1964 and 2010, which allowed us to examine the time-dependent relationship between appendectomy, tonsillectomy, and risk of PD. We used a rigorous matching design and adjusted for several covariates to reduce confounding. We performed sensitivity analysis restricted to primary PD diagnosis to test the robustness of the results. The study also has some limitations. First, PD register diagnoses are not perfect and there is a delay between onset of motor symptoms and first inpatient register PD diagnosis (21). However, the potential misclassification of PD is likely to be non-differential with regard to appendectomy or tonsillectomy, which would result in diluted estimates of the associations. Second, due to lack of information in the registers we were not able to control for potential confounding by lifestyle factors and medication use.

In conclusion, our data suggest that appendectomy and potentially also tonsillectomy are associated with a decreased risk of PD. A potential mechanism may be the surgical removal of alpha-synuclein redundancy in these organs. Our data provide additional evidence supporting the importance of the gut-to-brain axis in PD etiology.

DATA AVAILABILITY STATEMENT

The data analyzed in this study were obtained from the Swedish National Board of Health and Welfare and Statistics Sweden and because of Swedish privacy laws we cannot make the data publicly available. Requests to access these datasets should be directed to the Swedish National Board of Health and Welfare and Statistics Sweden after obtaining an ethical approval from a regional ethics review board.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethics Review Board, Stockholm, Sweden. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KW and FF were responsible for study concept, study design and funding. BL performed data management and statistical analysis as well as drafted the manuscript. All authors contributed to interpretation of results and critical revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00510/full#supplementary-material

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Genetic Architecture of Parkinson's Disease in the Indian Population: Harnessing Genetic Diversity to Address Critical Gaps in Parkinson's Disease Research

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Over the past two decades, our understanding of Parkinson's disease (PD) has been gleaned from the discoveries made in familial and/or sporadic forms of PD in the Caucasian population. The transferability and the clinical utility of genetic discoveries to other ethnically diverse populations are unknown. The Indian population has been under-represented in PD research. The Genetic Architecture of PD in India (GAP-India) project aims to develop one of the largest clinical/genomic bio-bank for PD in India. Specifically, GAP-India project aims to: (1) develop a pan-Indian deeply phenotyped

clinical repository of Indian PD patients; (2) perform whole-genome sequencing in 500 PD samples to catalog Indian genetic variability and to develop an Indian PD map for the scientific community; (3) perform a genome-wide association study to identify novel loci for PD and (4) develop a user-friendly web-portal to disseminate results for the scientific community. Our "hub-spoke" model follows an integrative approach to develop a pan-Indian outreach to develop a comprehensive cohort for PD research in India. The alignment of standard operating procedures for recruiting patients and collecting biospecimens with international standards ensures harmonization of data/bio-specimen collection at the beginning and also ensures stringent quality control parameters for sample processing. Data sharing and protection policies follow the guidelines established by local and national authorities.We are currently in the recruitment phase targeting recruitment of 10,200 PD patients and 10,200 healthy volunteers by the end of 2020. GAP-India project after its completion will fill a critical gap that exists in PD research and will contribute a comprehensive genetic catalog of the Indian PD population to identify novel targets for PD.

Keywords: Parkinson's disease, genetic diversity, genome-wide association study, common genetic variation, biobank

BACKGROUND

Parkinson's disease (PD) is the second most common neurodegenerative disorder in adults over the age of 60 years (1). According to the Global Burden of Disease study (2018), the worldwide burden of PD has more than doubled over the past two decades from 2.5 million patients in 1990–6.1 million patients in 2016 (2). India is home to nearly 0.58 million persons living with PD as estimated in 2016, with an expected major increase in prevalence in the coming years (2). Despite the large number of people affected with PD, insights into the underlying genetic and environmental risk factors specific to the Indian population are limited. This is in contrast to the Caucasian population in whom easy access to the patient cohort and the population homogeneity have driven initial large scale genome-wide studies (3, 4). Despite the success, the constraints of performing studies in a single homogenous population became apparent as well. This is because the Caucasian population contains only a subset of genetic diversity (5). Populations vary in terms of allele frequency, linkage disequilibrium (LD) patterns, and differences in effect estimates. This provides a scientific rationale that no single population is sufficient to fully uncover the variants underlying disease in all populations, and makes it imperative to pursue genetic research in diverse populations to capture the genetic diversity of a disease.

About 5–10% of PD is monogenic and inherited in an autosomal dominant or recessive manner. The large majority of patients have a sporadic disease. To date, 90 PD loci have been identified explaining a missing heritability in a range of 16–36% (3). It is also increasingly recognized that additional loci with varying degrees of minor allele frequency and effect size remain to be discovered which might account for the remaining missing heritability. Most of the PD loci have been identified in cohorts that are heavily biased toward persons with Caucasian ancestry (3, 4). This generates issues of reproducibility in a global context. For instance, variants in leucine-rich repeat kinase 2 (*LRRK2*), glucocerebrosidase (*GBA*), and alpha-synuclein (*SNCA*) genes identified in the western population have been shown to pose negligible risk to the Indian patients (6–9). Novel variants in the known genes or novel genes may be associated with PD risk in the genetically more diverse Indian population (10). Variations in allele frequency in genetically heterogeneous populations may provide adequate power to GWA studies with smaller sample sizes for the enriched loci. For example, the discovery of an association at a new putative locus at chr1 (PARK16) in the Japanese population for PD underscores the need to study ethnically diverse populations. The associated SNP, rs823128, which was shown to be protective against the development of PD specifically in the Asian population has a minor allele frequency ∼20% in the Japanese population as compared to only 3% in the Caucasian population (11). With this minor allele frequency, individual GWAS in the Caucasian populations had very little power to detect an association, even though the SNP was well-tagged with arrays. The 1,000 Genomes project which uses the combinatorial approach of exome and whole-genome sequencing suggests that individuals from different populations carry different profiles of rare and common variants and that low-frequency variants show substantial geographic differentiation, thus arguing in favor of diversifying genetic research especially in populations which have so far been underrepresented in gene mapping such as the Indian population (12). In addition to the potential for new gene discovery, the inclusion of ethnically diverse cohorts provides an opportunity to cross-validate newly identified loci, which has direct implications for the global applicability and scalability of potential novel therapeutic targets.

We initiated the Genetic Architecture of Parkinson disease in India (GAP-India) project to provide for the first time, a large-scale genetic catalog of the Indian PD population. This paper describes the design of the GAP-India project including the study sites, subject recruitment, clinical assessments, biospecimens processing, plan for data analysis and sharing, capacity building, and the ethical and regulatory frameworks within which we operate.

STUDY DESIGN

GAP-Indiastudy aims to understand the genetic architecture of PD in the Indian population through large scale sample collection and federated data analysis models. The study aims to collect pan-Indian genetic and phenotypic data and will develop one of the largest clinico-genomic PD resources for the scientific community from India. To achieve our objectives, we have formed a trilateral consortium, the Luxembourg-German-Indian Alliance on Neurodegenerative diseases and Therapeutics (Lux-GIANT) (**Figure 1**). The "knowledge-sharing" model aims to build capacity and exchange programs to integrate clinical/genetic centers and harmonize data collection with Luxembourg and German centers. Lux-GIANT follows a decentralized model and based on expertise, different cores have been created (**Figure 2**). For example, the central clinical core in India is established at the Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, Kerala. Given the diversity and vastness of India, apart from the central clinical core, three high volume academic movement disorders centers across India have been identified and established as nodal centers to recruit participants with a pan-India representation. These four clinical nodes are further connected to twenty clinical sub-centers which are spread throughout India. The central clinical core is responsible for coordinating patient recruitment and biospecimen collection with nodal centers. The nodal centers supervise the patient recruitment and biospecimen collection at the sub-centers. Similarly, genomics, functional and bioinformatics cores have been established in Luxembourg, Germany, and India. The functional core at the National Brain Research Center, Manesar, India, is mandated to develop the Lux-GIANT iPSCs biobank. The functional core in India coordinates its activities with the functional cores in Germany and Luxembourg. This has been done to share protocols and develop common functional protocols to perform functional studies. The array processing will be done at the Center for Cellular and Molecular Biology (CCMB), Hyderabad. The central genomics/bioinformatics core at Tubingen will coordinate data generation with the local center and subset of samples will be processed in Germany for quality control purposes.

PROJECTED COHORT AND STUDY SITES

The GAP-India study revolves around a network of clinical sites in India organized in a "hub and spoke" manner. Patient recruitment at each nodal or sub-center will be supervised by a neurologist with specific expertise in Movement Disorders. Subjects will be enrolled at all the sub-centers and the four nodal centers (SCTIMST, Trivandrum; All India Institute of Medical Sciences, New Delhi; National Institute for Mental Health and Neurosciences, Bengaluru and Nizams Institute of Medical Sciences, Hyderabad). The nodal centers are all high-volume academic centers with established movement disorders programs. The sub-centers include additional public sector teaching hospitals, larger multispecialty hospitals, and neurology clinics in the private sector.

Genetic evidence indicates that most Indians descended from a mixture of two divergent populations: Ancestral North Indians (related to Central Asians, Middle Easterners, and Europeans) and Ancestral South Indians (not closely related to other genetic groups) and almost all the current inhabitants are admixtures of these two broad groups to varying extents (13). Within the population, allele frequency changes between subgroups are larger than in European populations, owing to founder effects maintained by a transition to endogamy about 1,900–4,200 years ago (14). The 1,000 genomes project contains about 500 genomes from the Indian subcontinent (including India and geographically neighboring countries), from five diverse linguistic groups, yet the Ancestral North Indian component is prominent in this dataset (15). Within the linguistic groups too, population substructures were evident suggesting that careful matching of cases and controls from within the same ethno-linguistic groups is necessary to avoid false positive associations.

Geographical locations of the enrolling sites in India were chosen to consider this unique population structure and to enable a pan-Indian representation (**Supplementary Figure 1**). The study aims to enroll 10,200 PD patients and 10,200 healthy volunteers over 1 year. Furthermore, GAP-India aims to develop a cohort of 25,000 cases and 25,000 controls by 2024. The four nodal centers will directly enroll about 6,000 patients and the remaining subjects will be enrolled at the sub-centers. The sample size was chosen to take into consideration the statistical power to detect a risk associated variant in GWAS as well as the feasibility of attaining it within the timeframe of the project. The extensive multi-centric nature of the project helps in covering diverse genetic subgroups and meeting recruitment goals within the timelines.

SUBJECT RECRUITMENT

Subjects will be recruited from the Movement Disorder clinics or Neurology clinics run by the PIs of nodal and sub-centers. A detailed history and systemic and general neurological examination will be performed in all subjects. Research staff at all recruiting centers will be trained in the standard operating procedures including clinical assessments and familiarized with online data entry systems before site initiation. Subjects who meet all the following inclusion criteria will be recruited in the patient group: (1) clinical diagnosis of PD as per United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) diagnostic criteria (16), (2) age more than 18 years and (3) Asian Indian ethnicity. Subjects meeting any of the following criteria will be

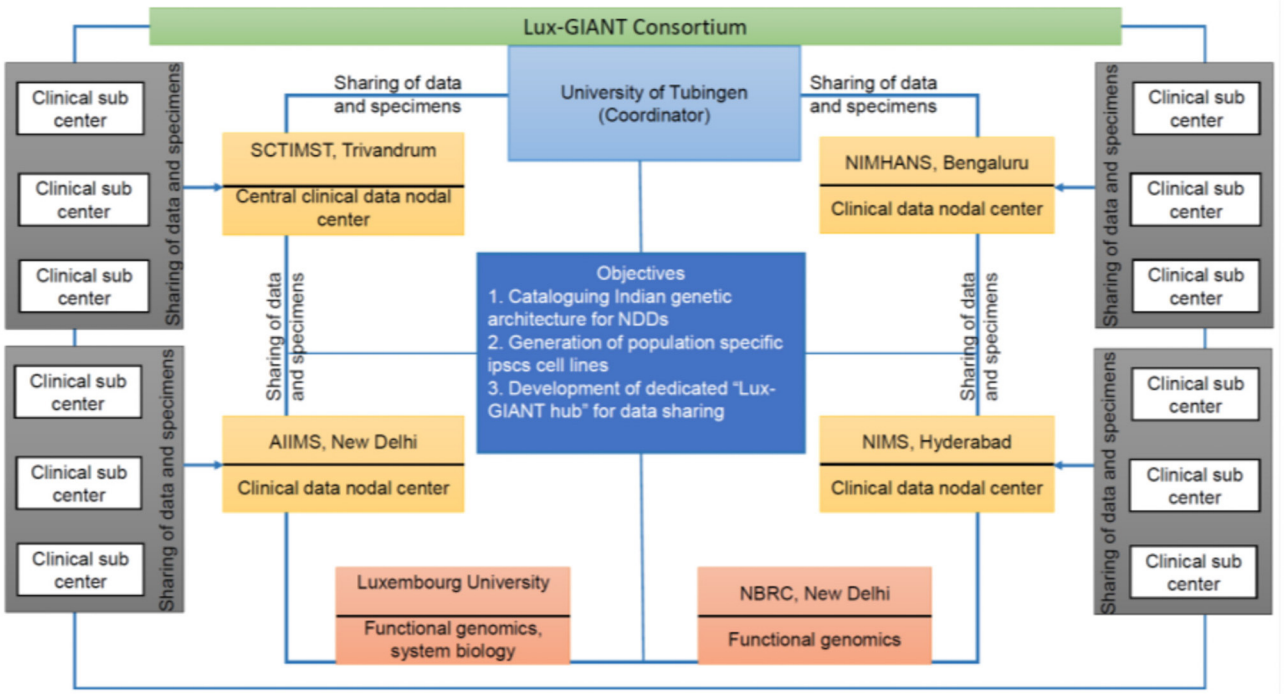

FIGURE 1 | Overall flow chart describing the details of the Lux-GIANT consortium. The consortium follows the "hub-spoke" model. The Lux-GIANT has established three main cores: genomics, clinical, and functional genomics. The University of Tubingen is the coordinator site. Luxembourg site aims to strengthen functional genomics and system biology. Functional core from India, National Brain Research Center, will be responsible for developing and maintaining iPSCs. Four main clinical-nodes capturing most of India are formed. These four nodes are connected to clinical sub-centers which span throughout India. The "central clinical node" aims to streamline the administrative process, which is required for clearance and sample shipment. The different nodes within India are connected with the main "central clinical node" for continuous update of cohort and members.

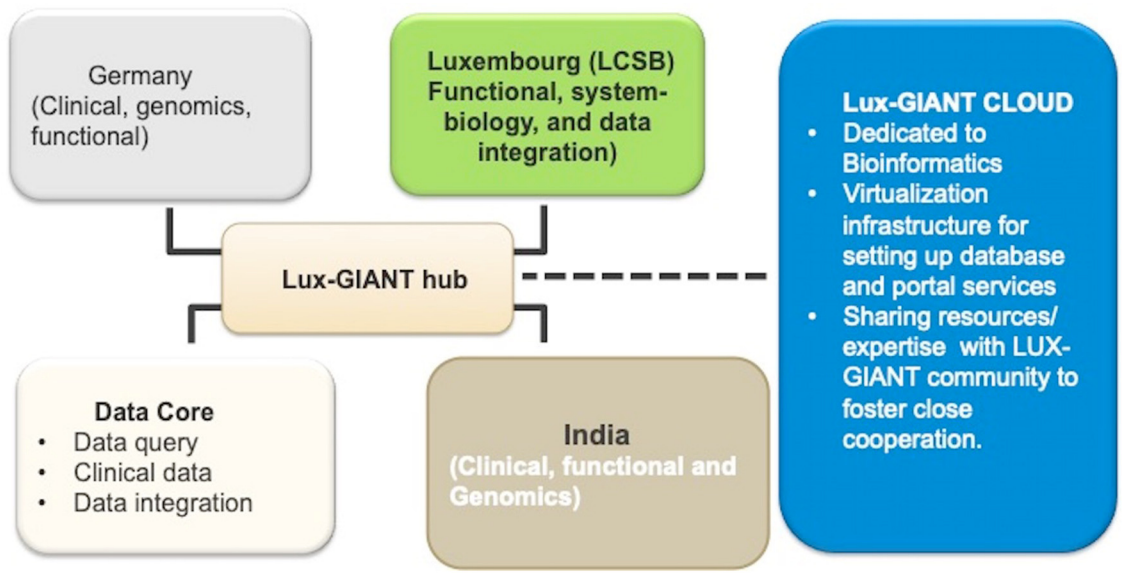

FIGURE 2 | Clinical, genomics, bioinformatics, and functional cores in the Lux-GIANT consortium.

excluded: (1) cognitive or psychiatric dysfunction sufficiently severe enough to impair the patient's ability to provide informed written consent (2) red flags or additional neurological signs raising suspicion of atypical Parkinsonism. Patients who have previously undergone surgical procedures such as pallidotomy, thalamotomy, or Deep Brain Stimulation will not be excluded. Healthy volunteers will be recruited through advertisements displayed on the hospital campus. They will be gender-matched and should belong to the same geographic–ethnic background as the patients. A detailed history and standard neurological examination will be performed before inclusion as controls. Volunteers with a family history of PD or other neurodegenerative diseases will be excluded from the control group. All subjects will be recruited after obtaining written informed consent and with the approval of the Institutional Ethics Committee. Centralized monitoring of recruitment rates and fidelity to operating procedures will be done by the clinical nodal center, SCTIMST.

CLINICAL ASSESSMENTS AND BIOSPECIMEN COLLECTION

Trained personnel at each clinical site will collect clinical and demographic data. The demographic data collected includes information on the geographical origin within India. Structured questions will capture information related to environmental exposures known to be associated with PD including pesticides, fungicides, insecticides, and other chemicals, smoking, caffeine, and head injury. Patients will be asked to report if they ever held a job requiring exposure to pesticides, herbicides, fungicides, insecticides, rodenticides, and fumigants and whether they were exposed to these chemicals at their place of residence through self-use or via another person. Lifetime smoking history of 100 or more cigarettes will be documented. History of head injury or concussion including falls, sporting activities, violence, and car or other accidents in childhood or adulthood will be queried. Patients will also be asked about exposure to caffeinated coffee in quantities more than once per week for 6 months or longer. Years of education will be documented. Furthermore, our environmental exposure data collection will align with the environmental questionnaire from the Genetic-Epidemiology of Parkinson disease (GEoPD) consortium to harmonize the dataset across the ongoing studies. A structured history and clinical examination will be conducted to collect data regarding onset symptoms, motor fluctuations, and dyskinesias, medications, and non-motor symptoms. Non-motor symptoms included in the interview are cognitive impairment, psychosis, depression, sweating abnormalities, seborrhea, sleep disorders including REM behavioral disorder, restless legs, hyposmia, orthostatic hypotension, constipation, dysphagia, and urinary/fecal incontinence. Data from Computerized Tomography (CT), Magnetic Resonance Imaging (MRI), Dopamine Active Transporter- Single Photon Emission Computed Tomography (DaT SPECT) will be collected if available. The family history will be probed for consanguinity and to identify any known relations with PD, dementia, tremor, or other neurological disorders. For patients who have undergone functional neurosurgery, the target, time since surgery and other details will be collected. The motor symptoms at the time of recruitment will be assessed by the Unified Parkinson's Disease Rating Scale (UPDRS Parts I- IV) (17). Subjects will be screened for cognitive dysfunction using the Montreal Cognitive Assessment (MoCA) and for depression using the Beck's Depression Inventory (BDI- II) (18, 19). Validated regional language versions of MoCA will be used for non-English speaking subjects. Demographic and risk factor information will be collected from the control group. Clinical terminologies have been standardized to enable data harmonization with existing research groups and also build a phenotypic information resource for this particular population of PD patients.

The whole blood samples will be collected at each recruiting center 10–15 ml of blood samples collected in EDTA tubes will be processed for DNA extraction using the *salting-out* method. The quantity and quality of DNA will be analyzed using a micro-volume UV/visible spectrophotometer (Nanodrop, Thermo Fischer). Additionally, the quality will also be checked by agarose gel electrophoresis. Those samples with an $A_{260/280}$ ratio of 1.8–2.0 and $A_{260/230}$ ratio of >2.0 will be stored in 1.2 ml screwcap cryovials barcoded with a unique sample ID at SCTIMST Biobank (−80°C), in aliquots. The samples received from the nodal centers and sub-centers will be again checked (using Nanodrop and agarose gel) at SCTIMST to ensure the quality and quantity of DNA that is required for the genotyping. For sequencing, 50 μl of 50–100 ng/μl DNA will be transported in 96-well microtiter plates sealed with peelable heat seals in a waterproof container and dry ice. At the time of collection, specimens will be de-identified by avoiding any personal identifiers on the label. Specimen labels and data collection instruments will be labeled by center-specific serial numbering. No direct personal identifiers will be stored in the online data capture system and quasi-identifiers like date of birth are flagged as such and de-identified by the system before export. Only the site PIs hold identifying information if required for re-identification at a later stage. The central clinical node and other investigators with access to the online database will not have access to direct personal identifiers. Biospecimens collected at the sub-centers will be transported to the nodal centers for DNA isolation. All biospecimens are finally routed to the clinical core at SCTIMST, Trivandrum for storage and in consenting subjects, longer-term bio-banking. In keeping with the existing regulatory framework in India, to promote capacity development, array processing and genotyping will be done at the Center for Cellular and Molecular Biology, Hyderabad. The genetic core at Tübingen will perform the bioinformatics analysis and a subset of specimens will be processed at the Lux-GIANT genotyping core facility in Munich for quality control purposes. All clinical and genetic data will be stored on a shared electronic platform with access restrictions and security protocols in place. Functional validation of putative pathogenic variants including patient-specific induced pluripotent stem cell modeling will be done at the National Brain Research Center, Gurgaon, India. In this way, the study is designed to comprehensively capture clinical and genetic information from a large Indian cohort in

a manner that enables integration with existing international cohorts. DNA isolation from whole blood will be done at the four nodal centers and centralized quality control monitoring at SCTIMST, Trivandrum. DNA specimens from consenting subjects will be maintained in a biorepository at SCTIMST for potential future research.

DATA ANALYSIS

For genetic analysis, a two-stage design will be followed. Currently, the arrays available for genotyping lack in-depth genetic variability information from the Indian population. GAP-India aims to address this issue by performing whole-genome sequencing (WGS) of around 500 subjects covering the north, south, east, and west of India.

Whole-Genome Sequencing

The data generated from whole-genome sequencing will be analyzed using the megSAP pipeline (https://github.com/imgag/megSAP) developed at the Institute of Medical Genetics and Applied Genomics, University Hospital of Tübingen (Tübingen, Germany). In brief, SeqPurge (v. 2020_03) will be used for adapter and quality trimming (20), Burrow-Wheeler Aligner mem (BWA mem (v.0.7.17) for read mapping (21), samblaster (v. 0.1.24) for duplicate removal (22), ABRA2 (v. 2.22) for indel-realignment (23), freebayes (v. 1.2.0) for calling of small variants (24), ClinCNV (v. 1.16.1) for CNV calling (25), Manta (v. 1.6.0) for structural variant calling (26), and Ensembl VEP (v. 96.3) for variant annotation (27). Furthermore, additional tools available from the ngs-bits toolset (https://github.com/imgag/ngs-bits) will be used for data cleaning.

GWAS Analysis

Study Population and Genotyping

The analysis cohort will represent ∼10,200 PD cases and 10,200 controls of Indian ancestry, genotyped with Illumina's Global Diversity Array (GDA) containing neurodegenerative specific content.

Quality Control

GenomeStudio will be used to cluster the genotyping array using the GenCall algorithm and preliminary QC will be implemented in GenomeStudio as described elsewhere (28). Data will be exported in the standard PLINK format and downstream QC procedures and statistical analysis will be conducted using the latest PLINK (http://pngu.mgh.harvard.edu/_purcell/plink) and R software packages (http://www.r-project.org/), installed on a Linux based computation resource (29). The post-GenomeStudio QC will be broadly divided into three main steps comprising of (i) Sample and genetic marker quality (ii) Population structure (iii) Genotyping consistency. Furthermore, QC will be implemented independently in each Indian subpopulation covering north, south, east, and west of India.

Sample and Genetic Marker Quality

Firstly, all samples and SNPs with missing rate>1% will be excluded. Concerning genetic marker quality, we would exclude SNPs with MAF<0.01 and HWE p-value $<1 \times 10^{-10}$ in cases as well as HWE $<1 \times 10^{-6}$ in controls (30). Allele frequencies will be checked with Indian sub-populations represented in the Haplotype Reference Consortium (HRC). Furthermore, allele frequency consistency across different batches of genotyping datasets will be checked to rule out the batch effect.

Population Structure

Individuals deviating ±3 SD from the samples' heterozygosity rate mean will be excluded. Only those males will be included which have an X chromosome homozygosity estimate of more than 0.8. On the other hand, only those females will be included which have an X chromosome homozygosity estimate of less than 0.2. Related samples will be filtered based on identity by descent (IBD) coefficient>0.1 (31). Principal component analysis (PCA) will be used to detect population outliers using the first ten principal components and the outlier samples will be removed. We identified five populations representing the Indian subcontinent in phase 3 1,000 Genomes Project (KGP): two from the northwestern region [Gujarati Indian in Houston, TX (GIH) and Punjabi in Lahore, Pakistan (PJL)], two from Southern region [Indian Telugu in the UK (ITU) and Sri Lankan Tamil in the UK (STU)] and one from Eastern region [Bengali in Bangladesh (BEB)]. The five Indian subcontinent populations marked as South Asain population in the PCA plot of the worldwide population showed a clear demarcation emphasizing the need to diversify the genomic research in under-represented populations to identify population-specific novel genetic loci for complex diseases (**Figure 3**).

Imputation

Imputation will be carried out using the Haplotype reference consortium (HRC) as a reference panel consisting of individuals from more than 26 worldwide populations (32). The SNPs with imputation info score of less than 0.7 will be discarded.

Association Analysis

Post-QC and imputation, association analysis will be conducted for each sub-population using binary logistic regression analysis assuming an additive genetic model adjusting for age, sex, and relevant principal components. A conventional genome-wide significance threshold of 5 × 10–8 will be used to identify the significant SNPs. The fixed meta-analyses inverse-variance weighting of log-ORs will be implemented in METAL to combine summary statistics across all the Indian sub-populations (33). Genome inflation factor λ will be computed using the median χ^2-statistics. Lastly, Manhattan and QQ plots will be constructed to visualize the results. All the summary statistics will be made available publicly. Heterogeneity in allelic effect sizes between different Indian sub-populations contributing to the meta-analysis will be assessed using Cochran's Q statistic.

Polygenic Prediction

We will further use genome-wide complex trait analysis (GCTA) to perform conditional and joint analysis to identify the top variants that account for heritable variation among different loci

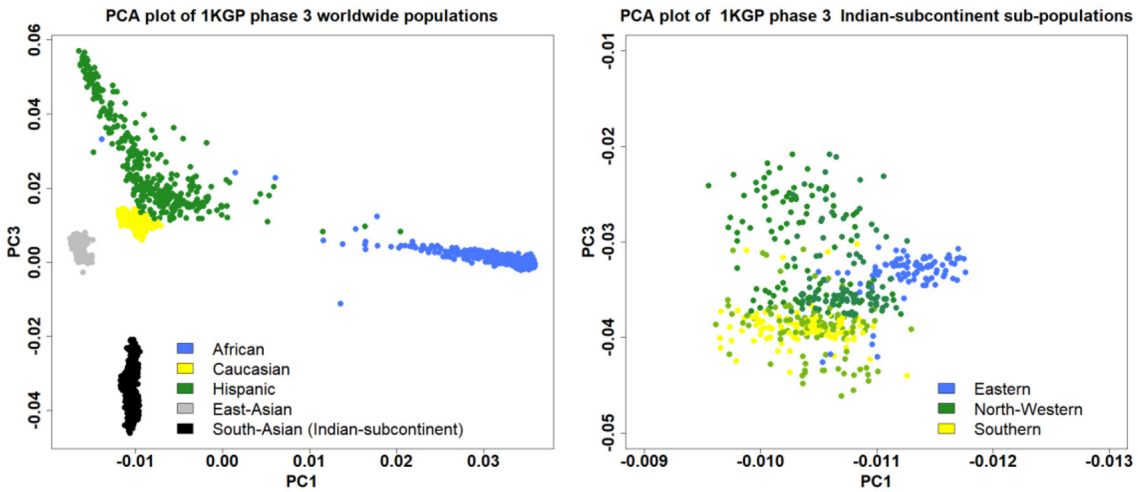

FIGURE 3 | The PCA plot using the phase 3 1,000 Genomes Project showing the distribution of the South Asian population among the worldwide populations (left), and sub-populations from different regions of the Indian subcontinent (right).

(34). Polygenic risk score profiling will be done in a standard weighted allele dose manner (35).

Biological Annotation

We will further integrate our GWAS summary statistics with expression and network data using Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA) to perform the tissue specificity and pathway enrichment analysis (36).

The genomics/bioinformatics core in Tubingen will be responsible for data integration and analysis.

Data Management and Sharing

To ensure seamless data exchange among Lux-GIANT partners, we have established a secure data management and analysis platform. At this moment this platform is equipped with REDCap, a state-of-the-art EDC (Electronic Data Capturing) system (https://www.project-redcap.org) widely used in various clinical and translational projects and is hosted at the "clinical core" site, SCTIMST. The Lux-GIANT REDCap instance is aligned with the Genetic Epidemiology of Parkinson disease (GEoPD) consortium minimal dataset Case Report Forms (CRFs). This will ensure uniform clinical data collection across various participating countries in GEoPD that are spread across five continents. This will facilitate cross-study data pooling and analysis for future multinational projects. All Indian nodal centers and sub-centers taking part in this study are collecting pseudonymized data into this secure and access-controlled instance centrally. All the identifiable information of each study participant stored separately at each site in a corresponding hospital system and only authorized clinical people from that site to have access to it. This setup is aligned with European general data protection regulations (GDPR) as well as Personal Data Protection Bill (PDP) 2019. This pseudonymized clinical data, as well as the corresponding molecular data, will be served to all Lux-GAINT partners via the "data core" site established at Tubingen by leveraging the infrastructure established by the German Network for Bioinformatics and Infrastructure (de.NBI, https://cloud.denbi.de). Through the dashboard of the de.NBI Cloud Site Tubingen the allocation of the desired resources (number of virtual CPU cores, number of virtual machines (VMs), amount of storage and RAM) will be covered. Furthermore, to provide secure access to the VMs to researchers, so-called security groups will be created, which can be seen as a VM specific firewall to control incoming and outgoing network traffic connections. To provide an additional layer of security to Lux-GIANT genomics data, a private network will be added to the Lux-GIANT cloud project to protect the network traffic from and to the volume where the genomics data reside. In addition to the network traffic protection, a certificate-based approach will be used to grant specific permissions (read, write) on a per-user base. The certificates will be distributed to the particular person using a state-of-the-art asymmetric encryption technique. The whole process starting with the project application is illustrated in **Figure 4**. For further details, please see (37). The system-infrastructure, as created, will provide a secure environment to handle and process sensitive patient data in a restrictive and responsible way using cloud resources. To complement our cloud-based activities, the "data integration" site, ELIXIR-Luxembourg Node (ELIXIR-LU) will FAIRify this data by making them Findable, Accessible, Interoperable, and Reusable (38). All the meta-data will be shared through the ELIXIR data catalog (https://datacatalog.elixir-luxembourg.org) that facilitates the Findability of the data. Both clinical and associated molecular data will be curated, harmonized, and integrated into a discovery analytics system—Ada (https://ada-discovery.github.io). It will be hosted in the de.NBI cloud Tubingen and will facilitate the data exploration and analysis through intuitive web interface rich with dynamic visual analytics and advanced machine learning (Deep Learning).

GAP-India aims to share data at the end of a 2-year embargo period, consistent with guidelines followed by other consortia's

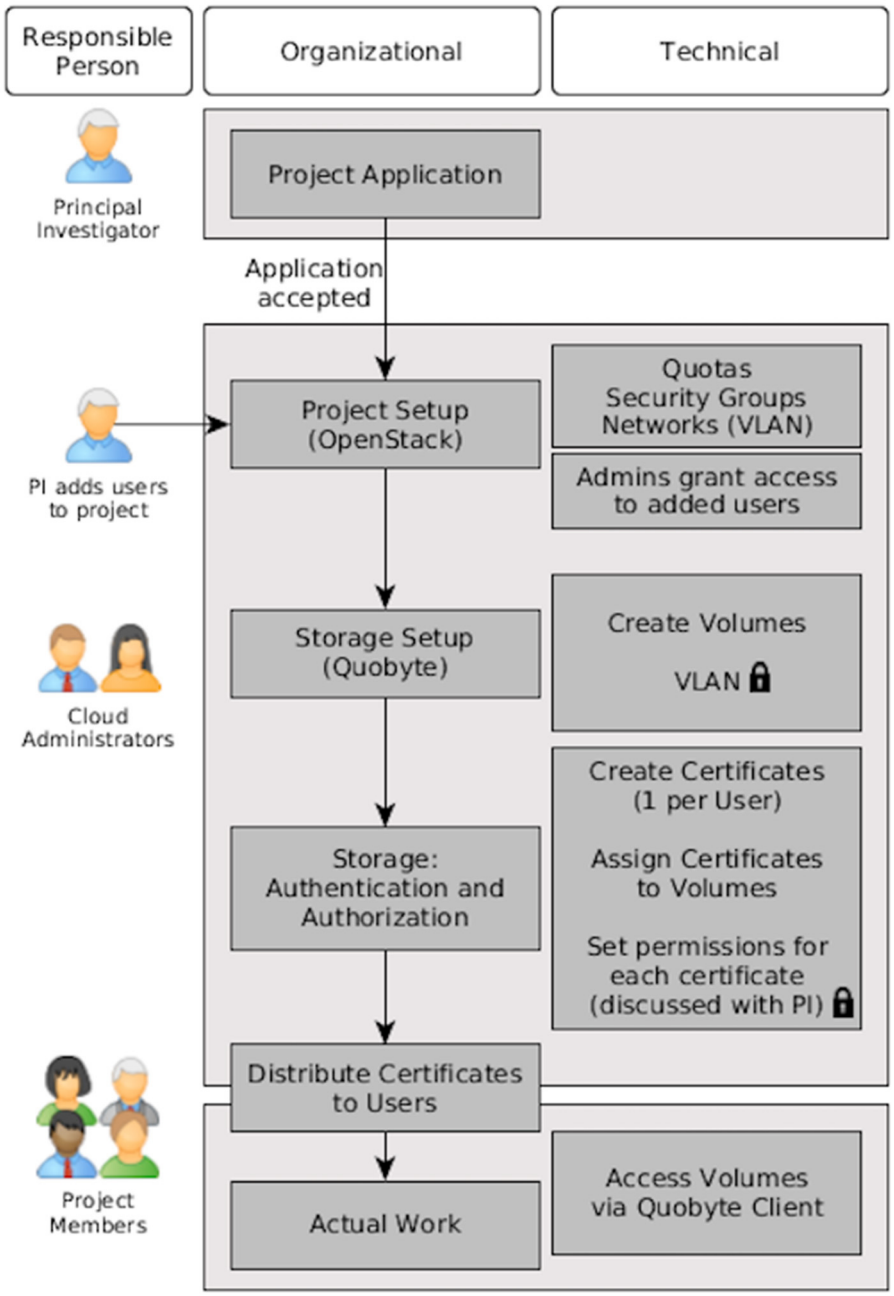

FIGURE 4 | Data management and access workflows in the Lux-GIANT network.

such as H3AfricaConsortium. The purpose of the 2-year embargo period is to give Lux-GIANT researchers a reasonable time-frame to analyze and publish their data before others do. The GAP-India project aims to develop an extensive data sharing plan designed to maximize the utility of its data for the scientific community. Lux-GIANT cloud portal through which GAP-India data will share data fall into two categories: (i) controlled access, and (ii) open access.

The controlled access via the Lux-GIANT portal hosted on the de.NBI Cloud will be given to researchers/institutes who will comply with the data protection and ethical regulations, as described in the GDPR, and PDP 2019. The open-access data which does not require prior ethical clearance will be made available to the scientific community either via the Lux-GIANT Portal or PDgene database.

Regulatory and Ethical Framework

GAP-India project aims to address two main issues: (1) To generate the most comprehensive PD genome-phenome catalog, including iPSC biobank of the Indian PD population, and (2)

to develop scientific and infrastructure capacities in India which have so far lagged in PD genomics research.

One of the major reasons that hinder the collaboration in the genomic era between various research consortia which are primarily led by institutions either in the USA and/or Europe and under-represented population such as India were concerns that data generated from the under-represented population will not be properly represented by the local stakeholders. GAP-India aims to dispel this notion of "scientific imperialism" by developing the "knowledge-sharing" model and also establishing the guidelines which adequately protect the interests of local investigators as well.

The data generated from the GAP-India project follows the strict ethical guidelines, as stipulated by the Indian Medical Council of Research (ICMR)- HMSC for international collaborative research and follows the provisions of ICMR guidelines for biomedical research in India (39). All the clinical recruiting sites obtain ethical approval from their specific ethics committee according to local protocols.

The GAP-India project involves multi-centers across India. There exists a considerable disparity in access to and protocols for regular health care among patients. Therefore, various ethical considerations have arisen during the development stage of the GAP-India project. Specifically, the following issues have been considered. (1) return of genetic results generated from the study, and how they will be received; (2) providing information about genetic findings to patients and care-providers; (3) concerns about stigmatization; and (4) ensuring equity and fairness in collaboration.

As per the Indian guidelines, we are mandated to return actionable results, with the potential to improve the health outcome of the participants. For this, a re-identification process will be followed through the PI of the recruiting center. Genetic counseling and guidance will be offered in case of such a return of results. Incidental findings that are not actionable will not be returned (39).

One of the major spin-offs from this study will create a core network of clinicians and researchers dedicated to PD genetics in India. A long-term biorepository and capacity building in terms of infrastructure and skill upgradation are additional advantages. Taken together, GAP-India aims to develop a dedicated pool of researchers and health care professionals to raise PD awareness in India.

The GAP-India study and the LUX-GIANT network aim to address a critical gap in knowledge regarding the genetic origins of PD, by leveraging the population diversity afforded by as a yet unaddressed population. We expect to generate novel data that may drive targeted therapies and make them applicable on a global scale.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committees of all participating clinical centers. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RR conceived, designed, organized and wrote the first draft, reviewed, and critically revised the manuscript. KD, RMK, and RY conceived the project, reviewed, and critically revised the manuscript. VS, UM, PA, NK, TF, HK, AS, KS, SM, SD, SK, LP, MB, PW, SR, MH, GW, and SP reviewed and critically revised the manuscript. FB, MH, JK, AK-S, SG, PL, MSt, and JR reviewed, and critically revised the manuscript. VB, GC, JS, PS, TG, OR, and VG reviewed and critically revised the manuscript. PP, RK, and RB organized the research project, and reviewed and critically revised the manuscript. KB, NC, RC, CT, MD, CG, HM, NK, SK, PM, CS, AKS, and DW reviewed and critically revised the manuscript. AK and MSh conceived, organized, and executed the research project; designed, executed, reviewed, and wrote the first draft; and reviewed and critically revised the manuscript.

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Parkinson's Disease Research on the African Continent: Obstacles and Opportunities

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The burden of Parkinson's disease (PD) is becoming increasingly important in the context of an aging African population. Although PD has been extensively investigated with respect to its environmental and genetic etiology in various populations across the globe, studies on the African continent remain limited. In this Perspective article, we review some of the obstacles that are limiting research and creating barriers for future studies. We summarize what research is being done in four sub-Saharan countries and what the key elements are that are needed to take research to the next level. We note that there is large variation in neurological and genetic research capacity across the continent, and many opportunities for unexplored areas in African PD research. Only a handful of countries possess appropriate infrastructure and personnel, whereas the majority have yet to develop such capacity. Resource-constrained environments strongly determines the possibilities of performing research locally, and unidirectional export of biological samples and genetic data remains a concern. Local-regional partnerships, in collaboration with global PD consortia, should form an ethically appropriate solution, which will lead to a reduction in inequality and promote capacity building on the African continent.

Keywords: Parkinson's disease, Africa, public health, awareness, epidemiology, genetics

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INTRODUCTION

Mirroring global trends, life expectancy on the African continent has greatly increased in recent decades, paralleling economic growth, and related to a decline in a number of infectious diseases. The World Health Organization reports that overall life expectancy at birth in Africa is currently 61.2 years (1). As a result of improved control of the HIV epidemic, malaria and diarrhoeal diseases, non-communicable disorders (NCD) have become increasingly important as a public health concern for Africa (2), which is a global pattern observed initially in higher income countries. The common movement disorder, Parkinson's disease (PD), is one example of an important neurological NCD in the aging African population.

Exploring the epidemiology and genetic etiology of NCDs is essential in order to dissect out patterns of disease susceptibility, environmental clustering, and medication responses at a population level, as well as on an individual basis. In this Perspective article, we provide an overview of the main difficulties that we consider to be hindering the progress of PD

epidemiological and genetic research on the African continent, and the solutions needed. We also provide summaries of the healthcare infrastructure in four African countries [represented by the four neurologists listed as authors; Tanzania (MD), Nigeria (MK), Mali (TC), and South Africa (JC)], and of the research done in these countries to illustrate the local obstacles but also the potential global opportunities for the field.

OBSTACLES TO PD RESEARCH ON THE AFRICAN CONTINENT

Limited Number of Neurologists

Epidemiological patterns are heavily dependent on the power of detection of disease, which is an interplay of diagnostic factors as well as the accuracy of determining the correct diagnosis in the general population. In the case of PD, the first and foremost factor in this is the availability of neurologists. On the African continent, there is a wide discrepancy in the number of neurologists and medical facilities between different countries.

Some urban centers may have comprehensive neurological and auxiliary services available, consisting of neurologists, neurosurgeons, neurophysiologists, and related equipment (such as electro-encephalography, electromyography, and nerve conduction studies). However, even in these centers, accessibility to such services by the general population is limited due to financial restrictions and barriers related to cultural perception of disease. Reviewing the situation in Africa as a whole, neurological services are either scarce or not available, as illustrated by a survey conducted in 2005 that included 11 countries that were entirely without neurological services (3). Currently, in most countries in Africa, there is still a dearth of neurologists, nurses, physiotherapists, and other allied professions due to limited training facilities for neurologists within Africa, as well as emigration of skilled personnel to more economically developed countries. Although, some research on the clinical and epidemiological aspect of PD has been conducted, genetics research of PD is limited in Africa, as a result of poor awareness and lack of facilities. The low number of scientific publications on PD mirrors the low density of neurological professionals (**Figure 1**). As can be seen in the figure, the Northern African Arabic countries bordering the Mediterranean Sea and South Africa at the tip of the continent are the two regions with better access to neurological surveillance and care than the remainder of Africa.

Public Health Education and PD

A potential obstacle for access to neurological care may be preconceived beliefs that exist among the community about neurological conditions. Erroneous beliefs may arise from the absence of knowledge and education regarding a particular disorder, as well as culturally determined perceptions. Public health education is therefore of considerable importance. Absence of such education, or the cultural inappropriateness of educational content (for instance by direct translation of information leaflets and videos from other global regions) might lead not only to ongoing lack of recognition of medical disorders but also to missing out on the benefits of effective treatments. If one is not attuned to the specific culturally appropriate requirements of a region, the impression might arise that there is a resistance toward receiving educational information. However, with an approach adjusted to the specific ethnic, geographical, or religious needs of a target population, the same information may be better understood and therefore accepted.

Stigma Associated With PD

In a Northern Tanzanian door-to-door survey in a semi urban setting, it appeared that many people suffering from PD met with various misconceptions about the disorder (4). Similarly, in a study conducted in South Africa, there was lack of knowledge about PD, with half of the members of the community believing that patients with PD should not live within the community (5). Ideas about guilt, witchcraft, and presumed mental disease all attribute to stigmatization. Such factors delay or prevent correct diagnosis or access to appropriate treatment for PD. The Tanzanian setting does have access to basic neurological services close to the survey area (6), which highlights the fact that targeting a community's perception of disease is potentially as important as is the improvement of structural facilities such as neurology clinics, laboratory diagnostics, and brain imaging. When educational material for patients and their caregivers is made available by a direct translation of quality material available from websites such as the International Parkinson and Movement Disorder Society (MDS; www.movementdisorders.org), it is expected to correctly reflect currently available evidence-based information. However, whether its contents will actually appeal to groups other than those in high-income regions, is less clear. In addition to appropriate translation, stigma due to superstitious beliefs and misconception (4, 5) also needs to be addressed in the educational material in order address target populations respectfully and effectively.

Consequently, we believe that some of the main challenges for PD research faced on the African continent are:

- Stigma of a visible impairment and the perception that the disease may be caused by a curse or is a bad omen.
- Delay in diagnosis and treatment due to traditional medicine being used as a first step for the majority of patients outside urbanized regions.
- Low rate of healthcare insurance coverage preventing affordability of long-term treatment in chronic disorders such as PD.
- Denial of a positive family history of a possibly genetic condition so as to prevent discredit to individuals or their relatives.

PD RESEARCH IN FOUR AFRICAN COUNTRIES

In this section, we highlight the situation regarding PD research in four countries, Mali, Nigeria, South Africa, and Tanzania to illustrate the obstacles and the opportunities. A summary of the resources and infrastructure currently available for PD studies in each country is provided in **Table 1**. This table clearly shows the severe shortages of suitable resources, infrastructure and

FIGURE 1 | A schematic diagram of African countries indicating the number of published articles on Parkinson's disease per country (source: https://www.ncbi.nlm.nih.gov/pubmed/). The search was conducted in December 2019 and the term "Parkinson" was used in addition to the country name for all countries located on the African continent (Algeria-Zimbabwe); the ensuing results were then reviewed for being appropriate as a publication related to Parkinson's disease. Duration of search extended from 2019 until 1952.

facilities in comparison to developed countries. However, despite this, high quality research has been done in these countries, as indicated below.

Table 1 also shows the problems with treatment strategies for PD in Africa. In this setting, after clinically diagnosing parkinsonism, drug treatment usually starts with a levodopa/carbidopa trial. Dopamine agonists are unavailable in the majority of African countries. Treatment can be called unsuccessful when about one gram of levodopa/carbidopa daily for a number of weeks does not elicit a significant treatment response. Practically however, the high cost of the treatment may necessitate patients to terminate this titration prematurely, or to reduce dosage frequency to once daily or very low dosages. There will be a proportion of patients who would have responded better had there been no financial limitations. Physiotherapy is also a useful treatment modality, but is best given in limited sessions due to long travel distances and low resources. Physiotherapy in lower income regions is aimed at education and low frequency follow up: patients and relatives may attend for a few days consecutively, perform home exercises and return a number of months later.

Tanzania

Tanzania, situated in East Africa, is one of the few countries in Sub-Saharan Africa where door-to-door prevalence data on PD are available from a survey of a semi urban and rural area (7). This survey also examined perception of disease, including that PD is considered to be an age-related phenomenon, which does not require treatment, or that it may be a punishment for having done something wrong (4, 7). The research group who conducted the survey has been funding levodopa therapy for newly diagnosed patients identified from the survey, in addition to following up the patients (8), and also studying physiotherapy interventions (9). A nearby tertiary referral center in Moshi, at the foot

TABLE 1 | Summary of healthcare and clinical resources available for the clinical management of Parkinson's disease in four sub-Saharan African countries.

	Mali	Nigeria	South Africa	Tanzania
Population size (millions)*	20	206	59	59
No. of neurologists**	20; (∼1 per million people)	200; (∼1 per million people)	Private sector 120; State sector 25; (∼2.5 per million people)	8 (∼1 per **7** million people)
No. of movement disorder specialists**	2	40 with special interest in movement disorders	Private sector, 15 with special interest in movement disorders; State sector, 2	None
Top three neurological conditions	Head trauma; stroke; CNS infections	Epilepsy; stroke; degenerative spinal cord disease	Stroke; epilepsy; peripheral neuropathy	Stroke; CNS infections; paraplegia
Prevalence of Parkinson's disease	Unknown	67/100,000 [community-based study; (13)]	Unknown	20/100,000 [community-based study; (7)]
Healthcare infrastructure	Three teaching hospitals; seven regional hospitals	85 tertiary hospitals (teaching hospitals and federal medical centres) of which 75 are public and 10 are private; 3,993 secondary hospitals of which ∼75% are private	400 state hospitals; 200 private hospitals	269 hospitals of which 120 public or parastatal. Of these, seven teaching hospitals are connected to a medical school including four zonal referral hospitals; six additional specialized hospitals.
Medication funding	TB, HIV-AIDS, malaria treatment, and cesarean delivery free; Health insurance rate below 20%, most out of pocket	TB, HIV-AIDS treatment, and vaccination free; Health insurance rate below 10%, most out of pocket	Three payment options i.e., full paying, partially subsidized or free of cost (based on income); <10% of population has health insurance	TB, HIV-AIDS treatment and vaccinations free; Health insurance rate below 10%, most out of pocket
No. of CT/MRI scanners**	20 CT scanners; 4 MRI scanners	100 CT scanners; 50 MRI scanners	265 CT scanners; 150 MRI scanners	22 CT scanners; 8 MRI scanners
No. of PET/DAT scanners**	Unavailable	Unavailable	Six PET scanners; DAT unavailable	One PET scanner; DAT unavailable
Levodopa medication availability	Available only in the capital city and some districts. There is no insurance coverage for L-dopa medication	Subject to global availability, predominantly 25/250 strength; <5% covered by Health Insurance	Widespread availability, predominantly 25/100 and 25/250 strength	Subject to global availability, ∼10% have coverage by healthcare insurance
DBS surgery availability	Unavailable	Unavailable	Available	Unavailable
No. of human geneticists	3	30–50	>200	Unavailable
No. of laboratories with human genetics expertise	Two labs [Neurosciences Department at the Point G Teaching Hospital, Faculty of Medicine, University of Sciences, Techniques and Technologies of Bamako (USTTB)]	30	>50	Two labs (Muhimbili National Hospital and Kilimanjaro Clinical Research Institute)

**Country population size taken from https://www.worldometers.info/population/countries-in-africa-by-population/.*

***Numbers are approximates as surveys have not been done.*

DAT, Dopamine transporter; DBS, Deep brain stimulation; CNS, central nervous system; CT, Computerized tomography; MRI, magnetic resonance imaging; PET, positron emission tomography.

of Mount Kilimanjaro, also has neurologists available for this patient population (6). However, a limited number of patients follow up to obtain levodopa maintenance therapy, illustrating that there are additional, obstacles to care in an African rural population over and above the availability of a neurologist.

A levodopa containing crop, Mucuna Pruriens, is presently being studied for its medicinal properties in Moshi. Its use as monotherapy or add-on medication in PD has proven to be successful in Bolivia and Ghana (10, 11). The crop is being grown, and will be roasted and ground at the hospital premises using readily available facilities since Moshi is known for its coffee industry, which uses the same procedures. The availability of locally sourced medication of this nature may also allow patients with chronic illness to grow their own medicine at home, and titrate it themselves for daily use. In the framework of the above study, an assessment on candidate and pharmacologically relevant genes (e.g., Catechol-*O*-methyltransferase; *COMT*) will be performed. To date, only one genetically confirmed PD kindred is known from the East African region, which was identified in North Tanzania and is due to a homozygous *PRKN* deletion (12).

Nigeria

Nigeria is the most populous and diverse nation in Africa with a growing population estimated to be over 200 million people, and home to many different ethnic groups speaking three major languages and over 250 other languages. Community based studies on the prevalence of PD obtained an age adjusted rate of 67 per 100,000 which is low compared to the frequency observed in African Americans (13). The clinical profile, etiology of Parkinsonism and PD and their complications have been described and are similar to the clinical profile in other regions of the world (14–16). Similarly, there have been studies on the non–motor features of neuropsychiatric impairment (17), cognitive impairment (18, 19), depression (20), gait instability (21), autonomic (22), gastrointestinal (23), and respiratory (24) involvement in PD. Studies to dissect the risk factors and etiology of PD among Nigerians include biochemical and pathological studies. Some authors observed the occurrence of Lewy bodies (25, 26), xenobiotics (27), and risk factors such as manganese among blacksmiths (28) and increased levels of trace metals (29). A few genetic studies have also been conducted and did not detect pathogenic mutations in *PRKN* (parkin), *LRRK*2, and *ATXN3* (30–32).

The challenges to care include low numbers of health care personnel, poor access to care, late presentation, as well as lack of medicine availability (33). However, new technologies, particularly telemedicine, have been identified as a promising area to improve access to care, especially for patients in rural communities (33). Educational campaigns and awareness efforts to tackle misconceptions as well as a multidisciplinary team care approach at the community level are anticipated to improve access and quality of care (34, 35).

Mali

Mali is situated in the midst of the Sahara Desert between North African and sub-Saharan African countries. The demography is diverse and consists of Sub-Saharan ethnic groups living in the southern part of the country (black African origins) and nomadic racial groups (Arabic-Berber origins) living in the northern part of the country (36). The two ethnic groups share similar historic, cultural and religious traditions with each other, and there are high rates of consanguinity. These features are also shared with neighboring countries, namely the North African countries across the Sahara Desert and the sub-Saharan countries in the South. Almost all facilities and health care personnel in Mali are located in a geographic area representing <10% of the country, where only 14% of the population live (37). PD is not regarded as an urgent health priority when compared to the disease burden of infectious diseases and other NCDs. Long-term availability of medication, follow up and patient education are also lacking.

Due to a lack of trained movement disorders specialists and severe constraints in health care infrastructure, only two hospital-based studies of PD have been conducted in Mali. From January 2012 to November 2013, all cases of PD were collected using in-patient and out-patient visit data at Point G Hospital in Bamako, which is the main teaching hospital in Mali. Among the 8,372 patients seen at the Neurology Department, 60 patients (0.7%) had PD (38). Mostly, individuals aged 61–80 years were affected, the frequency of young onset cases was 12.2%, and a positive family history of PD was present in 7.3%. Another study done in 2016 revealed non-motor signs in 90% of all patients with PD (39). To date, there are no published genetic studies on PD patients from Mali.

South Africa

South Africa has been described as a "melting pot," since the country is ethnically diverse due to its history, comprising people from a range of different ancestral backgrounds. South Africa has reasonably well-established healthcare and facilities for clinical management of PD but there are wide discrepancies in facilities between different provinces (largely as a legacy of the apartheid era) and between the urban and rural areas. The PD research group is based in Cape Town and was initiated in 2006. As the country has some of the best resources and infrastructure for human genetics studies on the continent, the focus of the PD research group is to study the genetic etiology of the different ethnic groups by establishing a DNA bank of clinically well-characterized PD patients. Initially, the group concentrated on familial and early onset PD, of all ethnic origins, but more recently, a focus has been on recruitment of South African patients of Black African ancestry. The group has identified pathogenic mutations, albeit at low frequencies, in all of the commonly associated PD genes, as elaborated on in the next section. Recently, a *PTRHD1* mutation was identified in a Xhosa family with Parkinsonism and intellectual disability (40).

GENETICS OF PD IN AFRICAN POPULATIONS

As has been highlighted by many previous reports, genetic studies on African populations have been very limited (35, 41–43). All of the published studies and their findings are summarized in

Supplementary Table 1. On the African continent, most of the work has been done on patients from North African Arabic countries where the frequency of the LRRK2 G2019S mutation was reported to be as high as 41% of patients (44) due to the presence of genetic founder effects. A number of studies have been conducted in South Africa but the mutation detection rate has been low (32, 40, 45–55). The other studies have been done in Nigeria (30–32), Tanzania (12), Zambia (56), and Ghana (57) but for the vast majority of the countries in Africa, no genetic studies have been reported. This is a striking omission since African populations have the oldest genomes and the greatest genetic diversity in the world, and are therefore likely to reveal novel insights into disease mechanisms and pathways underlying PD (58).

Notably, findings conducted on *LRRK2* and in particular, the G2019S mutation, have revealed interesting findings (**Supplementary Table 1**). Although common in North African Arabic populations, this mutation has not been identified in a single individual of Black African ancestry (30, 31, 49, 56, 57). A recent study conducted in South Africa found that 8 out of 647 patients screened were G2019S-carriers but all are of Ashkenazi Jewish origin except one (whose grandfather was German) (49). In a study on African Arabic patients in Tunisia, G2019S-carriers had similar PD symptoms to non-G2019S idiopathic PD cases but had a younger age at onset (AAO), a more benign phenotype and less cognitive impairment (59). In the South African study, the average AAO of the eight G2019S carriers was 56.6 years (SD 10.9), they had typical PD symptoms, and the homozygous mutation carrier did not exhibit a more severe disease to the others, although two patients had severe lower limb dystonia (49). It is plausible that patients of Black ancestry harbor other mutations in *LRRK2* but this would require comprehensive screening of all 51 exons of this gene.

In summary, genetic studies in African populations have the potential to be of great benefit for PD research globally but have largely been unexplored.

SOLUTIONS NEEDED

In order to tackle the major challenges and obstacles to care of PD patients and to facilitate more research on this disorder, we believe the following issues need to be urgently addressed: lack of awareness and wrong perceptions, lack of trained personnel and the unavailability of drugs. To tackle the lack of awareness, awareness campaigns, and culture specific educational materials need to be developed in the local languages. Governments should improve awareness and reduce stigma through the use of radio and television jingles, adverts, and drama. Celebrities in each country who have the disease could be encouraged to talk about PD. This will improve awareness in the populations and may encourage patients to seek care earlier than they do currently. Observance of World Parkinson's Day (on 11 April annually) in healthcare institutions as well as obtaining sponsorship for other events such as quiz competitions and arts and cultural activities amongst school learners would be important. It has been observed that school learners can help to raise awareness of neurological disorders among the older members of the family (60).

In addition, in the short term, training of multidisciplinary teams comprising primary care physicians, and geriatricians as well as training of neurology nurses has been established in some parts of Africa and should be encouraged. The training of other team members such physiotherapists, occupational and speech therapists, and dieticians, should be promoted through local neurological and international societies such as the International Parkinson and Movement Disorder Society. A previous review suggested that tele-neurology can be deployed for training of health care workers through local, regional, and intercontinental networks (61).

To tackle the problem of the non-availability and un-affordability of drugs, a multisectorial strategy involving governments, pharmaceutical organizations, and other key stakeholders is necessary. It will be important for governments across Africa to include drugs for PD in the National Drug Formulary and to enroll patients in the health insurance programme. Incorporating PD care into health insurance systems will also enable patients to have access to neuroimaging (62). Neuroimaging facilities are becoming more widely available in Africa, but the cost of investigation is not affordable for most patients.

Finally, a holistic approach to care could be developed and implemented. The organization of PD support groups and clubs as well as organization of community-based rehabilitation will help in the care of patients living in rural communities.

CONCLUSIONS

The genetic and environmental diversity across the African continent provides a wealth of information and opportunities for research into the epidemiological patterns of PD occurrence, its clinical phenotypes and the genetic and environmental causal factors. However, there is large variation in neurological and genetic research capacity across the African continent, and many unexplored areas in African PD research. Some countries are relatively well-equipped, but most are severely resource-constrained. A low resource environment strongly limits the possibilities of performing research locally, and therefore unidirectional export of genetic material to scientifically more developed countries remains a major concern. Local and regional partnerships can form an ethically appropriate solution, reducing inequalities, and promoting capacity building. There is also the possibility for collaboration of these partnerships with global consortia studying the genetic etiology of PD [Genetic Epidemiology of Parkinson's Disease (GEoPD; www.geopd.net) and The International Parkinson Disease Genomics Consortium (IPDGC; www.pdgenetics.org)], to provide training in genomics and bioinformatics to African scientists.

Furthermore, a major concern for the adequate treatment of PD patients is the availability of affordable levodopa-containing medication. Various African countries have difficulties in obtaining levodopa for their patients largely due to manufacturing capacity and supply chain constraints,

which has prompted the need for development of alternative therapies. The levodopa-containing plant Mucuna Pruriens thrives in the subtropics and can be grown by patients for their own use. Options such as these and others should be considered to provide African-based solutions to uniquely African problems when dealing with the emerging PD pandemic (63).

AUTHOR CONTRIBUTIONS

MD contributed to design, conception, writing of the first draft, and editing of the manuscript. TC contributed to writing sections of the manuscript. SB and OR contributed to conception and editing. JC contributed to editing and compiled the figure. MK contributed to writing sections and editing. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Genetic Analysis of *RAB39B* in an Early-Onset Parkinson's Disease Cohort

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Pathogenic variants in the gene encoding RAB39B, resulting in the loss of protein function, lead to the development of X-linked early-onset parkinsonism. The gene is located within a chromosomal region that is susceptible to genomic rearrangement, and while an increased dosage of *RAB39B* was previously associated with cognitive impairment, the potential role of dosage alterations in Parkinson's disease (PD) remains to be determined. This study aimed to investigate the contribution of the genetic variation in RAB39B to the development of early-onset PD. We performed gene dosage studies and sequence analysis in a cohort of 176 individuals with early-onset PD (age of onset ≤50 years) of unknown genetic etiology. An assessment of the copy number variation over both coding exons and the 3′ untranslated region (UTR) of *RAB39B* did not identify any alterations in gene dosage. An analysis of the UTRs identified two male individuals carrying single, likely benign, nucleotide variants in the 3′UTR (chrX:154489749-A-G and chrX:154489197-T-G). Furthermore, one novel variant of uncertain significance was identified in the 5′UTR, 229 bp upstream of the start codon (chrX:154493802-C-T). *In silico* analyses predicted that this variant disrupts a highly conserved transcription factor binding site and could impact *RAB39B* expression. The results of this study do not support a significant role for genetic variation in *RAB39B* as contributing to early-onset PD but do highlight that additional molecular studies are required to determine the mechanisms regulating *RAB39B* expression and their association with the disease. Genetic investigations in larger parkinsonism/PD cohorts and longitudinal studies of individuals with cognitive impairment due to an altered dosage of *RAB39B* will be required to fully delineate the contribution of RAB39B to parkinsonism.

Keywords: parkinson's disease, RAB39B, DNA polymorphisms, gene dosage, copy number variation

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative condition that manifests with a spectrum of motor symptoms including tremor, rigidity, bradykinesia, and gait disturbances. PD can be classified according to initial clinical presentation as early-onset PD (<50 years) or late-onset PD (>60 years). Despite a difference in disease onset, a post-mortem

TABLE 1 | Sequencing primers.

Primer name	Sequence	Amplicon size
hRAB39B 5′UTR F	TGGCAGTTTGAACGACAGAG	397 bp
hRAB39B 5′UTR R	GCTCTGCAGGTCTCCTTGG	
hRAB39B 3′UTR 1F	CATGCTCTCCTACTTGAACTGAA	1,000 bp
hRAB39B 3′UTR 1R	CCTGGCCAAGTGATTTTCAT	

examination of the central nervous system in both classifications demonstrates the hallmark pathological features of the disease, including neuron loss in the substantia nigra *pars compacta* and the presence of intraneuronal α-synuclein (α-syn)-positive inclusions, termed as Lewy bodies.

Currently, the molecular mechanisms underlying the development and the progression of PD remain largely unknown, and most disease cases are idiopathic. However, in a subset of ∼10% of cases, the disease etiology is genetic—the result of a monogenic mutation (1). Pathogenic variants in PD-associated genes can be point mutations or small in/dels that affect protein function or gene expression or can be larger copy number variants (CNV) that impact gene dosage. For example, protein-disrupting mutations and gene dosage alterations, which do not encompass the entire gene and result in loss of function, are an important mutation mechanism in recessive *parkin*-mediated PD (2). Similarly, multiplication of the entire gene encoding α-syn (SNCA), with associated increased dosage, expression, and elevated SNCA steady-state level, correlates with severity and disease progression in dominant PD (3–5). Genome-wide association studies have also identified additional risk loci contributing to the burden of the disease, including susceptibility alleles that can modulate the risk of developing PD through dysregulated gene expression. For example, the non-coding polymorphisms of the *SNCA* locus that impact promoter or enhancer activity correlate with a strong risk of developing sporadic PD (6–8).

Loss-of-function mutations in *RAB39B* were originally identified in two independent families who displayed the clinical features of early-onset Parkinson's disease (EOPD) with non-progressive intellectual disability and macrocephaly (9). RAB39B is a member of the RAB GTPase family with a putative role in vesicle trafficking. Several subsequent studies of the coding sequence and the splice junctions of *RAB39B* in large PD cohorts failed to identify additional pathogenic mutations, suggesting that the single-nucleotide variants in *RAB39B* that directly disrupt protein function are a rare cause of PD (10–14). However, genetic validation of the gene has been established by the identification of six additional causal *RAB39B* mutations, to date, in unrelated PD patients and families [reviewed in Ciammola et al. (15)]. Notably, a pedigree of European origin carrying a missense mutation in *RAB39B* (c.574G>A, p.G192R) manifested X-linked dominant PD in males, but the heterozygous females presented with later-onset parkinsonism and no intellectual disability (16). This potentially reduced penetrance in females suggests that the relative level of *RAB39B* expression may have an impact on the clinical presentation of PD.

RAB39B is located at Xq28 in a region flanked by low-copy repeats, making it susceptible to chromosomal aberrations mediated by a non-allelic homologous recombination. Indeed duplications at the Xq28 region, including the genes methyl CpG-binding protein 2 and GDP dissociation inhibitor 1, are frequently observed in males with intellectual disability and brain malformations (17). A single study investigating *RAB39B* copy number in a familial Chinese PD cohort ($n = 195$) did not identify any cases with dosage alterations (12). However, duplication and triplication of *RAB39B* have been previously reported to be associated with the development of X-linked intellectual disability (XLID) in male children (18, 19). It was not reported if the affected individuals presented with a movement disorder at the time of assessment.

The collective results, to date, have implicated *RAB39B* in the development of EOPD and parkinsonism. Although an altered dosage of *RAB39B* has been reported to cause XLID, it has not been associated with the development of PD to date. To further investigate the potential role of RAB39B in PD, we screened an EOPD cohort for CNV that could lead to an altered dosage of the gene. In addition, we performed sequence analysis of the untranslated regions (UTR) and immediately upstream of the putative transcription start site (TSS) to identify variants with the potential to dysregulate *RAB39B* expression.

MATERIALS AND METHODS

Patient Samples

Prior to commencing the study, appropriate institutional ethics approval and informed consent from patients were obtained. Genomic DNA isolated from the whole blood of 232 individuals diagnosed with EOPD (onset ≤50 years) was made available by author GDM. This EOPD cohort, consisting of 71 females and 161 males with mean age of onset of 42.7 ± 6.5 years, comprises participants in the Queensland Parkinson's Project in Queensland, Australia (20) and is representative of a Caucasian population. All patient DNA samples were collected under protocols approved by the Griffith University Human Research Ethics Committee (Project ESK/04/11/HREC). The samples were previously sequenced to exclude mutations in known PD-associated genes, including *SNCA* (MIM 163890), *PARK2* (MIM 602544), *DJ1* (MIM602533), *PINK1* (MIM 608309), and *LRRK2* (MIM 609007). The samples were also previously screened for variants in the coding region of *RAB39B* (MIM 300774). A subset of the cohort (176 individuals, consisting of 58 females and 118 males with mean age of onset of 42.6 ± 6.5 years) was utilized in this study.

Sequencing

We amplified genomic DNA corresponding to regions of the upstream regulatory region, the 5′UTR and the 3′UTR of *RAB39B*, using the primers detailed in **Table 1** and **Figure 1**. Sanger sequencing was performed using Big Dye Terminator v3.1 (Applied Biosystems, 4336697), according to the manufacturer's instructions, on 3730 Genetic Analyzer platform (Applied Biosystems). The sequences were aligned and analyzed using Sequencher 5.0 software (Genecodes). The detected variants

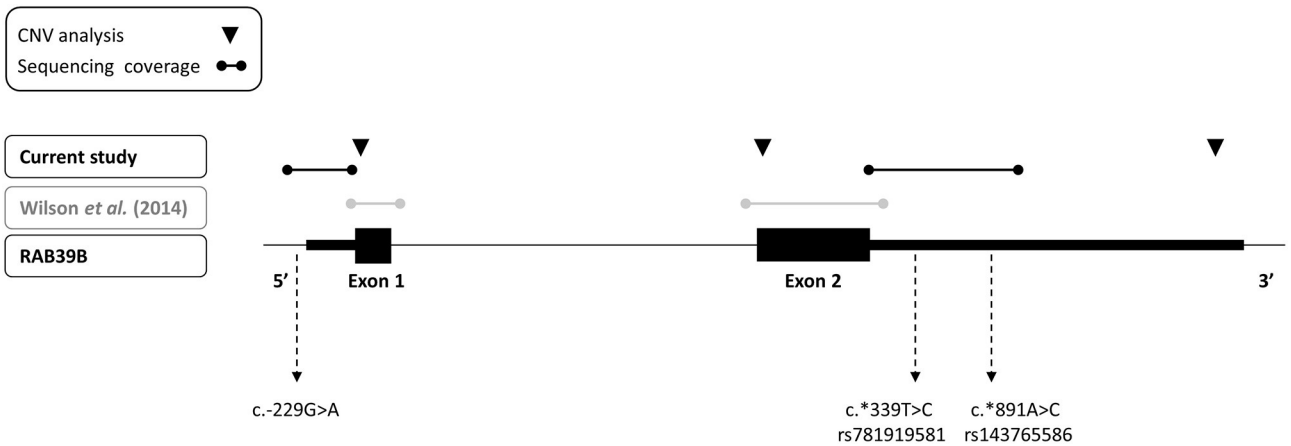

FIGURE 1 | Depiction of sequencing and copy number variant analyses performed for *RAB39B* in this study (black text) and previously (gray text) of an early-onset Parkinson's disease cohort. The three variants identified in the study are indicated.

were annotated using Varsome (https://varsome.com/) and filtered with GnomAD (https://gnomad.broadinstitute.org/). The variants not present in GnomAD were considered as novel. The pathogenicity of the variants was predicted using Combined Annotation Dependent Depletion (CADD) (https://cadd.gs.washington.edu/snv) and Deleterious Annotation of genetic variants using Neural Networks (DANN) (21), two *in silico* prediction tools designed to annotate both coding and non-coding variants. The reference cDNA and genomic sequences utilized for *RAB39B* were NM_171998.4 and GRCh37/hg19, respectively. The novel variants identified in this study have been submitted to the LOVD gene-specific database for *RAB39B* (https://www.lovd.nl/).

CNV Analysis

We performed an analysis of *RAB39B* CNV by quantitative real-time PCR (RT-PCR), utilizing commercially available Taqman assays interrogating exon 1, exon 2, or the 3′UTR of *RAB39B* (Life Technologies, Hs00817269_cn, Hs00745075_cn, and Hs02637133_cn, respectively; **Figure 1**). The reactions were duplexed with the human RNaseP copy number reference assay (Life Technologies, 4403326) and 10–20 ng gDNA amplified on a LightCycler LC480 II (Roche) according to the manufacturer's instructions. Each sample was assessed in triplicate. The threshold cycle was determined using LightCycler LC480 software 1.5.1.62 SP2, and *RAB39B* copy number was calculated using the ΔΔCT method.

RESULTS

We screened for CNVs over both coding exons of RAB39B and the 3′UTR by quantitative RT-PCR (**Figure 1**) but did not identify any variations in *RAB39B* exon or gene dosage. In addition, we analyzed 404 bp of sequence upstream of the initiating codon and 1,021 bp downstream of the termination codon for sequence variants in *RAB39B* in 176 individuals with EOPD of unknown genetic etiology (**Figure 1**). We identified three male individuals carrying single-nucleotide variants. One variant of uncertain significance (chrX:154493802-C-T) was identified in the 5′UTR, 229 bp upstream of the ATG start codon and close to the predicted TSS of Refseq NM_171998.4 (**Figure 2A**), of a male patient with a disease onset age of 50 years. This nucleotide is highly conserved (GERP 4.6) and the variant is predicted to disrupt a consensus activator protein-1 (AP-1) transcription factor binding site located within a DNase 1 hypersensitive peak (**Figure 2B**). This is a novel variant not previously identified in GnomAD, with *in silico* support of pathogenicity utilizing DANN (score 0.98) and CADD (score 21.2). Due to the study design of the Queensland Parkinson's Project (22), we were unable to test if the variant was *de novo* or perform functional studies of the variant in patient-derived cells. No intellectual issues were reported at the time of patient examination and there was no familial history of parkinsonism. Two likely benign variants were identified in the 3′UTR region of *RAB39B*. One variant was identified in a male patient with disease onset age of 48 years (NM_171998.4:c.*339T>C; chrX:154489749-A-G). This rare variant (rs781919581) has an average allele frequency of 0.00086 in GnomAD, with DANN and CADD scores of 0.75 and 5.29, respectively. The second variant was identified in a male patient with a disease onset age of 49 years (NM_171998.4:c.*891A>C; chrX-154489197-T-G). This rare variant (rs143765586) has an average allele frequency of 0.00087 in GnomAD, with DANN and CADD scores of 0.66 and 1.67, respectively.

DISCUSSION

RAB39B is a member of the RAB GTPase family with a putative role in vesicle trafficking in neurons. While there is considerable genetic and functional evidence demonstrating the loss of function mutations that cause an early-onset familial parkinsonian disorder in males, a potential broader role in idiopathic PD remains to be fully tested. Previously, we investigated a Caucasian EOPD cohort (n = 187) for

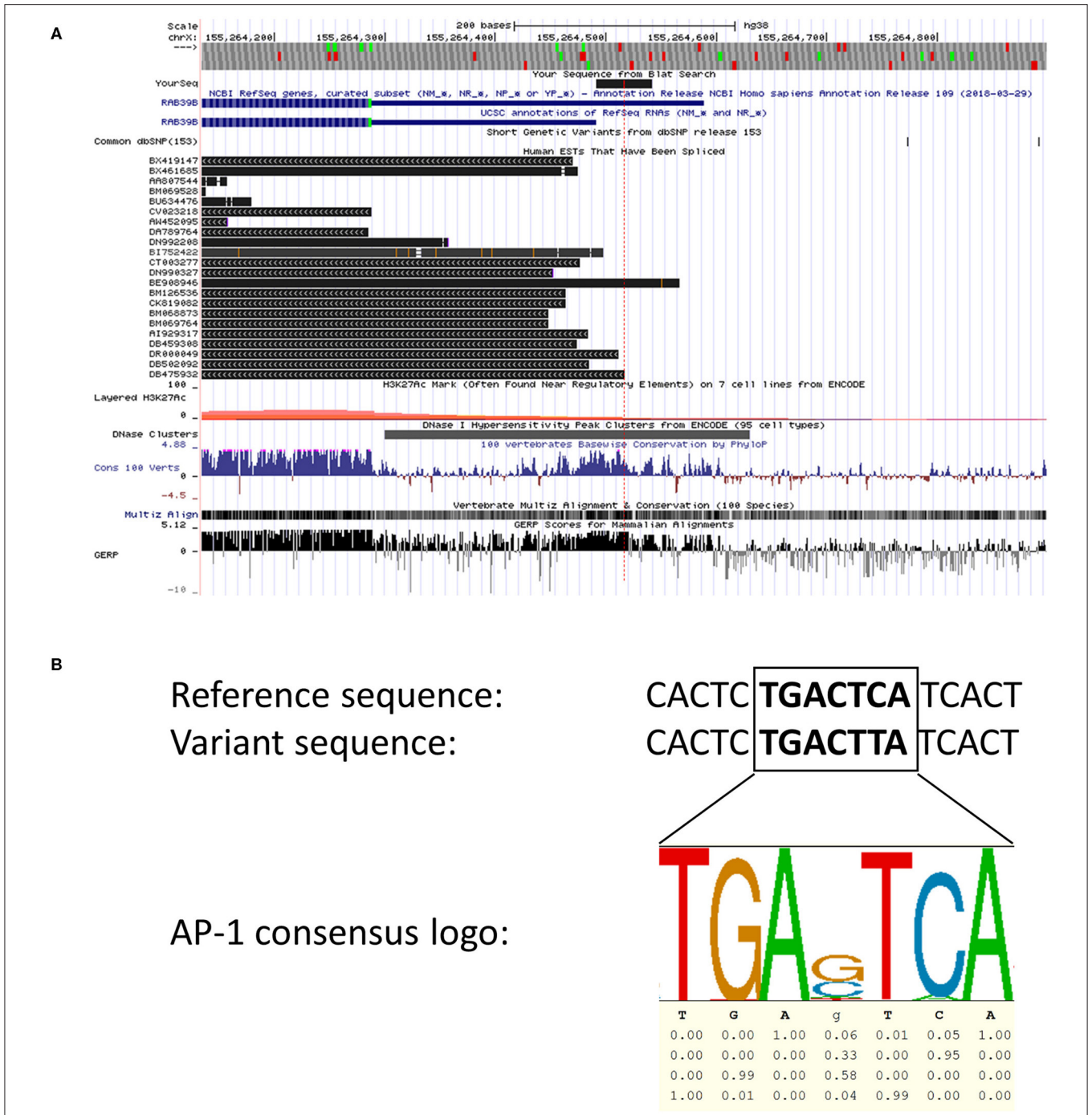

FIGURE 2 | *In silico* analyses of the 5′ region of *RAB39B*. **(A)** Screenshot of the UCSC browser (hg38, chrX:155,264,156-155,264,850) examining the 5′ region of *RAB39B*. The location of the chrX:154493802-C-T variant is depicted by the red dotted line. The blue sequences represent Refseq NM_171998.3 (outdated) and NM_171998.4, respectively. Spliced human expressed sequence tags are shown in black. The lower tracks demonstrate that the variant is located within a region of DNase 1 sensitivity and displays high vertebrate conservation compared to immediate flanking sequence. The final track demonstrating GERP scores represents an analysis of the corresponding sequence using the hg19 dataset. **(B)** An alignment of the 5′ region of *RAB39B* showing the reference genomic sequence (top) with the variant sequence (middle). The predicted AP-1 transcription factor binding site is in bold highlight. The sequence logo (bottom) generated from ENCODE data demonstrates the core AP-1 consensus sequence and the conservation of each nucleotide.

alterations in the coding regions of *RAB39B* and found no variants of significance (9). In this study, we investigated a subset of this EOPD cohort for CNVs and non-coding variants that could potentially result in the dysregulated expression of *RAB39B*. Although the non-coding variants may not directly impact protein function, they can alter the protein levels in

neurodegenerative diseases such as PD by modulating mRNA synthesis, stability, localization, and translation. Non-coding polymorphisms in *SNCA*, *PARKIN*, and *DJ1* have all been previously identified to be associated with the development of PD in cohort screens (23–27). For example, while protein-disrupting mutations in *parkin* are a common cause of recessive EOPD (28), the variants in the promoter/5′UTR region that affect *parkin* expression are associated with idiopathic PD (29).

We did not identify any CNV alterations in the 176 samples analyzed, suggesting that increased *RAB39B* dosage may not be associated with EOPD. While screening additional large cohorts will further test this hypothesis, longitudinal studies of individuals with XLID secondary to duplication and triplication of *RAB39B* (18, 19) will also inform whether an increased dosage of RAB39B can cause a parkinsonian phenotype. Given that the PD phenotype associated with the loss of RAB39B function appears to manifest later in life compared to intellectual disability [>20 years; (15)], it is probable that if the affected individuals are going to develop parkinsonism, it will be at a later age than the time of report.

Screening of the non-coding regions of *RAB39B* revealed three variants, one immediately proximal to the TSS and two within the 3′UTR. Both UTR variants were classified as likely benign according to the ACMG guidelines (30). In contrast, an *in silico* analysis identified that the upstream variant chrX:154493802-C-T was novel, with predictions supportive of pathogenicity. Our analysis of both expressed sequence tags and genomic conservation around the variant suggests that it disrupts a highly conserved AP-1 transcription factor binding site. We hypothesize that this motif is important for regulating the *RAB39B* expression, and the variant likely downregulates the expression by preventing the binding of important transcription factors such as AP-1. The AP-1 family of transcriptional factors can modulate a wide range of molecular functions, one of which is neuronal plasticity (31). The AP-1 regulation of neuron-enriched RAB GTPases has not been previously reported, although one study demonstrated that AP-1 can regulate *RAB11A* promoter activity and thus endosomal recycling (32). Interestingly, a phylogenetic analysis of the RAB GTPase family shows that RAB39 shares the most recent common ancestor with RAB11 (33), suggesting that the transcriptional regulation of some RAB GTPases may be evolutionarily conserved. Currently, knowledge of the transcriptional regulation of *RAB39B* is lacking. Specifically, the promoter region, primary TSS, and important transcription factors for *RAB39B* have yet to be identified and functionally characterized. Therefore, while our analysis of the chrX:154493802-C-T variant is consistent with a potential effect on *RAB39B* expression, in the absence of functional validation, the significance of the variant remains uncertain.

Overall our results are consistent with previous reports suggesting that the genetic variation in *RAB39B* is a rare cause of EOPD. A genetic analysis of the UTRs and the regulatory regions of *RAB39B* has not been reported previously; our identification of a novel 5′ variant, with *in silico* predictions supporting pathogenicity, warrants further investigation. Moreover, a recent study in a small cohort of individuals with idiopathic PD suggested that steady-state levels of RAB39B in brain tissue might be decreased (34). Therefore, further genetic and functional studies are required to determine the consequences of dysregulated *RAB39B* expression and test its potential role as a susceptibility gene associated with PD or parkinsonism more broadly.

DATA AVAILABILITY STATEMENT

Details of variants identified in this study have been submitted to the LOVD gene specific database for RAB39B https://databases.lovd.nl/shared/genes/RAB39B.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Griffith University human research ethics committee (project ESK/04/11/HREC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG and GW contributed to study design, execution, and data analysis. NS and AR contributed to study execution and data analysis. GM provided materials and contributed to study design. SS contributed to study design and data analysis. PL designed and funded the study and contributed to study conception and data analysis. YG and PL wrote the first draft of the manuscript. All the authors contributed to manuscript revision and read and approved the submitted version.

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Association Between Glucocerebrosidase Mutations and Parkinson's Disease in Ireland

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Multiple studies implicate heterozygous *GBA* mutations as a major genetic risk factor for Parkinson's disease (PD); however, the frequency of mutations has never been examined in PD patients from the Irish population. We prospectively recruited 314 unrelated Irish PD patients (UK Brain Bank Criteria) and 96 Irish healthy controls (without any signs or family history of parkinsonism) attending. The Dublin Neurological Institute (DNI). Complete exon *GBA* Sanger sequencing analysis with flanking intronic regions was performed. The *GBA* carrier frequency was 8.3% in PD and 3.1% in controls. We identified a number of potentially pathogenic mutations including a p.G195E substitution and a p.G377C variant, previously described in a case study of Gaucher's disease in Ireland. On genotype–phenotype assessment hallucinations, dyskinesia, and dystonia were more prevalent in *GBA*-PD. The genetic etiology of PD in Ireland differs from the continental Europe as seen with the lower *LRRK2* and higher than in most European countries *GBA* mutation frequency. Determining genetic risk factors in different ethnicities will be critical for future personalized therapeutic approach.

Keywords: *GBA*, glucocerebrosidase, Ireland, sequencing, Parkinson's disease

INTRODUCTION

Glucocerebrosidase gene (*GBA*) encodes *B*-glucocerebrosidase enzyme hydrolyzing glucocerebroside to glucose and ceramide. While homozygous or compound heterozygous *GBA* mutations alter glucocerebrosidase activity and result in a recessive lysosomal-storage disorder, Gaucher's disease, heterozygous variants have been implicated in Parkinson's disease (PD) pathogenesis (1–3). In fact, *GBA* mutations have been identified as one of the strongest known genetic risk factors for PD (5–20% of PD patients are reported to harbor *GBA* mutations depending on ethnicity) (2). Initially, *GBA*-related PD was thought to reflect the clinical phenotype of idiopathic PD (3); however, current evidence suggests unique motor (e.g., frequent falls, freezing of gait, dyskinesia, faster progression), non-motor [autonomic symptoms, younger age at onset (2.6–0.9 year earlier age at onset)] (4), increased prevalence of rapid eye movement (REM) sleep behavior disorder (RBD) and daytime sleepiness (5) features and cognitive impairment (frequent cognitive decline and hallucinations) (5–7).

The *GBA* gene is located on chromosome 1q22 and consists of 11 coding exons. The existence of a highly homologous pseudogene (GBAP1) (sharing 96% of exonic sequence) located 16 kb downstream of the functional *GBA* gene makes *GBA* sequencing studies challenging (8). Sequencing of all exons and using long-range PCR primers specific for the functional *GBA* gene is the most reliable method for genetic screening. However, many centers continue to restrict the analysis to the most common variants or exons where most of the mutations are reported (exons 9 and 10) (9). *GBA* p.L444P and p.N370S are the two most common pathogenic substitutions among 335 reported, responsible for 50–70% of cases (1). Based on homozygous and compound heterozygous mutations resulting in specific subtypes of Gaucher's disease, *GBA* mutations can be classified as "mild" (p.N370S and p.R496H) and "severe" [p.L444P, p.D380A, p.R131C, p.D409H, p.R463C, p.R257Q, p.V394L, c.1263-1317del, and RecNciI-a recombinant allele (p.L444P-A456P-V460V)] (10). Carriers of mild mutations are reported to have 2.2-fold higher risk of PD and mean age at onset of 58.1 (±10.6), while carriers of severe mutations have 9.92- to 21.29-fold increased risk of PD and mean age at onset of 52.1 (±11.2) (10, 11). *GBA* p.E326K homozygous and compound heterozygous mutations do not cause Gaucher's disease; thus, there may be a distinct mechanism predisposing to PD in carriers of p.E326K. Until recently, believed to be a benign polymorphism, now p.E326K is an established risk factor (large meta-analyses) causing rapid motor progression of PD ($\beta = 3.42$; 95% CI, 0.66–6.17; $p = 0.02$) (12, 13), cognitive decline, and the development of RBD among those who did not have the disorder at baseline (5, 6, 11). Similarly, there is an emerging trend in the literature to classify p.T369M as a risk variant (with the reported effect size similar to that of p.E326K, baseline RBD, associated cognitive decline, and higher hazard ratio of reaching H&Y3) (4, 6, 14, 15).

The prevalence of *GBA* mutations can vary according to ethnicity (10) (e.g., mutations are common in Ashkenazi Jewish populations). Locally derived data are important for further genetic characterization, development of local guidelines, enrolment in clinical trials, and search for the disease-modifying treatments. Large Irish families, small population size of the country (Republic of Ireland population, 4.79 million in 2017, as per the most recent census) (16), and relative isolation from the continental Europe make the Irish population ideal for genetic studies. We and others reported that genetics of PD in the Irish population differs from that in the continental Europe, e.g., *LRRK2* mutations are rare (17). We have shown in a recent epidemiological study that the population structure in Ireland differs from the rest of the Europe, and this may be related to the Celtic ancestry (18). We have also demonstrated that the prevalence of another neurological condition, amyotrophic lateral sclerosis (ALS) in Ireland, differs from other European countries (19). The prevalence of *GBA* in PD and genotype-phenotype correlation has never been studied in Ireland, and we hypothesized that similarly to *LRRK2*, it may differ from that in other populations.

METHODS

Ethical Approval

The ethical approval (1/378/1,300) was granted by the Mater Misericordiae University Hospital (MMUH), Dublin, Ireland. Informed written consent was obtained.

Design

This is an observational cross-sectional study.

Participants

Three hundred fourteen unrelated Irish patients over age 18 diagnosed with PD (UK Brain Bank Criteria) were recruited from a tertiary referral center, Dublin Neurological Institute (DNI), at the MMUH, Dublin, Ireland. Patient's attending the DNI reside in the Dublin city or rural areas of the country. To expand the diversity of participants, an additional notice about the study recruitment was posted on the Irish Parkinson's Association website. Secondary and atypical forms of parkinsonism were excluded. Ninety-six healthy controls (friends or spouses of patients) without any signs or family history of parkinsonism, over age 18 were recruited from the DNI.

Sequencing and Data Analysis

Genomic DNA was isolated from peripheral blood lymphocytes using QIAmp Blood Midi Kit. Genetic analysis was performed at the Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA. All PD patients were prescreened for the most common genetic causes of autosomal dominant disease (*LRRK2*, *SNCA*, *VPS35*) and patients with young onset PD (YOPD) (age, <50) also for autosomal recessive PD (*PARKIN*, *PINK1*, *DJ1*). PD patients ($n = 314$) were tested for specific point mutations; LRRK2 p.G2019S, SNCA p. A53T, and p.A30P and VPS35 p.D620N (TaqMan Allelic Discrimination Assay, on Quant Studio System Real-Time PCR System). Multiplex ligation-dependent probe amplification (MLPA) analysis was employed to determine the dosage alterations in *SNCA*. PD patients with YOPD ($n = 81$) were investigated by Sanger sequencing of all coding exons plus 25 base pairs of exon–intron boundary of *PARKIN* (12 exons), *PINK1* (8 exons), and *DJ1* (6 exons) genes. MLPA analysis was also performed.

GBA sequencing of all 11 coding exons plus 25 base pairs of exon–intron boundary was performed on all patients and 96 controls and confirmed bidirectionally. PCR and primer sequences are available in **Supplementary Tables 1, 2**. The pathogenicity of the variants was determined based on the current literature and *in silico* tools [Polyphen v2 (20), SIFT (21), Mutation Taster (22)] (**Table 1**). Results are presented in **Table 2**. Both newer and well-established historical nomenclature (GBA protein −39 amino acids) for *GBA* variants annotation was used.

Samples from two patients were tested for β-glucosidase enzyme level and chitoriosidase level by the use of a validated functional assay (fluorimetric assay method) (23) at the Guy's Hospital, London, United Kingdom.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, Version 22.0. Cohort characteristics were assessed using

TABLE 1 | Pathogenicity prediction results for *GBA* genotypes found in Irish Parkinson's disease (PD) patients and controls: pathogenic/risk variants, predicted pathogenic, and VUS genotypes.

GBA Protein	GBA Protein (-39aa)	Genotype	rs #	Exon	Mutation Taster	Polyphen 2	SIFT	dbSNP	ClinVar	Literature	Irish Study
Pathogenic/risk GBA variants											
E365K	E326K	G > A	rs2230288	8	Path	Benign	Tolerated	Path	Risk	Risk	Risk
T408M	T369M	C > T	rs75548401	8	Polymorphism	Benign	Tolerated	VUS	VUS	Risk	Risk
F255Y	F216Y	T > A	rs74500255	7	Path	Probably Path	Tolerated	Path	Path	Path	Path
N409S	N370S	A > G	rs76763715	9	Path	Possibly Path	Path	Path	Path	Path	Path
D448H	D409H	G > C	rs1064651	9	Path	Benign	Tolerated	Path	Path	Path	Path
L483P	RecNcil	T > C	rs421016	10	Path	Path	Path/Tolerated	Path	Path	Path	Path
A495P	Recombinant	G > C	rs368060								
V499V		G > C	rs1135675								
Predicted pathogenic											
G416C	G377C	G > T	No rs	9	Path	Probably Path	Path	N/A	N/A	1 patient Gaucher's	Predicted Path
Variants of unknown significance											
G234E	G195E	G > A	rs74462743	6	Path	Probably Path	Path	SNV	N/A	1 patient Gaucher's	VUS
R301H		G > A	rs140955685	7	Polymorphism	Benign	Tolerated	SNV	N/A		VUS
R368C		C > T	rs374306700	8	Path	Probably Path	Path	VUS	VUS		VUS

GBA Protein (−39aa) the conventional nomenclature for GBA alleles following Human Genome Variation Society (HGVS) recommendation referring to the processed protein and excluding the 39-residue signal peptide. GBA Protein, the alternative nomenclature; Path, pathogenic; VUS, variant of unknown significance; SNV, single-nucleotide variant.

descriptive statistics. Bivariate associations between categorical variables were calculated using Pearson chi-square tests (X^2) or Fisher's exact tests (when expected cell counts ≤5 observations, e.g., genotype frequencies comparison). Where continuous variables were normally distributed, independent sample *t*-tests were used, and where not, Mann–Whitney *U*-tests. The results were deemed statistically significant where $p < 0.05$. Two hypotheses were tested. The first was that the presence of a *GBA* variant increased the risk of PD in the Irish population. Logistic regression model was used to control for age and gender. The second hypothesis was that the *GBA* variant carriers have earlier age-at-PD onset (14). A linear regression model was fitted with age at onset as the dependent variable and the presence or absence of a *GBA* variant as the independent variable (gender as a covariate). Linear and logistic regression models were used to test whether the frequency of the motor complications, wearing off, dyskinesia, dystonia, freezing of gait, hallucinations, dementia, and Unified Parkinson's Disease Rating Score part III (UPDRS-III) differed between carriers and non-carriers. Gender, age, and disease duration were considered as covariates. In the sensitivity analysis, each of the motor complications was also controlled for medications, UPDRS-III score, and disease subtype.

RESULTS

We screened 314 Irish patients with PD of which 62.4% (n = 196) were male and 37.6% (n = 118) were female, with mean age at inclusion of 64.94 ± 10.69 years, mean age at onset of 56.23 ± 12.04 years, and mean disease duration of 8.62 ± 6.97 years. The majority had tremor-predominant disease (55.7%), followed by postural instability gait disorder (PIGD) (34.1%) and mixed subtypes (10.2%). Motor complications were seen in 43.3%. Family history of PD was present in 173 patients (55.1%). A group of 96 Irish controls [51% men (n = 49), 49% women (n = 47), mean age at inclusion (61.15 ± 14.5)] was studied to assess the mutation frequency in the ethnically matched population.

We examined 314 patients for genes associated with autosomal dominant PD. Among 314 patients, we identified one PD patient with LRRK2 G2019S mutation positive for *GBA* benign intronic variant (exon 7−18 bp). We did not identify any carriers of p.A53T or p.A30P in SNCA or p.D620N in VPS35. There were no patients with *SNCA* dosage alterations.

On examination of the 81 patients with YOPD, we identified one homozygote carrier (p.G430D/p.G430D), three compound heterozygote (p.Leu112fsX163/p.R275W; p.G430D/Ex 4&5del; p.R275W/Ex3 del), and three heterozygote carriers [p.R275W/wt. (n = 1); p.P437L/wt (n = 2)] in *PARKIN* gene. None of the *PARKIN* gene carriers had variants in *GBA* detected. No homozygous or compound heterozygous carriers of *PINK1* or *DJ1* were detected.

We detected 26 carriers of *GBA* pathogenic/risk variants in PD and 3 in controls [p.E326K, P.T369M, p.N370S, p.F216Y, p.D409H, and RecNcil (p.L444P-A456P-V460V)] (**Tables 2, 3**). These variants were found in 8.3% of PD patients and 3.1% of controls ($p = 0.08$, X^2). The result remained non-significant after controlling for age and gender [odd's ratio (OR), 3.2 ($p = 0.06$; 95%CI, 0.94–10.97). RecNcil and p.N370S carrier frequency in PD was 1.9% [RecNcil, $n = 3$, minor allele frequency

TABLE 2 | Pathogenic/risk variants and variant of unknown significance (VUS) genotypes, carrier frequency, and minor allele frequency in Irish Parkinson's disease (PD) patients and controls and comparison to ExAC and GnomAD databases.

GBA protein	GBA protein (-39aa)	Genotype	rs #	Exon	PD het/homo	PD carrier frequency	PD MAF	Controls het/homo	PD carrier frequency	PD MAF	ExAC MAF	GnomAD MAF
Pathogenic/risk GBA variants												
T408M	T369M	C > T	rs75548401	8	6/0	1.92%	0.96%	4/0	2.37%	1.18%	0.98%	0.94%
E365K	E326K	G > A	rs2230288	8	12/1	4.14%	2.23%	3/1	2.37%	1.48%	1.20%	1.23%
F255Y	F216Y	T > A	rs74500255	7	1/0	0.33%	0.16%	0/0	0%	0%	0.02%	0.00%
N409S	N370S	A > G	rs76763715	9	3/0	0.96%	0.48%	0/0	0%	0%	0.36%	0.20%
D448H	D409H	G > C	rs1064651	9	1/0	0.32%	0.16%	0/0	0%	0%	0.01%	0.02%
L483P	RecNcil recombinant	T > C	rs421016	10	3/0	0.96%	0.48%	0/0	0%	0%	0.39%	0.14%
A495P		G > C	rs368060		3/0	0.96%	0.48%	0/0	0%	0%	0.01%	0.01%
V499V		G > C	rs1135675		3/0	0.96%	0.48%	0/0	0%	0%	0.02%	0.03%
Predicted pathogenic												
G416C	G377C	G > T	No rs	9	1/0	0.32%	0.16%	0/0	0%	0%	Not reported	Not reported
Variants of unknown significance												
G234E	G195E	G > A	rs74462743	6	1/0	0.32%	0.16%	0/0	0%	0%	–	0.00%
R301H		G > A	rs140955685	7	0/1	0.32%	0.32%	0/0	0%	0%	0.01%	0.02%
R368C		C > T	rs374306700	8	1/0	0.32%	0.16%	0/0	0%	0%	0.00%	0.00%

GBA Protein (–39aa) the conventional nomenclature for GBA alleles following Human Genome Variation Society (HGVS) recommendation referring to the processed protein and excluding the 39-residue signal peptide. GBA Protein, the alternative nomenclature; MAF, minor allele frequency; het, heterozygote; homo, homozygote; n, number.

(MAF) = 0.48%; p.N370S, $n = 3$, MAF = 0.478%); these variants represented 18.2% (6/33) of the pathogenic/risk variants and were not present in controls. The frequency of *GBA* mutations in familial PD was 8.1% ($n = 14$) and 8.6% ($n = 12$) in sporadic PD. The most common variants detected in PD were the risk variants: p.E326K (12 heterozygotes including p.E326K/G377C and p.E326K/T369M and 1 homozygote, p.E326K/E326K) and p.T369M ($n = 6$, including mentioned above p.E326K/T369M), followed by the pathogenic variants: RecNcil ($n = 3$) and p.N370S ($n = 3$) (**Tables 2–4**). Additionally, we detected three variants of unknown significance (VUS), p.G195E, p.R301H, and p.R368C, in three PD patients and none in controls (**Tables 1, 4**). We also identified 11 carriers of benign, known intronic variants (rs140335079, T > A: three heterozygotes, five homozygotes; exon 7–17 bp, G > C: one homozygote; rs377143075, T > C: two heterozygotes).

The mean age at onset for the mild mutation carriers (p.N370S $n = 3$) was 66 (±4.58) years, while for the severe mutations (p.D409H, $n = 1$; RecNcil, $n = 3$), it was 50 (±13.56) years ($p = 0.11$, independent t-test). The mean age at onset for the risk variants carriers (p.E326K, $n = 12$; p.T369M, $n = 6$ including p.E326K/p.T369M) was 54.66 (±11.15).

We found a VUS p.R301H variant, which has not been previously reported either in ExAC (24) or gnomAD (24) databases in a homozygous state. The affected was a man with PIGD from age 57 years, normal cognition, and Hoehn and Yahr (H&Y) stage 3 who was asymptomatic for Gaucher's disease. We detected two very interesting variants. p.G195E reported in Gaucher's disease, but not in PD, predicted to be pathogenic by *in silico* tools (25) in a 69-year-old man. β-Glucosidase enzyme level was measured, and it was at 10.3 nmol/h/mg protein (normal range, 8.4–32.8), and plasma chitoriosidase level was normal. The patient had unilateral, tremor-predominant, levodopa-responsive PD and normal cognition from age 54 years (**Figure 1**3-1). Later on, his cognition declined (MoCA score was 25/30: visuospatial/executive abilities and delayed recall were affected), but hallucinations were not present. He had mild drooling of saliva during the nighttime and REM sleep behavior disorder (RBD). His medications included carbidopa/levodopa 25/100 mg four times daily, mirabegron 50 mg for urinary frequency, and citalopram 15 mg for well-controlled depression. He was also on a continuous positive airway pressure (CPAP) machine for an obstructive sleep apnea. His MRI brain was normal, and dopamine transporter single photon emission computerized tomography (DaT scan) showed a decreased dopamine tracer uptake more pronounced on the left side of the brain. On examination he was hypophonic, had slight rigidity in all limbs, and was bradykinetic more on the right than on the left side. He had bilateral postural and kinetic hand tremor without rest tremor. He did not have any other motor complications.

There was a PD history in his maternal aunt (deceased) (**Figure 1**2-13), maternal uncle (deceased) (**Figure 1**2-10) and in his 67-year-old cousin (deceased) (**Figure 1**3-8). He had tremor-predominant PD since age 51 years and was positive for the same variant. Initially, he was started on mirapexin for 2 years. He developed hallucinations at age 56 (benign, mainly animals) treated with quetiapine 25 mg in the morning and 50 mg at night. He became forgetful, developed dementia (MoCA score, 7/30), and was commenced on donepezil 10 mg once a day and memantine 10 mg twice a day. He then was switched to carbidopa/levodopa preparation 25/100 mg two tablets three times daily. His other non-motor symptoms

TABLE 3 | Genotype–phenotype correlation in risk variants of GBA carriers with Parkinson's disease (PD).

ID	Genotype	Sex	Familial PD	Age (years)	Onset (years)	First symptom	Duration (years)	Subtype	UPDRSIII	H&Y	Cognitive decline	Motor	Hallucinations	FOG
1	E326K/wt	M	–	69	62	Bradykinesia	7	PIGD	51	2	+	Dyskinesia wearing off	–	–
2	E326K/wt	M	–	64	59	Bradykinesia	5	PIGD	10	2	–	–	–	–
3	E326K/wt	M	–	51	43	Tremor	8	Tremor	15	1	–	Wearing off	–	–
4	E326K/wt	F	–	75	73	Tremor	2	Tremor	17	2	+	–	–	–
5	E326K/wt	F	+	72	50	Tremor	22	PIGD	25	3	+	Dyskinesia wearing off dystonia	+	-
6	E326K/wt	F	+	64	64	Tremor	0	Tremor	13	1	–	–		
7	E326K/wt	F	+	71	65	Pain	6	PIGD	24	2	–	Dyskinesia		
8	E326K/wt	M	+	65	43	Gait	22	Mixed	9	2	PDD	Dyskinesia wearing off	–	–
9	E326K/wt	F	+	61	56	Writing	5	Tremor	20	1	–	–	–	–
10	E326K/wt	F	+	62	58	Tremor	4	Tremor	14	1	–	–	–	–
11	E326K/E326K	F	–	65	49	Bradykinesia	16	PIGD	20	2	+	Dyskinesia	+	+
12	E326K/T369M	F	–	77	62	Tremor	15	Mixed	missing	3	PDD	Dyskinesia wearing off	+	+
13	E326K/G377C	M	+	54	39	Tremor	15	Mixed	7	1	–	Dyskinesia Wearing off	–	–
14	T369M/wt	M	–	54	44	Tremor	10	Tremor	22	2	–	–	–	–
15	T369M/wt	M	–	79	69	Tremor	10	Tremor	54	3	–	Dyskinesia wearing off	–	–
16	T369M/wt	M	–	86	61	Tremor	25	Tremor	44	2	–	–	+	–
17	T369M/wt	F	–	61	55	Tremor	6	Tremor	27	2	–	–	–	–
18	T369M/wt	M	+	38	32	Bradykinesia	6	PIGD	36	2	–	Dyskinesia wearing off dystonia	–	–

FOG, freezing of gait; H&Y, Hoehn and Yahr score.

TABLE 4 | Genotype–phenotype correlation in GBA pathogenic variants and variants of unknown significance carriers with Parkinson's disease (PD).

ID	Genotype	Sex	Familial PD	Age (years)	Onset (years)	First symptom	Duration (years)	Subtype	UPDRSIII	H&Y	Cognitive decline	Motor	Hallucinations	FOG
Pathogenic variants														
19	F216Y/wt	M	+	56	53	Tremor	3	Tremor	28	2	–	–	–	–
20	N370S/wt	F	+	82	67	Tremor	15	Tremor	31	2	+	–	–	–
21	N370S/wt	M	–	64	61	Tremor	2.5	Mixed	46	2	PDD	–	–	–
22	N370S/wt	F	+	78	70	Tremor	8	PIGD	9	2	–	Dyskinesia	–	–
23	RecNcil	F	–	61	56	Tremor	5	Tremor	9	2	–	–	–	–
24	RecNcil	F	+	59	54	Tremor	5	PIGD	missing	2	PDD	Wearing off	+	-
25	RecNcil	F	+	33	30	Bradykinesia	3	Mixed	13	1	–	Dyskinesia wearing off dystonia	–	–
26	D409H/wt	M	+	64	60	Tremor	3.5	Tremor	24	1	–	Wearing off	–	–
Variants of unknown significance														
27	R368C/wt	M	–	61	55	Bradykinesia	7	PIGD	28	2	–	–	–	–
28	R301H/R301H	M	–	59	57	Bradykinesia	2.5	PIGD	37	3	–	–	–	–
29	G195E/wt	M	+	69	54	Tremor	15	Tremor	28	1	+	–	–	–

FOG, freezing of gait; H&Y, Hoehn and Yahr scale.

included depression—stable on venlafaxine (effexor XL) 150 and 75 mg, and RBD. He was unable to turn in bed and get out of a chair without help and had freezing of gait episodes. There was no wearing off or dyskinesia present. He needed assistance with cutting food, dressing up, and hygiene. His swallow became affected with occasional choking episodes; therefore, he was commenced on thickened fluid diet. He had a urinary catheter *in situ* due to the difficulties arising from the urinary urgency.

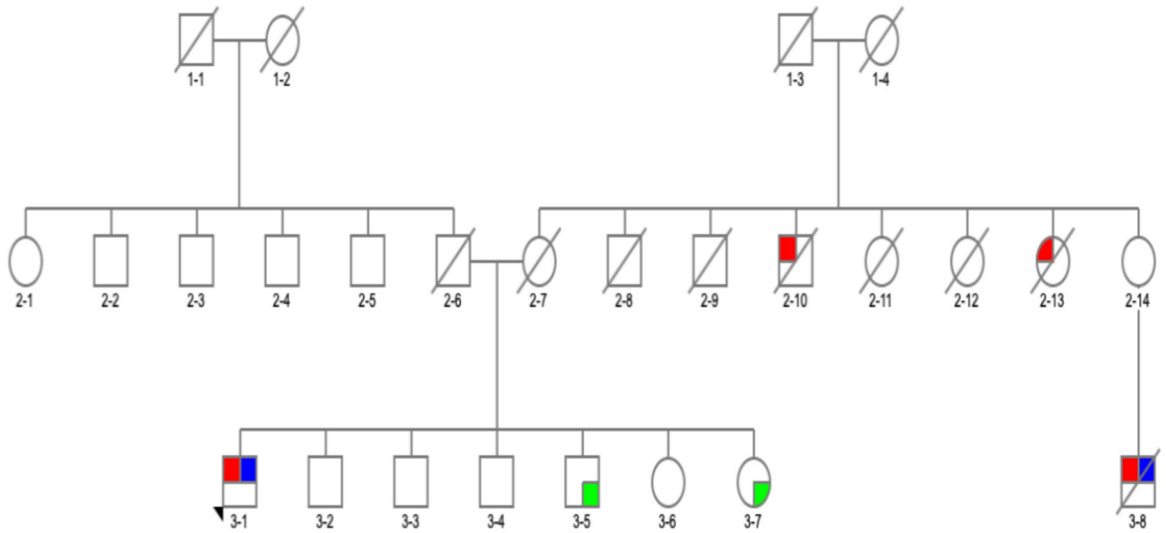

FIGURE 1 | Family pedigree of the patient with *GBA* p.G195E variant in exon 6, rs74462743: red, affected with Parkinson's disease (PD); blue, tested and positive for p.G195E variant; green, tested and negative for p.G195E variant; arrow, proband; diagonal line, deceased.

The biggest problem was agitation in the evening. At the time of recruitment, he was wheelchair bound, and his UPDRS-III score was 28. On examination, there was slight, however ongoing, resting tremor and bilateral, severe bradykinesia, and rigidity. The dopamine uptake on the DaT scan at age 65 was reported as profoundly decreased. The variant was absent in two asymptomatic siblings of the proband age 57 and 67 (**Table 3**). Neurological examination was normal in both cases, and MoCA test was 30/30 in the sister (**Figure 1**3-7) and 29/30 in the brother (**Figure 1**3-5). There were no subtle signs of Gaucher's disease with normal hematology, biochemistry, and liver tests.

The second interesting variant found was p.G377C (c.1246 G > T) variant (not reported in databases), predicted to be pathogenic by *in silico* tools (**Tables 1**, **2**) in a 54-year-old man (**Figure 2**3-1). On further analysis, the β-glucosidase enzyme level was 10.8 nmol/h/mg protein (normal range, 8.4–32.8). The enzyme level in simultaneous controls was 13.6, 14.2, and 14.4. The level of plasma chitotriosidase was normal (as expected in a heterozygous asymptomatic for Gaucher's disease patient). The patient had tremor-predominant PD and normal cognition since age 39 years. There were no hallucinations. At 42 years old, he developed dyskinesia, which was treated by a deep brain stimulator (DBS) at age 49. He also had dystonia and micrographia (the majority of the words was not legible when written). At the time of recruitment, he was on amantadine 100 mg twice a day, selegiline 5 mg, slow release levodopa preparation at night (half-sinemet CR), and carbidopa/levodopa 50/12.5 mg five times a day. On examination, there was hypophonia, moderately stooped posture, and slow gait. PD was present in his mother diagnosed at age 45 (**Figure 2**2-6), who died at 73, maternal aunt diagnosed in her 70s (deceased at 78) (**Figure 2**2-9), and maternal grandfather (**Figure 2**1-3).

On genotype-phenotype assessment between 26 mutation/risk variant carriers and 285 non-carriers, the median age at onset in both groups was the same (57 years), carriers were more likely to be a female (53.8% carrier women vs. 46.2% carrier men, $p = 0.08$, X^2), and the majority (65.4%) of carriers had late onset PD ($n = 17$, $p = 0.31$, X^2) and reported having a positive PD family history (53.8%, $n = 14$, $p = 0.87$, X^2) (**Table 5**). However, these results were statistically non-significant (**Table 1**). Cognitive problems were present in 34.6% of carriers in comparison to 29.8% in non-carriers; however, the p-value was non-significant. Hallucinations were four times more prevalent in carriers than non-carriers [$p = 0.01$, OR 3.97 (95%CI 1.434–11.015), Fisher's exact test]. This remained true after adjusting for age, gender, disease duration, dementia, cognitive impairment, and medications. While additionally controlled for the presence of *Parkin* homozygous/compound heterozygous or *LRRK2* mutations, the prevalence of hallucinations in *GBA* mutation carriers remained significantly higher than in *GBA* non-carriers [$p = 0.04$, OR 4 (95%CI 1.1–15.3)]. In terms of motor complications dyskinesia [$p = 0.003$, OR 3.36 (95%CI 1.46–7.75, X^2 test)] and dystonia [$p = 0.009$, OR 12.261 (95% CI 2.34–64.212, Fisher's exact test)] were more prevalent in carriers vs. non-carriers and were independently associated with the carrier status after controlling for age, gender, disease duration, medications, UPDRS III score, and subtypes. When the presence of dyskinesia and dystonia was controlled for the presence of *PARKIN* homozygous/compound heterozygous or *LRRK2* mutations, the result for dyskinesia remained significant [dyskinesia: $p = 0.007$, OR 4.37 (95%CI 1.49–12.85)], but it became non-significant for dystonia ($p = 0.088$). There was no association found between other variables and *GBA* status (**Table 5**).

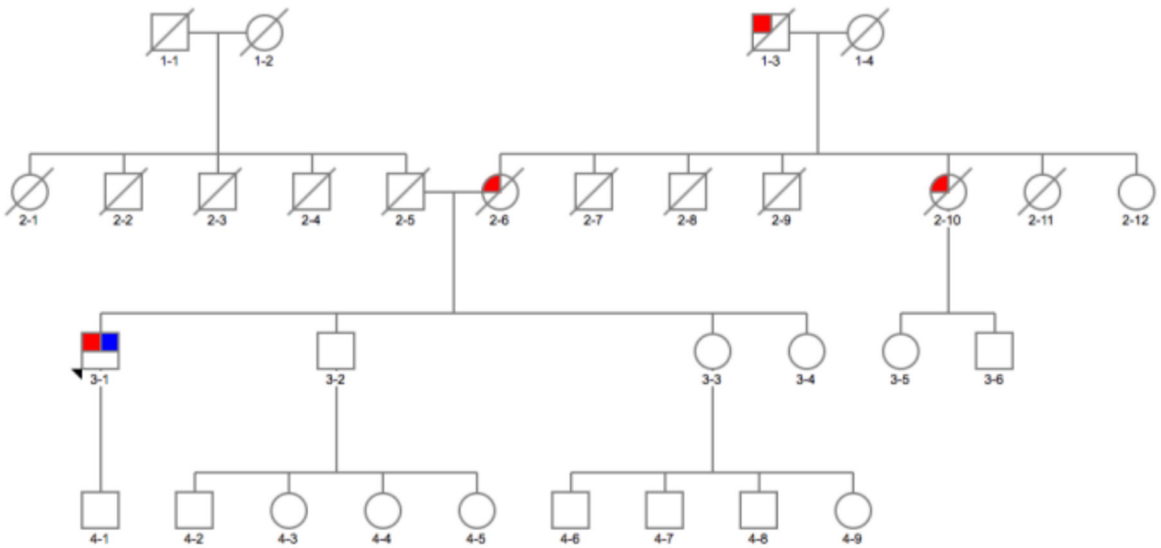

FIGURE 2 | Family pedigree of the patient with *GBA* p.E326K variant in exon 8 and p.G377C variant in exon 9: red, affected with Parkinson's disease (PD); blue, tested and positive for p.E326K/G377C variant; arrow, proband; diagonal line, deceased.

DISCUSSION

GBA mutations carried in the heterozygous state are a strong risk factor for developing α-synucleinopathy including PD. *GBA* variants also appear to act as phenotypic modifiers affecting cognition and motor progression of PD. The frequency of *GBA* variants differs across populations (9, 26, 27), and herein, we show that patients with PD in Ireland have a relatively high frequency of *GBA* mutations (PD, 8.3%; controls, 3.1%). The frequency is higher than that of the Portuguese (6.1%, 14/230) (28), Greek (4.7%, 8/172) (29), Flanders–Belgian population (4.5%, 12/266) (30), and British (4.2%, 33/790) (10), and slightly above the average European carrier frequency (6.7%, 76/1,130) (16). The carrier frequency both in Irish patients and controls was high when compared to that reported in most European studies with the exception of Spain (9.8%, 22/225) (31). The higher *GBA* frequency in Spain could be related to the higher rate of Jewish ancestry in the Iberian Peninsula (32).

While the number of p.N370S and RecNcil carriers was equal in Irish PD (with no carriers of p.L444P found), it has been reported that p.N370S is more prevalent in the Europeans and Ashkenazi Jews (70%), and p.L444P is the most common mutation in Chinese (62%) (27, 33). It could be argued that p.T369M should not be included in our pathogenic/risk group, as the number of p.T369M carriers was greater in the control group p.T369M ($n = 6$) than in the PD group ($n = 5$ heterozygotes and $n = 1$ compound heterozygote with p.E326K); however, we did include p.T369M variant in the pathogenic/risk group in agreement with the most recent literature (4, 6, 14, 15).

We detected a p.G377C (p.G416C, c.1246G > T) variant. The G > T nucleotide change in position 416 has not been found in the available databases; however, a change G > A (p.G416S, rs121908311) in the same position is classified as pathogenic. The β-glucosidase enzyme level in a heterozygote carrier within the normal range found in our patient is consistent with the report by Alcalay et al. (26) (p.E326K does not cause Gaucher's disease even in a homozygote state, and the enzymatic level is also within the normal range) (26). The p.G377C variant was described in one case study of Gaucher's disease from Northern Ireland (34) as a compound heterozygote, but not in PD. Only homozygous or compound heterozygous variants cause Gaucher's disease; therefore, p.G377C was necessary to contribute to the disease in the case report by Illingworth (34). Moreover, the level of plasma chitotriosidase was 8,000 in the Illingworth (34) case, further supporting symptomatic Gaucher's disease (the level of chitotriosidase is only elevated in symptomatic patients with Gaucher's disease). Taken together, these evidence support the likely pathogenicity of this variant.

We also identified a p.G195E variant implicated in Gaucher's disease (25) that cosegregated with PD, which merits further investigation (the at-risk siblings are now older than the affected, but we cannot exclude the development of PD at a later age due to the intrafamilial PD heterogeneity). This finding may suggest a more significant role of p.G195E in PD; however, this variant needs to be further explored.

There was no association of gender with pathogenic/risk variants in PD in keeping with findings from Lesage et al. where there was no difference detected (9). Male/female ratio was also reported 5:1–3:2 in other studies (35). Combined MCI-PD ($n = 5/26$) and PDD ($n = 4/26$) occurred in 34.6%. This is in keeping with other studies (24–48%) (36). The more common cognitive decline (six-fold increased dementia risk) has been reported in the literature (5, 6, 31), but the prevalence of cognitive impairment and dementia analyzed either separately or as one group in our study did not differ between carriers and non-carriers (however, the sample was small). The *GBA*

TABLE 5 | Genotype–phenotype assessment in GBA pathogenic/risk variants carriers with Parkinson's disease.

	GBA pathogenic and risk variants carriers *n* = 26	Non-carriers *n* = 285	*p*-value
Gender	*n* (%)	*n* (%)	
Women	14 (53.8%)	104 (36.5%)	
Men	12 (46.2%)	181 (63.5%)	0.08[a]
	Median (range)	Median (range)	
Age at assessment	64 (33–86)	67 (32–89)	0.58[c]
Age at onset	57 (30–73)	57 (16–85)	0.68[c]
	n (%)	*n* (%)	
Early onset PD < 50	7 (26.9%)	74 (26%)	0.91[a]
	Median (range)	Median (range)	
Disease duration	6 (0–25)	7 (0.5–50)	0.95[c]
Family history	*n* (%)	*n* (%)	
Yes	14 (53.8%)	158 (55.4%)	
No	12 (46.2%)	127 (44.6%)	0.88[a]
Cognition	*n* (%)	*n* (%)	
MCI-PD	5 (19.2%)	54 (18.9%)	1[b]
PDD	4 (15.4%)	31 (10.9%)	0.51[b]
Hallucinations	6 (23.1%)	20 (7%)	**0.01**[b]
Motor complications	14 (53.8%)	122 (42.8%)	0.27[a]
Wearing off	11 (42.3%)	92 (32.3%)	0.29[a]
Dyskinesia	11 (42.3%)	51 (17.9%)	**0.003**[a]
Dystonia4	3 (11.5 %)	3 (1.1%)	**0.009**[b]
Freezing of gait	3 (11.5%)	31 (10.9%)	1[b]
DBS *in situ*	1 (3.8%)	6 (2.1%)	1[b]
	n = 24	*n* = 278	
	Median (range)	Median (range)	
UPDRS-III score	21 (7–54)	22 (3–83)	0.18[c]
UPDRS-III categories	*n* (%)	*n* (%)	
<32 (mild)	19 (79.2%)	224 (80.6%)	
33–58 (moderate)	5 (20.5%)	52 (18.7%)	
> 58 (severe)	0 (0%)	2 (0.7%)	0.1[d]
Falls	3 (11.5%)	25 (8.8%)	0.71[b]
Medications	*n* (%)	*n* (%)	
Levodopa	22 (84.6%)	230 (80.7%)	0.79[b]
MAOBI	16 (61.5%)	169 (59.3%)	0.82[a]
Dopamine agonists	11 (42.3%)	131 (46%)	0.72[a]
Madopar (levodopa+benserazide)	1 (3.8%)	26 (9.1%)	0.7[b]
Anticholinergics	1 (3.8%)	23 (8.1%)	0.51[b]
Amantadine	2 (7.7%)	28 (9.8%)	0.76[b]
Duodopa	0 (0%)	4 (1.4%)	1[b]
Apomorphine	1 (12.5%)	7 (2.5%)	1[b]
Subtype			
Tremor-predominant	13 (50%)	161 (56.5%)	0.52[a]
PIGD	8 (30.8%)	97 (34%)	0.73[a]
Mixed	5 (19.2%)	27 (9.5%)	0.16[b]
First symptom			
Tremor	18 (69.2%)	190 (66.7%)	0.79[a]
Bradykinesia	5 (19.2%)	59 (20.7%)	0.85[a]
Stiffness	0 (0%)	6 (2.1%)	0.67[b]
Writing difficulties	1 (3.8%)	5 (1.8%)	1[b]
Pain	1 (3.8%)	1 (0.4%)	0.16[b]
Speech problems	0 (0%)	2 (0.7%)	1[b]
Gait problems (all)	1 (3.8%)	22 (7.7%)	1[b]
Loss of arm swing	0 (0%)	3 (1.1%)	1[b]
Shuffling	0 (0%)	12 (4.2%)	0.4[b]
Dragging a leg	1 (3.8%)	6 (2.1%)	1[b]
Balance problems	0 (0%)	1 (0.4%)	1[b]

[a]Pearson chi square test, [b]Fisher exact test, [c]Mann–Whitney U-test, [d]Linear by linear Armitage exact trend test. Bold value indicates statistically significant.

mutation/risk variant presence was independently associated with hallucinations in our study in keeping with other studies (31). The three-fold higher occurrence of dyskinesia in the *GBA* carrier group in comparison to non-carriers in our study is in keeping with other reports. The higher occurrence of dyskinesia suggests that *GBA* carriers may be more sensitive to medications and genetic assessment in the appropriate patient should be taken into consideration (5, 9, 36) (similarly to the levodopa sensitivity and more frequent dyskinesia resulting in small levodopa doses being used for parkin mutations carriers). While the number of patients with YOPD in the study ($n = 81$) may seem high, and this group is interesting in itself from the genetic perspective, these were neither specifically preselected for the recruitment nor were they related in any way (only probands were reported in this study).

There are several strengths of our study including the examination of the *GBA* prevalence in Irish PD for the first time, the full sequencing of coding regions (and exon-intron boundaries) of the *GBA* gene on the 3.14% of the Irish PD population (314 PD patients recruited/10,000 number of PD patients in Ireland) (limited screening for p.N370S and p.L444P would result in 81.8% of the mutations being missed in our study), comparison to the ethnically matched control group with a high genotyping success rate, and comprehensive investigation of other causes of autosomal dominant and recessive PD (making this study the most comprehensive and up-to-date report of genetics of PD in the Irish population).

Our study also has a few limitations: it was an observational cross-sectional study; therefore, the progression of the disease could not be assessed, and a longitudinal study should be performed in the future. It should be noted that we were unable to reliably determine phase for the two individuals with a presumed compound heterozygous carrier state, both carried the risk variant E326K (E326K/T369M, $n = 1$; E326K/G377C, $n = 1$); however, these subjects would fall into the risk/pathogenic group regardless of being in "cis" or in "trans" status. While we acknowledge the small sample size of the control group in comparison to the PD group most likely leading

to the non-significance of the *GBA* prevalence data, in our opinion, having a control group is an important part of any *GBA* study.

With the advent of new screening technologies, *GBA* carriers will be encountered more frequently in our clinics, and it will be essential to prepare a mutation-specific approach to the management of PD. Efforts to find a disease-modifying treatment for *GBA* carriers with PD are ongoing. There are currently two clinical trials underway, one trial examining ambroxol, an over-the-counter medication used to reduce mucus production in respiratory tract disorders, and its influence on motor and cognitive progression in *GBA* carriers, and a placebo controlled phase 2 double-blind study (MOVES-PD) of GZ/SAR4027671—a molecule capable of crossing brain-blood barrier (37, 38). Another recently completed trial showed that the target engagement and CSF penetration were accomplished in PD patients treated with oral ambroxol. While the CSF glucocerebrosidase activity decreased, the glucocerebrosidase protein levels and alpha-synuclein levels increased and UPDRS-III score improved by a mean of 6.8 points. Notably, these changes occurred both in patients with and without *GBA* mutations (39). There are no recommendations of how to proceed, where the risk of PD is disclosed, but preventative approaches may be rapidly approaching. Patients should be informed about the increased possibility of cognitive decline, depression, falls, autonomic vulnerability, family (35) planning, and disease progression [carriers are reported to die earlier at 75.7 years (SD 5.5) than non-carriers 80.9 (6.6)] (40). Treatment options for *GBA* mutation carriers should be a little bit distinct and focus on the avoidance of medications increasing the risk of falls (lowering blood pressure), worsening cognitive status, and deferral of levodopa therapy (36). Further research is required, but personalized therapeutic approach for PD may be closer than we might think.

CONCLUSION

We present the most comprehensive and up-to-date overview of genetics of PD in the Irish population. We, for the first time, showed the link between *GBA* and PD in Ireland. In agreement with our hypothesis, the findings of our study suggest that the *GBA* prevalence in PD is higher than in most European countries, and genetic background of Irish PD patients warrants further studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mater Misericordiae University Hospital, Dublin, Ireland 1/378/1300. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DO: study idea and design, study recruitment and phenotypic assessment, DNA extraction, GBA sequencing, and laboratory work, interpretation of the results and statistical analysis, preparation of the first draft of manuscript, correction, and approval of the final draft. AM: study recruitment and phenotypic assessment, DNA extraction, critique, and approval of the final draft. AS-B and RW: laboratory work, critique, and approval of the final draft. BM: study recruitment, critique, and approval of the final draft. RM and OH: critique and approval of the final draft. OR: study idea, lead of the laboratory work and expertise, overlooking the laboratory work at the Mayo Clinic, expertise in the interpretation of the results, critique, and approval of the final draft. TL: study idea and design, lead of the neurological expertise, overlooking the study in the Dublin Neurological Institute, critique, and approval of the final draft. All authors: read and approved the final version on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00527/full#supplementary-material

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Genomic Analysis Identifies New Loci Associated With Motor Complications in Parkinson's Disease

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Background: Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by a clinical symptomatology involving both motor and non-motor symptoms. Motor complications associated with long-term dopaminergic treatment include motor fluctuations and levodopa-induced dyskinesia (LID), which may have a major impact on the quality of life. The clinical features and onset time of motor complications in the disease course are heterogeneous, and the etiology remains unknown.

Objective: We aimed to identify genomic variants associated with the development of motor fluctuations and LID at 5 years after the onset of PD.

Methods: Genomic data were obtained using Affymetrix Axiom KORV1.1 array, including an imputation genome-wide association study (GWAS) grid and other GWAS loci; functional variants of the non-synonymous exome; pharmacogenetic variants; variants in genes involved in absorption, distribution, metabolism, and excretion of drugs; and expression quantitative trait loci in 741 patients with PD.

Results: *FAM129B* single-nucleotide polymorphism (SNP) rs10760490 was nominally associated with the occurrence of motor fluctuations at 5 years after the onset of PD [odds ratio (OR) = 2.9, 95% confidence interval (CI) = 1.8–4.8, $P = 6.5 \times 10^{-6}$]. *GALNT14* SNP rs144125291 was significantly associated with the occurrence of LID (OR = 5.5, 95% CI = 2.9–10.3, $P = 7.88 \times 10^{-9}$) and was still significant after Bonferroni correction. Several other genetic variants were associated with the occurrence of motor fluctuations or LID, but the associations were not significant after Bonferroni correction.

Conclusion: This study identified new loci associated with the occurrence of motor fluctuations and LID at 5 years after the onset of PD. However, further studies are needed to confirm our findings.

Keywords: genome-wide association study, genomic variants, Parkinson's disease, motor fluctuations, levodopa-induced dyskinesia

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INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder characterized by a heterogeneous clinical symptomatology involving both motor and non-motor symptoms (1–3). The pathological hallmarks of PD are abnormal accumulation of alpha-synuclein (α-syn) aggregates, Lewy bodies, and Lewy neurites (4, 5). The α-synucleinopathy in PD involves not only dopaminergic neurons in the substantia nigra pars compacta of the midbrain but also other vulnerable neurotransmitter systems in the central nervous system (6, 7).

Levodopa is the most effective and potent medication for the treatment of motor symptoms of PD (8), and early treatment with levodopa increases life expectancy (9). However, long-term treatment of patients with PD with levodopa can result in the occurrence of motor fluctuations and dyskinesias. These late motor complications can become major causes of disability and reduce the quality of life of patients (10). To date, the pathophysiological mechanisms underlying motor fluctuations and levodopa-induced dyskinesia (LID) in patients with PD remain unclear.

Over the last two decades, rare variants of more than 20 genes have been reported to cause genetic PD (11). The common genetic risk factors for sporadic PD have been identified by genome-wide association studies (GWAS). To date, 90 independent genetic variants have been identified as risk factors for sporadic PD (12). Although previous GWAS and other genetic studies have indicated the importance of genetic contribution to the development of PD, the contribution of genetic factors to specific phenotypes of PD has not been well-studied. Identification of genetic risk factors for the major clinical phenotypes of PD may provide important insights into the underlying molecular mechanisms and valuable information for potential adjustments to overcome genetic heterogeneity in clinical trials. This GWAS aimed to identify the genetic variants associated with the occurrence of motor fluctuations and LID in patients with sporadic PD.

MATERIALS AND METHODS

Patients

We included 741 patients who were diagnosed with PD (**Supplementary Figure 1**). Experienced movement disorder specialists (SJC, HSR, MJK, JK, and YJK) made the diagnosis of PD using the clinical diagnostic criteria of the United Kingdom Parkinson's Disease Society Brain Bank (13). All patients were enrolled from the clinical practice of the Department of Neurology of the Asan Medical Center, Seoul, South Korea, between January 1, 2011 and April 30, 2016. All patients were born and resided in South Korea. All patients were unrelated and ethnic Koreans without any foreign ancestry. The Institutional Review Board (IRB) of Asan Medical Center approved the study, and all patients provided an informed consent in accordance with the IRB regulations.

Clinical Assessment

Motor fluctuations were defined as alternating between periods of good motor symptom control (on-time) and periods of reduced motor symptom control (off-time), which were dependent on the scheduled intake time of levodopa and other dopaminergic medications (14). The time between the onset of PD motor symptoms and the occurrence of motor fluctuations was assessed in each patient.

LID was defined as involuntary choreiform or dystonic body movements, which occur most frequently when levodopa concentrations are at its highest (peak-dose dyskinesia) or, less commonly, at the beginning or end of levodopa administration, or both (diphasic dyskinesia) (14). The time between the onset of PD motor symptoms and the occurrence of LID was assessed in each patient. PD onset was defined as the onset of first motor symptoms in patients with PD.

The presence of motor fluctuations or LID was determined using the clinical history and Unified Parkinson's Disease Rating Scale (UPDRS) part IV. Dystonia that occurred in the morning before taking a medication was not considered as LID (15).

Genomic Analysis

Genotype data were obtained using the Korean Chip (K-CHIP), obtained from the K-CHIP consortium. K-CHIP was designed by the Center for Genome Science, Korea National Institute

TABLE 1 | Demographic and clinical characteristics of patients.

Characteristic	Patients
Total sample, *n*	741
Men, *n* (%)	325 (43.9)
Women, *n* (%)	416 (56.1)
Age at onset of PD, years, mean ± SD (range)	57.1 ± 0.1 (28–87)
Disease duration, years, mean ± SD (range)	10.8 ± 4.5 (5–31)
Patients with motor fluctuations, *n* (%)	554 (74.8)
Duration between PD onset and development of motor fluctuations, years, mean ± SD (range)	6.9 ± 3.4 (1–24)
Patients with levodopa-induced dyskinesia, *n* (%)	496 (66.8)
Duration between PD onset and development of levodopa-induced dyskinesia, years, mean ± SD (range)	7.2 ± 3.4 (1–21)
Patients with motor fluctuations at 5 years after PD onset, *n* (%)	219 (29.6)
Duration between PD onset and development of motor fluctuations, years, mean ± SD (range)	3.9 ± 1.1 (1–5)
Patients with levodopa-induced dyskinesia at 5 years after PD onset, *n* (%)	172 (23.2)
Duration between PD onset and development of levodopa-induced dyskinesia, years, mean ± SD (range)	3.9 ± 1.1 (1–5)
MMSE score (range)	26.1 ± 3.2 (10–30)
MoCA score (range)	22.6 ± 5.6 (3–30)

PD, Parkinson's disease; SD, standard deviation; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment.

of Health, Korea (4845-301, 3000-3031) (www.cdc.go.kr). K-CHIP uses Affymetrix Axiom Customized Biobank Genotyping Arrays (Affymetrix, Santa Clara, CA, USA) and contains 827,783 variants. K-CHIP consists of an imputation GWAS grid [505,000 Asian-based grid with minor allele frequency (MAF) >5% in Asians]; exome contents [84,000 Korean-based grid with MAF >5%, in Koreans; 149,000 coding single-nucleotide polymorphisms (cSNPs); and insertions and deletions on the basis of data from 2000 whole exome sequences and 400 whole genome sequences with MAF> 0.1%]; new exome/loss of function contents (44,000 variants); expression quantitative trait loci (17,000 variants); absorption, distribution, metabolism, and excretion genes; and other miscellaneous variants.

Sample Quality Controls

The primary sample quality control was as follows: samples with low call rate (<0.95%) were excluded from the analysis because of the possibility of low DNA quality or experimental error; high heterozygosity was excluded from the analysis because of low DNA quality or possible contamination of samples. The entire sample distribution was checked, and low-quality samples were excluded if they deviated significantly from the entire sample distribution. SNP pruning was also performed. Because cryptic first-degree relative and multidimensional scaling (MDS) analyses are very time consuming when using whole data, only representative SNP information based on linkage disequilibrium were selected from the data. Due to the possibility of population stratification, samples that deviated from the whole sample were excluded from the analysis by assessing the MDS. If there were more than a certain number of SNPs with only one sample, the possibility of errors due to DNA quality and technical artifacts was excluded.

Secondary sample quality control consisted of genotype calling, excluding samples deemed to be of low quality based on the primary sample quality control criteria and sex-inconsistent samples. Samples that did not satisfy the quality control criteria after a repeat sample quality control were excluded. SNP data were excluded from the cryptic first-degree relative analysis

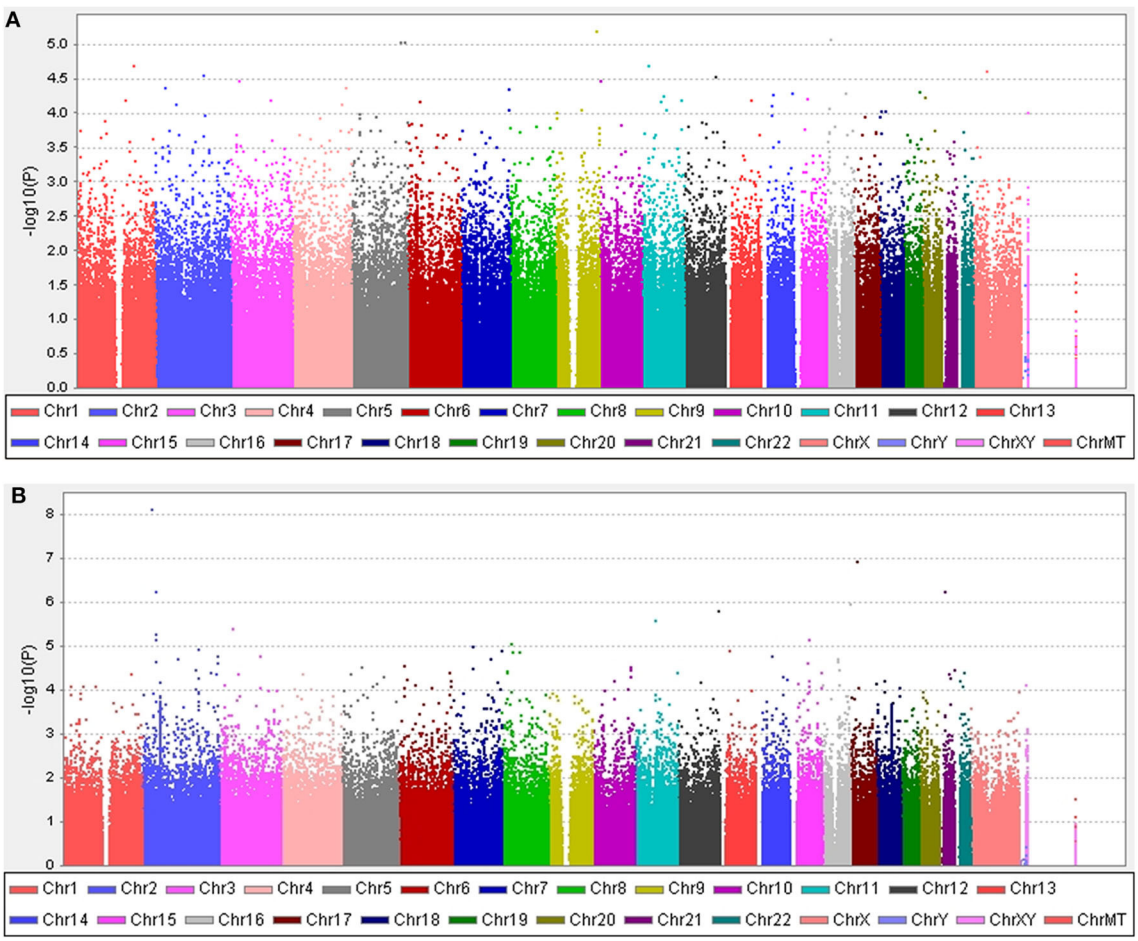

FIGURE 1 | Manhattan plots. **(A)** The plot shows *P*-values for association analyses between 583,535 SNPs and the occurrence of motor fluctuations 5 years after the onset of Parkinson's disease (PD). The most significant association observed is with a locus on chromosome 9 (rs10760490). **(B)** The plot shows *P*-values for association analyses between 583,535 SNPs and the occurrence of levodopa-induced dyskinesia 5 years after the onset of PD. The most significant association observed is with a locus on chromosome 2 (rs144125291). *P*-values are log-transformed (*y*-axis) and plotted against chromosomal position (*x*-axis).

because statistical analysis assumes the independence for each sample in most cases.

SNP Quality Controls

An SNPolisher analysis was performed to exclude low-quality SNPs. SNPs with low call rates were excluded when the call rate was <95% because errors in the calling process can occur due to probe design and clustering analysis problems. If the Hardy–Weinberg equilibrium (HWE) test *P*-value of a specific SNP is low, it indicates a probable error in the genotype clustering process; therefore, the HWE $P < 10^{-6}$. If the frequency of a genetic variation is extremely different from that in Korean and Asian populations, there may be a genotype clustering error. Therefore, we excluded cases where the difference in MAF was >0.2. Both cases and controls were excluded if the MAF was <1%.

Statistical Analysis

The associations of each genetic variant with the occurrence of motor fluctuations and LID were investigated using multiple logistic regression models. We used the Cochran–Armitage trend test and the Jonckheere–Terpstra test, and adjusted all analyses by sex and age at onset of PD. For each genetic variant, we calculated the odds ratio (OR), 95% confidence interval (CI), and two-tailed *P*-value. For sensitivity analyses, similar analyses were performed for patients aged ≥50 years at onset of PD to further adjust for the effects of age at onset of PD on the occurrence of motor fluctuations and LID. The *P*-values from the primary analyses were assessed for significance using the Bonferroni correction for multiple comparisons. Clustering quality control was performed by visual inspection of analytic data of SNPs with a $P < 0.0001$. Markers that did not clearly separate between different genotypes and were not closely located in the same genotype were excluded (**Supplementary Figure 2**). Manhattan plots and quantile–quantile plots were constructed for *P*-values for all genotyped variants that passed quality controls.

The statistical analysis was performed using the PLINK program (version 1.90, NIH-NIDDK Laboratory of Biological Modeling, Bethesda, MD, USA), Haploview (version 4.2, Daly Lab at the Broad Institute, Cambridge, MA, USA), LocusZoom (version 1.4, University of Michigan, Department of Biostatistics, Center for Statistical Genetics, Ann Arbor, MI, USA), and R (version 3.1.2, Free Software Foundation, Inc., Boston, MA, USA).

RESULTS

Patients

Clinical and genotyping data were obtained from 741 patients with PD who were followed for at least 5 years after the onset of PD. The demographic and clinical features of study patients are summarized in **Table 1**. The study group consisted of 325 men

TABLE 2 | Top 20 genomic variants associated with the occurrence of motor fluctuations, in decreasing order of statistical significance.

Gene	SNP	Chr	Position	Region relative to gene	Allele (minor/major)	Minor allele frequency (case/control)	OR (95% CI)	*P*-value
FAM129B	rs10760490	9	130335418	Intron	A/G	0.08/0.03	2.93 (1.80, 4.77)	6.50E−06
SNX29	rs150380018	16	12569788	Intron	G/T	0.04/0.01	6.53 (2.54, 16.79)	8.35E−06
C5orf52	rs10051838	5	157102159	Missense	A/G	0.17/0.09	2.09 (1.50, 2.91)	9.07E−06
STK10	rs77462941	5	171598434	Intron	C/T	0.13/0.23	0.50 (0.36, 0.68)	9.41E−06
FAM163A	rs6680679	1	179753147	Intron	G/A	0.14/0.07	2.15 (1.50, 3.07)	2.01E−05
NAV2	rs7949975	11	19985339	Intron	G/C	0.33/0.23	1.71 (1.33, 2.19)	2.05E−05
LOC392452	rs2022502	23	45540415	Upstream, downstream	C/T	0.20/0.10	2.39 (1.58, 3.62)	2.48E−05
GALNT13	rs6710932	2	154872606	Intron	A/G	0.08/0.16	0.45 (0.31, 0.66)	2.81E−05
NFYB	rs75845252	12	104539534	Upstream	T/C	0.09/0.04	2.62 (1.64, 4.19)	2.97E−05
RBMS3-AS3	rs13068014	3	29170975	Downstream	A/C	0.30/0.20	1.71 (1.33, 2.20)	3.31E−05
AKR1C4	rs191812506	10	5272947	Downstream, upstream	C/T	0.05/0.01	4.14 (2.01, 8.55)	3.31E−05
GALNTL6	rs77688865	4	172563203	Upstream, downstream	G/T	0.05/0.01	3.69 (1.90, 7.19)	4.17E−05
GALNT14	rs144125291	2	31106055	Downstream, upstream	T/C	0.05/0.02	3.47 (1.84, 6.52)	4.35E−05
DPP6	rs59309371	7	153938863	Intron	T/C	0.25/0.36	0.59 (0.46, 0.76)	4.51E−05
CTU1	rs117770234	19	51614232	Upstream	A/G	0.04/0.01	4.43 (2.03, 9.68)	4.78E−05
CDH8	rs138852987	16	61482087	Downstream, upstream	C/T	0.06/0.02	3.29 (1.79, 6.05)	5.01E−05
DIO3	rs11624718	14	102069522	Downstream, upstream	G/A	0.39/0.50	0.63 (0.50, 0.79)	5.20E−05
SLC25A21	rs8010937	14	37324893	Intron	A/C	0.13/0.07	2.12 (1.46, 3.07)	5.32E−05
PPP6R3	rs61188641	11	68336714	Intron	G/A	0.04/0.01	4.63 (2.05, 10.47)	5.58E−05
LOC339593	rs6040792	20	11597971	Upstream, downstream	C/T	0.25/0.16	1.74 (1.33, 2.28)	5.83E−05

SNP, single-nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

TABLE 3 | Top 20 genomic variants associated with the occurrence of levodopa-induced dyskinesia, in decreasing order of statistical significance.

Gene	SNP	Chr	Position	Region relative to gene	Allele (minor/major)	Minor allele frequency (case/control)	OR (95% CI)	*P*-value
GALNT14	rs144125291	2	31106055	Downstream, upstream	T/C	0.07/0.01	5.45 (2.87, 10.33)	7.88E−09
C17orf51	rs139221627	17	21715699	Upstream	T/C	0.07/0.02	4.68 (2.51, 8.73)	1.20E−07
C21orf37	rs208892	21	18813490	Intron	A/G	0.40/0.26	1.90 (1.47, 2.45)	5.74E−07
LRPPRC	rs10495912	2	44305461	Upstream, downstream	A/G	0.07/0.02	4.03 (2.24, 7.24)	5.81E−07
CBFA2T3	rs150854091	16	89028784	Intron	A/G	0.08/0.02	3.64 (2.10, 6.33)	1.12E−06
TMEM132C	rs1531246	12	128999121	Intron	G/C	0.18/0.09	2.28 (1.62, 3.21)	1.60E−06
SCGB1D4	rs953169	11	62083542	Upstream	G/A	0.45/0.31	1.80 (1.41, 2.31)	2.57E−06
TMEM158	rs118109628	3	45279523	Downstream, upstream	A/G	0.03/0.003	9.35 (2.96, 29.55)	3.91E−06
LRPPRC	rs12185607	2	44296280	Upstream, downstream	T/G	0.10/0.04	2.86 (1.79, 4.57)	5.25E−06
ADAM10	rs118049686	15	58895720	Intron	A/G	0.06/0.02	4.07 (2.11, 7.86)	6.93E−06
LRPPRC	rs17031893	2	44283172	Upstream, downstream	G/A	0.08/0.03	3.24 (1.89, 5.55)	6.96E−06
EXTL3	rs73564758	8	28521861	Intron, downstream	G/A	0.05/0.01	4.58 (2.20, 9.52)	8.96E−06
ZNF138	rs117999072	7	64228326	Upstream, downstream	A/G	0.08/0.03	3.15 (1.84, 5.37)	1.06E−05
TTC30B	rs6737342	2	178419117	Upstream, downstream	G/A	0.07/0.02	3.37 (1.90, 5.98)	1.19E−05
HSPH1	rs143639498	13	31696395	Downstream	C/T	0.05/0.01	4.48 (2.15, 9.32)	1.25E−05
LOC389602	rs10281583	7	155811374	Downstream	A/G	0.18/0.09	2.11 (1.50, 2.97)	1.30E−05
DUSP26	rs147270897	8	34132814	Intron, upstream	C/T	0.04/0.01	5.39 (2.31, 12.57)	1.36E−05
SOX17	rs183607239	8	55390249	Downstream, upstream	G/A	0.03/0.004	7.50 (2.59, 21.75)	1.40E−05
RPL32P3	rs6795866	3	129064722	Exon, downstream	G/A	0.05/0.01	4.59 (2.15, 9.80)	1.70E−05
TMX1	rs10129471	14	51782967	Intron, downstream, upstream	T/C	0.08/0.03	3.12 (1.81, 5.38)	1.71E−05

SNP, single-nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

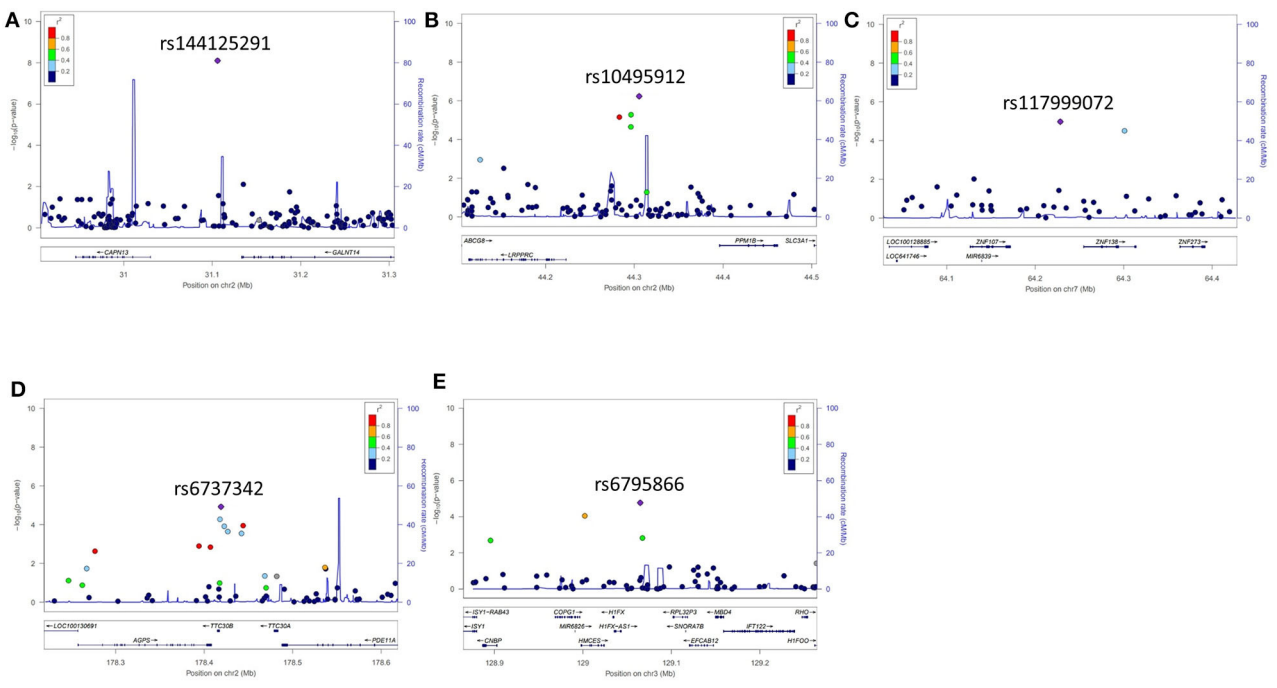

FIGURE 2 | Representative regional plots for genetic variants that showed associations with the occurrence of levodopa-induced dyskinesia 5 years after the onset of Parkinson's disease. **(A)** rs144125291, **(B)** rs10495912, **(C)** rs117999072, **(D)** rs6737342, and **(E)** rs6795866.

TABLE 4 | Demographic and clinical characteristics of patients aged ≥50 years at onset of Parkinson's disease.

Characteristic	Patients
Total sample, *n*	578
Men, *n* (%)	247 (42.7)
Women, *n* (%)	331 (57.3)
Age at onset of PD, years, mean ± SD (range)	61.1 ± 7.1 (50–87)
Disease duration, years, mean ± SD (range)	10.3 ± 3.9 (5–27)
Patients with motor fluctuations, *n* (%)	403 (69.7)
Duration between PD onset and development of motor fluctuations, years, mean ± SD (range)	7.1 ± 3.2 (1–20)
Patients with levodopa-induced dyskinesia, *n* (%)	354 (61.1)
Duration between PD onset and development of levodopa-induced dyskinesia, years, mean ± SD (range)	7.3 ± 3.2 (1–19)
Patients with motor fluctuations 5 years after PD onset, *n* (%)	141 (24.4)
Duration between PD onset and development of motor fluctuations, years, mean ± SD (range)	3.9 ± 1.2 (1–5)
Patients with levodopa-induced dyskinesia 5 years after PD onset, *n* (%)	115 (19.9)
Duration between PD onset and development of levodopa-induced dyskinesia, years, mean ± SD (range)	4.0 ± 1.1 (1–5)
MMSE score (range)	25.8 ± 3.2 (10–30)
MoCA score (range)	21.8 ± 5.7 (3–30)

PD, Parkinson's disease; SD, standard deviation; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment.

(43.9%) and 416 women (56.1%). The mean age at onset of PD was 57.1 years, while the mean disease duration from the onset of PD to the last follow-up was 10.8 ± 4.5 years.

Motor Fluctuations

Five years after the onset of PD, 219 (29.6%) patients exhibited motor fluctuations. No difference was observed between patients with PD with motor fluctuations (92 men, 42.0%) and those without motor fluctuations (233 men, 44.6%) ($P = 0.480$) in terms of sex. The mean age at onset of PD was lower in patients with motor fluctuations than in those without motor fluctuations (54.0 ± 10.3 years vs. 58.4 ± 9.7 years, $P < 0.001$). The mean disease duration between the onset of PD and the last follow-up was shorter in patients with motor fluctuations than in those without motor fluctuations (9.5 ± 4.0 years vs. 11.3 ± 4.6 years, $P < 0.001$; **Supplementary Table 1**). The 583,535 SNPs that passed quality controls were genotyped and analyzed. Quantile–quantile plots were made for the presence of LID at 5 years after onset of PD (**Supplementary Figure 3A**), and a Manhattan plot is described in **Figure 1A**. The top 20 SNPs associated with the occurrence of motor fluctuations are listed in **Table 2**. *FAM129B* SNP rs10760490 was nominally associated with the occurrence of motor fluctuations at 5 years after onset of PD (OR = 2.9, 95% CI = 1.8–4.8, $P = 6.5 \times 10^{-6}$). However, *FAM129B* SNP rs10760490 and other SNPs were not significant after Bonferroni correction (**Table 2**).

Levodopa-Induced Dyskinesia

Five years after the onset of PD, 172 patients had LID (23.2%). No difference was observed between patients with LID (75 men, 43.6%) and those without LID (250 men, 43.9%) ($P = 0.892$) in terms of sex. The mean age at onset of PD was lower in patients with LID than in those without (55.2 ± 10.7 years vs. 57.7 ± 9.8 years, $P = 0.007$). The mean duration between disease onset and the last follow-up was shorter in patients with LID than in those without LID (9.1 ± 3.5 years vs. 11.3 ± 4.6 years, $P < 0.001$; **Supplementary Table 2**). After quality controls, 583,379 SNPs were genotyped and analyzed. Quantile–quantile plots were made for the occurrence of LID (**Supplementary Figure 3B**), and a Manhattan plot is described in **Figure 1B**. The top 20 SNPs associated with the occurrence of LID 5 years after the onset of PD are listed in **Table 3**. The *GALNT14* SNP rs144125291 had the lowest *P*-value and was significantly associated with LID even after Bonferroni correction (OR = 5.5, 95% CI = 2.9–10.3, $P = 7.88 \times 10^{-9}$; **Table 3**). The representative regional association plots of rs10495912, rs117999072, rs6737342, and rs6795866 showed other risk variants within 150 kb (**Figures 2A–E**).

Sensitivity Analysis for Patients With PD Aged ≥50 Years at the Onset of PD

The clinical features of patients with PD are presented in **Table 4** and **Supplementary Tables 3, 4**. Five years after the onset of PD, 141 (24.4%) of 578 patients with PD exhibited motor fluctuations. A Manhattan plot is described in **Figure 3A**. The top 20 SNPs associated with the occurrence of motor fluctuations are listed in **Table 5**. *RABL6* SNP rs191519045 had the lowest *P*-value, but none of the SNPs were significant after Bonferroni correction. Representative regional association plots of rs72850586, rs76767606, and rs12408511 showed other risk variants within 150 kb (**Supplementary Figures 4A–C**).

Five years after the onset of PD, 115 (19.9%) of 578 patients with PD had LID. A Manhattan plot is described in **Figure 3B**. The 20 SNPs associated with the occurrence of LID are listed in **Table 6**. None of these SNPs were significant after Bonferroni correction. Regional association plots of rs117999072, rs149201992, and rs6907129 showed other risk variants within 150 kb (**Supplementary Figures 4D–F**).

DISCUSSION

We found several genetic variants that showed associations with motor fluctuations and LID in patients with PD. The occurrence of motor fluctuations was associated with genetic variants in *FAM129B*, *SNX29*, *C5orf52*, and *STK10* with $P < 1.0 \times 10^{-5}$, although the associations were not significant after Bonferroni correction. The occurrence of LID was most significantly associated with *GALNT14* SNP rs144125291, and this association was significant after Bonferroni correction.

The pathophysiology of LID in PD is not well-understood. The functional state of the basal ganglia may be characterized by changes in the neuronal firing rate and oscillatory neuronal activity, which become excessive and possibly have a pathogenic role in the occurrence of abnormal corticostriatal

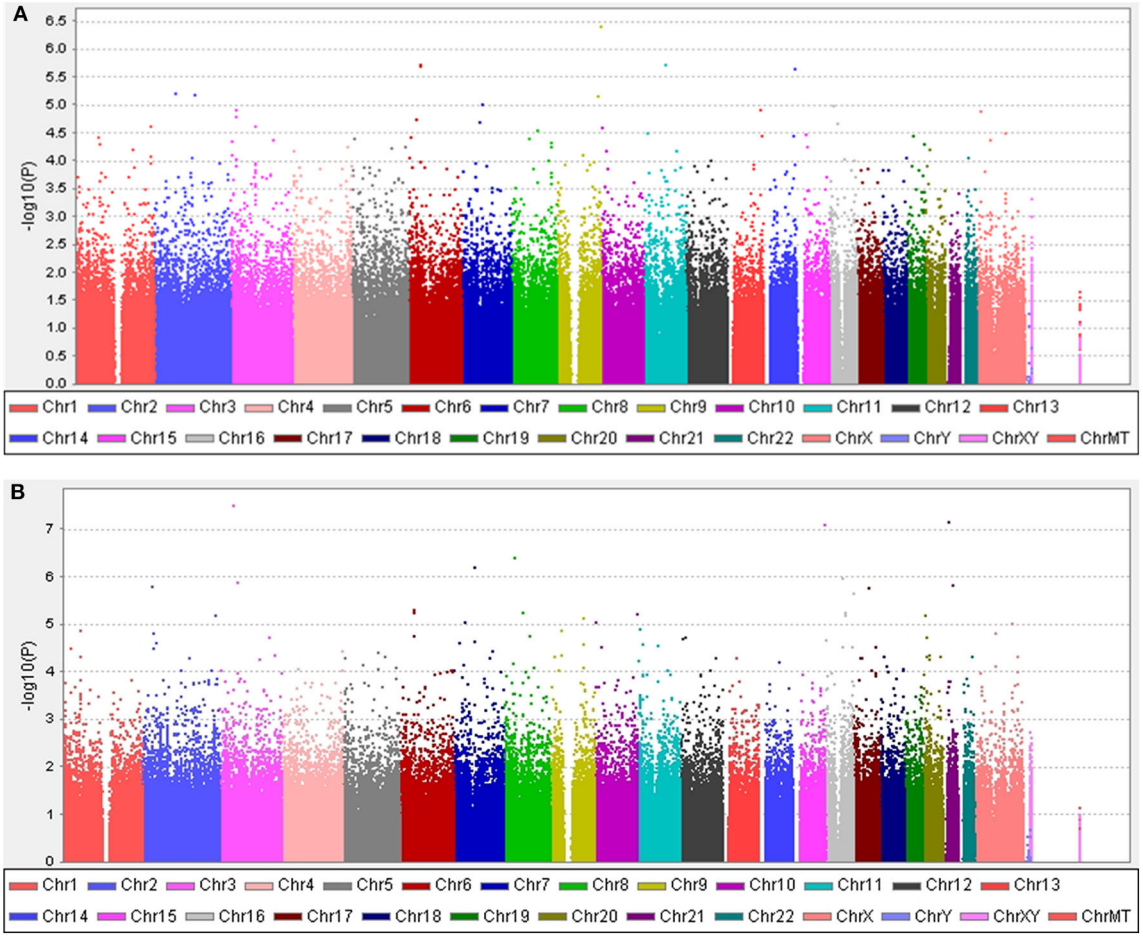

FIGURE 3 | Manhattan plots. **(A)** The plot shows the *P*-values for association analyses between 580,128 SNPs and the occurrence of motor fluctuations 5 years after Parkinson's disease (PD) onset in patients aged ≥50 years at disease onset. The most significant association observed is with a locus on chromosome 9 (rs191519045). **(B)** The plot shows *P*-values for association analyses between 579,399 SNPs and the occurrence of levodopa-induced dyskinesia 5 years after onset of PD in patients aged ≥50 years at disease onset. The most significant association observed is with a locus on chromosome 3 (rs118109628). *P*-values are log-transformed (*y*-axis) and plotted against chromosomal position (*x*-axis).

connectivity (16). These mechanisms have been implicated in the pathophysiology of LID in PD. A polymorphism in brain-derived neurotrophic factor, recognized as modulating human cortical plasticity, affects the time to onset of LID in PD in addition to the response to rTMS (17, 18). Further studies using non-invasive brain stimulation techniques may be warranted to clarify the role of those genetic variants in LID.

GALNT14 SNP rs144125291 is located in the intergenic region 27,276 bases downstream of the gene variant for *GALNT14*. The *GALNT14* gene encodes a Golgi protein that is a member of the polypeptide *N*-acetylgalactosaminyltransferase protein family (19). This enzyme catalyzes the transfer of *N*-acetyl-D-galactosamine to the hydroxyl group on serines and threonines in target peptides (19). Alterations in this gene may play a role in cancer progression and response to chemotherapy in several types of cancer (20–26). Some genes, such as *LRRK2* and *PRKN*, may be associated with both cancer and PD (27–30). *GALNT14* contributes to breast cancer invasion by altering cell proliferation and motility, by altering the expression levels of *EMT* genes, and by stimulating MMP-2 activity (31). MMP-2 is reported to play a role in the inflammatory response (32). *GALNT14* may also cause abundant post-translational modifications, such as glycosylation, which is closely related to tumor growth and metastasis as well as resistance to chemotherapy (33). The development of LID in patients with PD is also related to altered post-synaptic transcription factors and maladaptive plasticity in the nigrostriatal neurons (34). Although the precise pathogenic mechanisms of LID remain unclear, chronic inflammation in the brain and altered post-synaptic plasticity may play key roles in the development of LID (34–36). *GALNT14* SNP rs144125291 may affect the basal level of neuroinflammation in the brain or maladaptive post-synaptic plasticity. However, further functional studies are needed to elucidate the precise role of *GALNT14* in LID.

Several other genes also showed possible association with the occurrence of LID, including *LRPPRC*. *LRPPRC* SNP

TABLE 5 | Top 20 genomic variants associated with the occurrence of motor fluctuations in patients aged ≥50 years at onset of Parkinson's disease.

Gene	SNP	Chr	Position	Region relative to gene	Allele (minor/major)	Minor allele frequency (case/control)	OR (95% CI)	*P*-value
RABL6	rs191519045	9	139707344	Intron, exon	G/A	0.04/0.002	17.66 (3.89, 80.15)	3.81E−07
PPP6R3	rs61188641	11	68336714	Intron	G/A	0.05/0.01	8.40 (2.97, 23.79)	1.91E−06
SAYSD1	rs72850586	6	39153495	Upstream, downstream	G/A	0.05/0.01	8.40 (2.97, 23.77)	1.92E−06
SAYSD1	rs72850539	6	39137869	Upstream, downstream	A/G	0.05/0.01	8.36 (2.95, 23.66)	2.05E−06
DIO3	rs11624718	14	102069522	Downstream, upstream	G/A	0.35/0.49	0.51 (0.39, 0.68)	2.28E−06
ANTXR1	rs56216132	2	69377298	Intron	A/C	0.50/0.35	1.87 (1.42, 2.46)	6.07E−06
MAP3K2	rs147429309	2	128136163	Intron, upstream	T/G	0.05/0.01	6.99 (2.63, 18.57)	6.61E−06
FAM129B	rs10760490	9	130335418	Intron	A/G	0.10/0.03	3.24 (1.89, 5.55)	7.10E−06
ZNF92	rs190170956	7	65066217	Upstream, downstream	C/T	0.04/0.003	10.62 (2.90, 38.88)	9.74E−06
SNX29	rs150380018	16	12569788	Intron	G/T	0.04/0.004	8.75 (2.76, 27.69)	1.00E−05
TBC1D5	rs73817453	3	18117165	Intron	A/G	0.05/0.01	5.38 (2.33, 12.42)	1.20E−05
LINC00460	rs117816291	13	106915837	Upstream, downstream	A/G	0.05/0.01	5.38 (2.33, 12.42)	1.20E−05
GRPR	rs12009947	23	16108832	Upstream, downstream	T/C	0.22/0.41	0.41 (0.27, 0.62)	1.31E−05
TBC1D5	rs76767606	3	18064472	Intron	A/G	0.05/0.01	5.63 (2.34, 13.56)	1.61E−05
CMAHP	rs6456661	6	25214720	Upstream, downstream	A/G	0.05/0.01	6.04 (2.38, 15.29)	1.79E−05
NUPR1L	rs146088024	7	56232344	Downstream, upstream	C/A	0.06/0.01	4.98 (2.21, 11.21)	1.99E−05
XPO6	rs142186210	16	28138044	Intron, exon	G/A	0.05/0.01	5.96 (2.35, 15.09)	2.12E−05
GBE1	rs6798680	3	81905441	Intron, upstream, downstream	A/C	0.38/0.48	0.56 (0.42, 0.73)	2.40E−05
RYR2	rs12408511	1	237842915	Intron	T/A	0.10/0.04	2.92 (1.74, 4.91)	2.47E−05
PFKP	rs117516530	10	2966617	Upstream, downstream	G/A	0.17/0.08	2.31 (1.55, 3.43)	2.58E−05

SNP, single-nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

rs10495912 showed a possible association with LID and is an intergenic variant located 60,028 bases upstream of *LRPPRC*. *LRPPRC* encodes a leucine-rich pentatricopeptide motif-containing protein that predominantly localizes to the mitochondria. The pentatricopeptide repeat (PPR) protein family plays a major role in RNA stability, regulation, processing, splicing, translation, and editing (37). LRPPRC regulates energy metabolism, and the maturation and export of nuclear mRNA. *LRPPRC* mutations have been found to cause Leigh syndrome in a French–Canadian population and are associated with reduced levels of LRPPRC and lower steady-state levels of mitochondrial transcripts (38). Leigh syndrome is an inherited neurometabolic disorder characterized by the occurrence of severe and deadly acidotic crises due to a tissue-specific deficiency in cytochrome c oxidase (38). An *LRPPRC* intronic variant can affect the normal splicing of *LRPPRC* and has been associated with susceptibility to PD (39). Mitochondrial susceptibility in the putamen is reported to play a role in the development of dyskinesia in patients with PD (40), suggesting that abnormal energy metabolism caused by *LRPPRC* variants may be associated with the occurrence of LID. However, further genetic and functional studies are needed to elucidate the role of *LRPPRC* in the development of LID.

Of the genes associated with the occurrence of motor fluctuations, *FAM129B* showed the lowest *P*-value (OR = 2.93, 95% CI = 1.8–4.8, $P = 6.5 \times 10^{-6}$). Knockdown of FAM129B in HeLa cells accelerates the onset of apoptosis induced by TNF-α (41). Activation of the inflammatory response is closely associated with the pathogenesis of PD, and the increased release of pro-inflammatory cytokines such as TNF-α, interleukin-1β, and interferon-γ has been observed in the post-mortem brain of a PD patient (42). In addition to susceptibility to PD, neuroinflammation in the striatum as well as in the substantia nigra pars compacta may play an important role in the development of motor fluctuations in PD via presynaptic and post-synaptic mechanisms. The storage hypothesis for motor fluctuations posits that the loss of presynaptic dopaminergic terminals reduces the capacity for storage of dopamine in the striatum, thereby inhibiting the ability to compensate for oscillations in plasma levodopa levels, and neuroinflammation may contribute to this effect (43). Neuroinflammation and chronic overproduction and abnormal release of TNF-α by microglia may also contribute to the post-synaptic mechanisms of motor fluctuations, which may be associated with complex striatal functional abnormalities in basal ganglia motor circuits (44). Further functional studies are necessary to investigate the precise role of *FAM129B* in neuroinflammation in PD.

TBC1D5, which showed a possible association with the occurrence of motor fluctuations in patients with PD aged over 50 years, functions as a GTPase-activating protein for RAB7 and inhibits recruitment of the VPS35/VPS29/VPS26 subcomplex to membranes (45). The retromer complex is a key component of the endosomal protein sorting machinery and mediates cargo selection through a trimeric complex comprising VPS35/VPS29/VPS26, which is recruited to endosomes by

TABLE 6 | Top 20 genomic variants associated with the occurrence of levodopa-induced dyskinesia in patients aged ≥50 years at the onset of Parkinson's disease.

Gene	SNP	Chr	Position	Region relative to gene	Allele (minor/major)	Minor allele frequency (case/control)	OR (95% CI)	*P*-value
TMEM158	rs118109628	3	45279523	Downstream, upstream	A/G	0.04/0.002	20.95 (4.56, 96.32)	3.26E−08
C21orf37	rs208892	21	18813490	Intron	A/G	0.43/0.25	2.26 (1.67, 3.05)	6.84E−08
PCSK6	rs12908851	15	102042815	Intron, upstream, downstream	T/C	0.07/0.01	6.82 (3.05, 15.24)	8.15E−08
DUSP26	rs147270897	8	34132814	Intron, upstream	C/T	0.05/0.01	8.62 (3.20, 23.24)	3.89E−07
ZNF138	rs117999072	7	64228326	Upstream, downstream	A/G	0.09/0.02	4.33 (2.32, 8.08)	6.42E−07
CHD9	rs149201992	16	52837183	Downstream, upstream	C/T	0.07/0.01	5.81 (2.63, 12.82)	1.04E−06
HESX1	rs191751991	3	57241967	Intron, upstream	G/A	0.04/0.003	12.65 (3.40, 47.12)	1.35E−06
EVA1C	rs141704048	21	33771938	Upstream, downstream	G/A	0.04/0.003	12.53 (3.36, 46.66)	1.54E−06
GALNT14	rs144125291	2	31106055	Downstream, upstream	T/C	0.07/0.01	5.23 (2.48, 11.03)	1.56E−06
LOC284080	rs75357358	17	48123894	Downstream, upstream	A/C	0.04/0.003	12.45 (3.34, 46.38)	1.68E−06
CBFA2T3	rs150854091	16	89028784	Intron	A/G	0.08/0.02	4.30 (2.24, 8.26)	2.28E−06
CYP39A1	rs6907129	6	46597608	Intron	T/G	0.04/0.01	8.36 (2.83, 24.69)	5.10E−06
CYP39A1	rs6905960	6	46597262	Intron	G/A	0.04/0.01	8.34 (2.82, 24.64)	5.25E−06
CYP39A1	rs7749491	6	46598263	Intron	G/A	0.04/0.01	8.32 (2.82, 24.58)	5.41E−06
CYP39A1	rs16874881	6	46596379	Intron	T/A	0.04/0.01	8.30 (2.81, 24.53)	5.57E−06
RNU6-21P	rs11648356	16	62206339	Downstream, upstream	T/C	0.07/0.01	4.94 (2.31, 10.53)	5.58E−06
CA8	rs72661489	8	60908452	Downstream, upstream	T/C	0.05/0.01	6.32 (2.55, 15.64)	5.85E−06
MIR378C	rs60808734	10	132367515	Downstream	C/A	0.05/0.01	6.30 (2.55, 15.61)	6.04E−06
CDH8	rs58952871	16	62050053	Intron	C/T	0.07/0.01	4.89 (2.29, 10.43)	6.45E−06
PLCB1	rs58120268	20	8120394	Intron	G/A	0.07/0.01	4.89 (2.29, 10.43)	6.45E−06

SNP, single-nucleotide polymorphism; Chr, chromosome; OR, odds ratio; CI, confidence interval.

binding to RAB7a and SNX3 (46). This retromer function is closely linked to PD. *VPS35* mutations are a rare cause of autosomal dominant late-onset PD. The clinical features of PD with *VPS35* mutations were as follows: lower onset age, good response to levodopa, and motor complications (47). *VPS13C* mutations are a rare cause of autosomal recessive early-onset PD. The clinical features of PD with *VPS13C* mutations suggested that the progression is rapid and severe (48). Thus, *VPS*-related variants might be associated with motor complications in patients with PD. *RYR2*, which also associates with the occurrence of motor fluctuations in patients with PD aged over 50 years ($P = 2.5 \times 10^{-5}$), encodes a ryanodine receptor. Ryanodine receptors are intracellular calcium release channels found in the endoplasmic reticulum of all cells, with RYR2 predominating among the three isoforms (RYR1, RYR2, and RYR3) (49). When cellular Ca^{2+}-regulating systems are compromised, synaptic dysfunction, impaired plasticity, and neuronal degeneration occur, such as in PD (50). Functional studies are needed to clarify the roles of *TBC1D5* and *RYR2* in the occurrence of motor fluctuations in PD.

The genetic association studies using a small number of pre-specified genetic region were able to determine the genetic risk variants for LID. A previous study reported that the Val158Met variant of catechol-*O*-methyltransferase was associated with LID (51). In another previous study, 229 (45.5%) of 503 Korean patients with PD experienced LID during the mean disease duration of 10.9 years (52). In their candidate gene association study, only the p.S9G variant of dopamine receptor D3 was associated with the occurrence of diphasic dyskinesia (52). However, these studies had limitations as only a limited number of candidate genes were selected due to their incomplete understanding of the pathophysiology of motor complications. Our GWAS investigated a genome-wide set of genetic variants, and this hypothesis-free GWAS may provide a comprehensive evaluation of genetic risk factors for motor complications.

This study has limitations. First, our study used retrospective clinical data. Motor fluctuations and LID are closely related to the pattern and dosage of dopaminergic medications, which were not randomized due to the inherent limitations of a retrospective study. The prevalence of motor fluctuations (29.6%) and LID (23.2%) was slightly lower in the present study than in the previous clinical studies (53, 54); however, this rate of motor complications may be dependent on the patterns of prescribing dopaminergic medications (55, 56). Recently, the prevalence of motor complications is now ∼20–28%, which is comparable to what we observed (57, 58). Motor fluctuations and LID are complex phenomena where several factors may contribute to their development and further studies are required to better understand their pathophysiology (59). Second, we assessed the UPDRS for the evaluation of LID, but we did not use more specific assessment tools, such as Unified Dyskinesia Rating Scale, due to practical issues. Hence, future studies should perform a more detailed clinical assessment of LID. Third, our

sample size was small compared with that of the traditional GWAS. Deep phenotyping in larger samples is challenging; thus, a well-designed GWAS on clinically important issues should be conducted.

In conclusion, this study provides new insights into the genetic contributions to motor fluctuations and LID in PD. Future collaborative longitudinal genomic studies are needed to further investigate the genetic risk factors associated with motor fluctuations and LID in patients with PD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories: https://www.ncbi.nlm.nih.gov/SNP/snp_viewBatch.cgi?sbid=1063124. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of Asan Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SC contributed to the conception, organization, execution of the research project, design, execution, review, critique of the statistical analysis, writing of the first draft, and review and critique of the manuscript. H-SR contributed to the execution of the research project, design, execution, review, critique of the statistical analysis, writing of the first draft, and review and critique of the manuscript. KP, NC, JiK, Y-MP, SJ, M-JK, YK, JuK, KK, and S-BK contributed to the execution of the research project, design, execution, review, critique of the statistical analysis, and review and critique of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00570/full#supplementary-material

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Longitudinal Monitoring of Parkinson's Disease in Different Ethnic Cohorts: The DodoNA and LONG-PD Study

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Background: Different factors influence severity, progression, and outcomes in Parkinson's disease (PD). Lack of standardized clinical assessment limits comparison of outcomes and availability of well-characterized cohorts for collaborative studies.

Methods: Structured clinical documentation support (SCDS) was developed within the DNA Predictions to Improve Neurological Health (DodoNA) project to standardize clinical assessment and identify molecular predictors of disease progression. The Longitudinal Clinical and Genetic Study of Parkinson's Disease (LONG-PD) was launched within the Genetic Epidemiology of Parkinson's disease (GEoPD) consortium using a Research Electronic Data Capture (REDCap) format mirroring the DodoNA SCDS. Demographics, education, exposures, age at onset (AAO), Unified Parkinson's Disease Rating Scale (UPDRS) parts I-VI or Movement Disorders Society (MDS)–UPDRS, Montreal Cognitive Assessment (MoCA)/Short Test of Mental Status (STMS)/Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS), Epworth Sleepiness Scale (ESS), dopaminergic therapy, family history, nursing home placement, death and blood samples were collected. DodoNA participants (396) with 6 years of follow-up and 346 LONG-PD participants with up to 3 years of follow-up were analyzed using group-based trajectory modeling (GBTM) focused on: AAO, education, family history, MMSE/MoCA/STMS, UPDRS II-II, UPDRS-III tremor and bradykinesia sub-scores, Hoehn and Yahr staging (H&Y) stage, disease subtype, dopaminergic therapy, and presence of autonomic symptoms. The analysis was performed with either cohort as the training/test set.

Results: Patients are classified into slowly and rapidly progressing courses by AAO, MMSE score, H &Y stage, UPDRS-III tremor and bradykinesia sub-scores relatively early in the disease course. Late AAO and male sex assigned patients to the rapidly progressing group, whereas tremor to the slower progressing group. Classification is independent of which cohort serves as the training set. Frequencies of disease-causing variants in *LRRK2* and *GBA* were 1.89 and 2.96%, respectively.

Conclusions: Standardized clinical assessment provides accurate phenotypic characterization in pragmatic clinical settings. Trajectory analysis identified two different trajectories of disease progression and determinants of classification. Accurate phenotypic characterization is essential in interpreting genomic information that is generated within consortia, such as the GEoPD, formed to understand the genetic epidemiology of PD. Furthermore, the LONGPD study protocol has served as the prototype for collecting standardized phenotypic information at GEoPD sites. With genomic analysis, this will elucidate disease etiology and lead to targeted therapies that can improve disease outcomes.

Keywords: longitudinal monitoring, Parkinson's disease, structured clinical documentation, motor symptoms, non-motor symptoms

INTRODUCTION

Parkinson's disease (PD), the second most common neurodegenerative disease has an insidious onset and a long pre-symptomatic and symptomatic course. Four cardinal features that include resting tremor, bradykinesia, rigidity, and postural instability define the motor aspects of the disease. Different disease subtypes have been described including a tremor-predominant, akinetic/rigid predominant and mixed subtype (1). Non-motor features, including cognitive dysfunction, anosmia, anxiety, depression, sleep disorders, and autonomic dysfunction are also observed either alone or in varying combinations. Simuni et al. reported that for the Primary Progression Markers Initiative (PPMI) cohort, the higher baseline non-motor scores were associated with female sex and a more severe motor phenotype (2). Longitudinal increase in non-motor score severity was associated with older age and lower CSF aβ1–42 at baseline.

The temporal profile of the motor symptom appearance and progression is rather variable. A number of different patient cohorts have been followed longitudinally for different lengths of time and identified predictors of disease progression. Mollenhauer et al. analyzing the *De Novo* Parkinson (DeNOPA) cohort reported that baseline predictors of worse progression of motor symptoms included male sex, orthostatic blood pressure drop, diagnosis of coronary artery disease, arterial hypertension, elevated serum uric acid, and CSF neurofilament light chain (3). In the DeNOPA cohort, predictors of cognitive decline in PD were previous heavy alcohol abuse, current diagnoses of diabetes mellitus, arterial hypertension, elevated periodic limb movement index during sleep, decreased hippocampal volume by MRI, higher baseline levels of uric acid, C-reactive protein, high density lipoprotein (HDL) cholesterol, and glucose levels. In their cohort, risk markers for faster disease progression included cardiovascular risk factors, deregulated blood glucose, uric acid metabolism, and inflammation. Lawton et al. reported four clusters from the Tracking Parkinson's and Discovery cohorts: one with fast motor progression and symmetrical motor disease, poor olfaction, cognition, and postural hypotension; a second with mild motor and non-motor disease and intermediate motor progression; a third with severe motor disease, poor psychological well-being, and poor sleep with an intermediate motor progression; and a fourth with slow motor progression with tremor-dominant, unilateral disease (4). From the PPMI cohort, Aleksovski et al. reported that the postural instability gait disorder (PIGD) subtype was characterized by more severe disease manifestations at diagnosis, greater cognitive progression, and more frequent psychosis than tremor predominant patients (5). From the PPMI cohort, Latourelle et al. identified higher baseline MDS-UPDRS motor score, male sex, and increased age, as well as a novel Parkinson's disease-specific epistatic interaction, as indicative of faster motor progression (6). De Pablo-Fernandez et al. reported that the presence of autonomic symptoms contributed to a more rapid and severe disease course (7).

Comparing the findings of the different reported cohorts indicates partially overlapping clinical predictors. At the same time though, they reveal a variable clinical assessment. Here, we present an analysis of disease trajectory by GBTM in two large PD patient cohorts from five different countries followed at a routine clinical practice setting using identical clinical measures (8). We find that over an interval of 13 years, there are two trajectories, one with a more benign and another with a more severe disease progression. Patients can be reliably assigned to either group relatively early in their disease course.

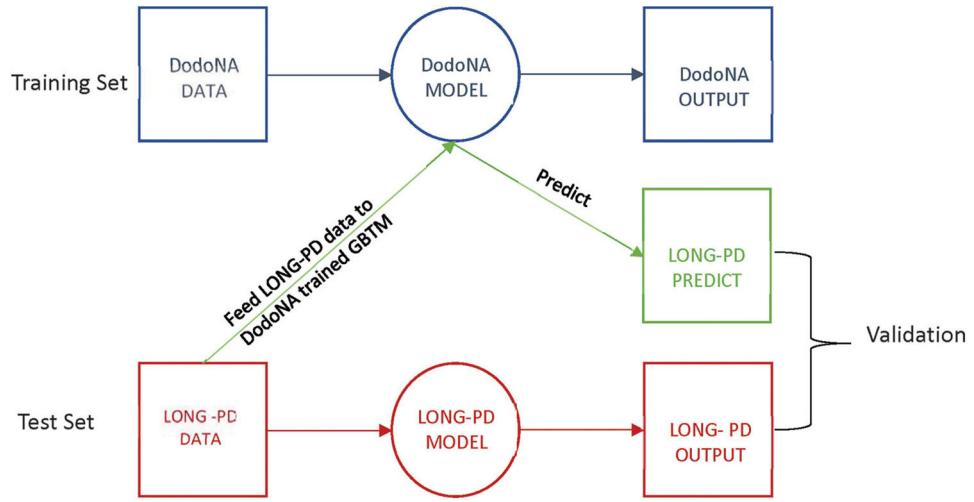
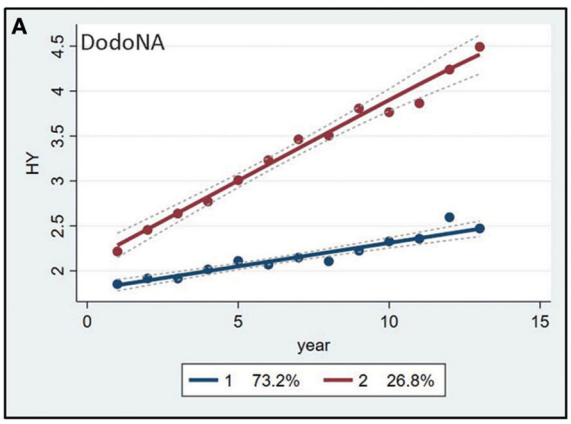
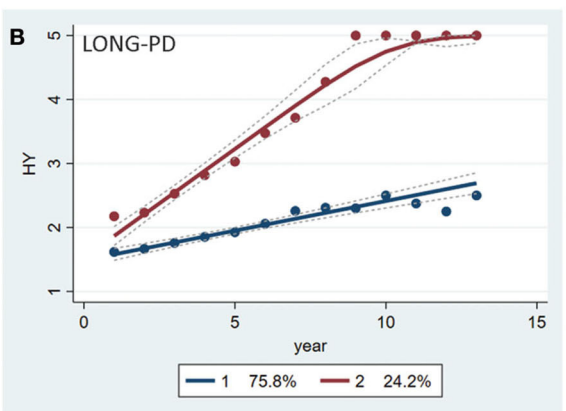


FIGURE 1 | Group-based Trajectory Modeling flowchart.





C

Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	263	16
2	6	61

Accuracy:	0.9364
95% CI:	(0.9053, 0.9597)
Sensitivity:	0.9777
Specificity:	0.7922
Pos Pred Value:	0.9427
Neg Pred Value:	0.9104
Prevalence:	0.7775
Detection Rate:	0.7601

FIGURE 2 | Hoehn and Yahr (H&Y) stage groups in the DNA Predictions to Improve Neurological Health (DodoNA) and Longitudinal Clinical and Genetic Study of Parkinson's Disease (LONG-PD) cohorts. **(A)** The model trained on DodoNA data (training set). **(B)** The model trained on the LONG-PD data (test set). **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.

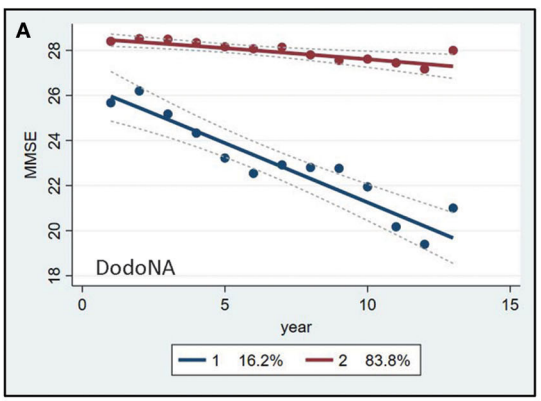
METHODS

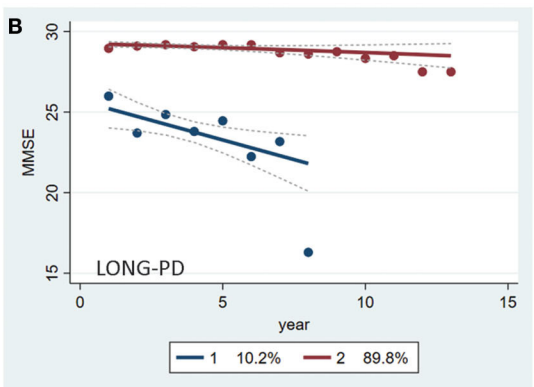
Clinical Information

Two patient cohorts with PD were included in the study: (1) the DNA Predictions to Improve Neurological Health (DodoNA) cohort, which includes patients that are followed longitudinally in the Department of Neurology at NorthShore University HealthSystem in Evanston, Illinois and (2) the Longitudinal Clinical and Genetic Study of Parkinson's Disease (LONG-PD) cohort that includes PD patients enrolled by clinician investigators from Norway, Greece, South Korea, and Sweden. These investigators entered their clinical data through REDCap, a web-based database. The patient information that was submitted from the four different sites is referred to as the LONG-PD cohort in aggregate. The cohorts included both previously diagnosed and naïve patients. A copy of the study protocol is available in the **Supplemental Information**.

The DodoNA Cohort

The goal of interpreting variation in DNA to predict neurological disease led to naming the NorthShore cohort as the "DodoNA" cohort after the Dodona oracle of ancient Greece. The content of the electronic medical record-based (EPIC systems) SCDS toolkit was developed through frequent movement disorder neurologist meetings aimed to reach a consensus on the essential data elements that conform to Best Practices in the treatment of PD, parkinsonism, or tremor patients, taking into consideration relevant literature and American Academy of Neurology (AAN) guidelines (9), and the International Consortium for Health Outcomes Measurement (ICHOM) guidelines (10). The criteria for which rating scales and score test measures to include in the toolkit were: (a) to obtain clinically relevant information in a standardized manner that can be performed at regular intervals; and (b) that the standardized assessment can be performed during an office visit within the time limitations that are imposed by a routine office visit. The toolkit content consists of discretized fields that record detailed information regarding initial and current symptoms, medication history and treatment response, and imaging results, as well as score test measures, including the Geriatric Depression Scale (GDS) (11), Epworth Sleepiness Scale (ESS) (12), United Parkinson's Disease Rating Scale (UPDRS) (13), Part I—Mentation, Behavior and Mood, UPDRS Part II—Activities of Daily Living (ADLs), UPDRS-Part III—Motor Score, UPDRS-IV—Complications of Therapy (COT), UPDRS-Part V—Hoehn and Yahr staging (H&Y), UPDRS-Part VI—Schwab & England Score (S&E), and the Short Test of Mental Status (STMS) (14) that are autoscored. For cognitive assessment, initially, the MoCA (Montreal Cognitive Assessment) (15) was used. However, due to licensing permissions, the STMS





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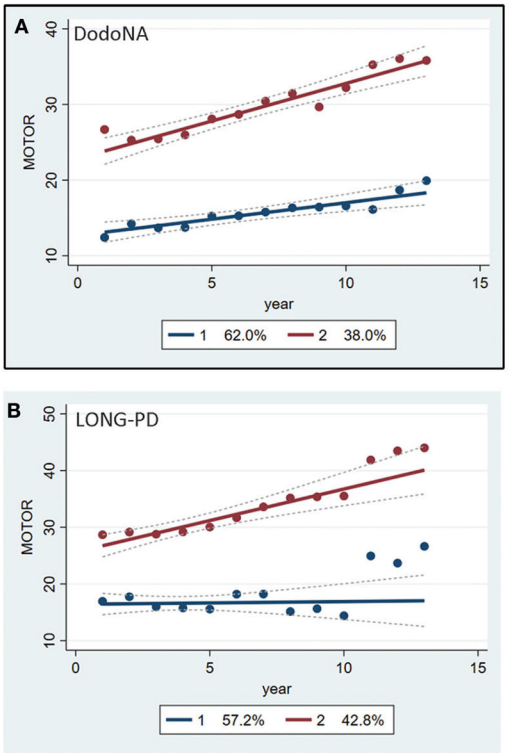
Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	19	1
2	16	310

Accuracy:	0.9509
95% CI:	(0.9225,0.9711)
Sensitivity:	0.54286
Specificity:	0.99678
Pos Pred Value:	0.95000
Neg Pred Value:	0.95092
Prevalence:	0.10116
Balanced Accuracy:	0.76982

FIGURE 3 | Mini-Mental State Examination (MMSE) score groups in the DodoNA and LONG-PD cohorts. **(A)** The model trained on DodoNA data (training set). **(B)** The model trained on the LONG-PD data (test set). **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.



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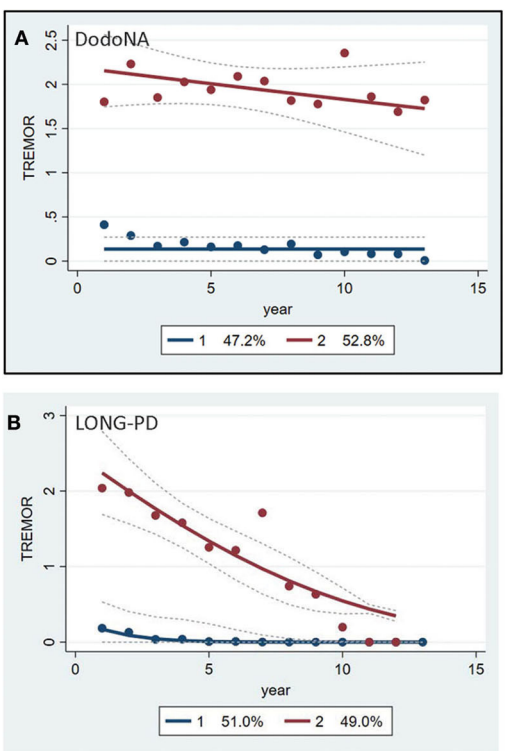
Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	169	8
2	33	136

Accuracy:	0.8815
95% CI:	(0.8427, 0.9136)
Sensitivity:	0.8366
Specificity:	0.9444
Pos Pred Value:	0.9548
Neg Pred Value:	0.8047
Prevalence:	0.5838
Balanced Accuracy:	0.8905

FIGURE 4 | Motor Score groups in the DodoNA and LONG-PD cohorts. **(A)** The model trained on DodoNA data (training set). **(B)** The model trained on the LONG-PD data (test set). **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.



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Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	143	90
2	39	73

Accuracy:	0.6261
95% CI:	(0.5727, 0.6773)
Sensitivity:	0.7857
Specificity:	0.4479
Pos Pred Value:	0.6137
Neg Pred Value:	0.6518
Prevalence:	0.5275
Balanced Accuracy:	0.6168

FIGURE 5 | Unified Parkinson's Disease Rating Scale (UPDRS) (UPDRS)-III Tremor sub-score groups in the DodoNA and LONG-PD cohorts. **(A)** The model trained on DodoNA data (training set). **(B)** The model trained on the LONG-PD data (test set). **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.

was subsequently used. Both scores (MoCA and STMS) can be converted to the Mini-Mental State Examination (MMSE) published nomograms (16) (unpublished data, with permission of Dr. Bradley Boeve, Mayo Clinic, Rochester, MN, USA), and therefore, all cognitive scores are recorded as the MMSE converted score.

The implementation of the toolkit has been cost effective, and the annual follow-up rates using the toolkit exceeded 85%.

The LONG-PD Cohort

The clinical information for the LONG-PD cohort was entered by the neurologists from the four participant sites in the REDCap web-based tool designed for the electronic capture and sharing of data (http://project-redcap.org/). NorthShore built a REDCap form mirroring the DodoNA SCDS toolkit. A working group refined the form and defined required fields for all sites. The finalized form was presented at the annual meeting of the Genetic Epidemiology of Parkinson's Disease (GEoPD) Consortium in Vancouver, Canada (2015). All of the teams (DodoNA project, LONG-PD) are members of GEoPD. The REDCap format was chosen because it provides an easily accessible Interface for collecting and validating data, as well as automated data export to statistical packages in a secure, de-identified manner (SPSS, SAS, Stata, R).

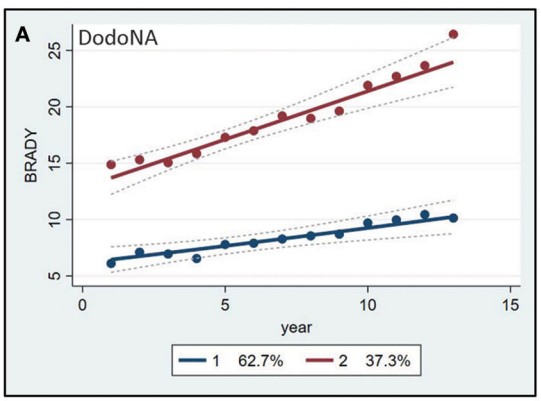
Data Treatment

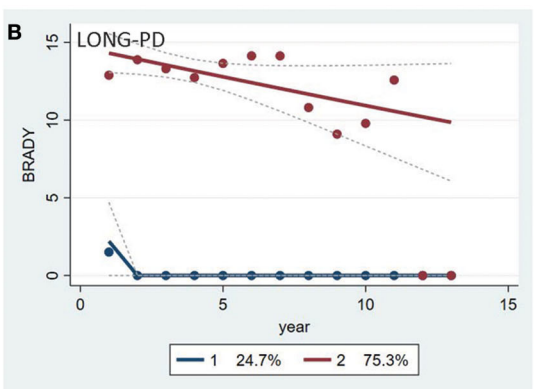
Subjects were excluded that experienced onset of symptoms 10 years or more prior to their initial visit or that had less than two valid visits (at 1 year or greater intervals). Prior to assessment, subject scores were assumed to be unknown, and the study was limited to a 13-year period covering all patient visits in the cohort. Missing motor assessment scores were imputed as zero for calculation of patient tremor and bradykinesia sub-scores.

Statistical Analysis

Group-based trajectory modeling (GBTM) was applied to identify latent subgroups within the patient cohorts, given their covariates and assessment scores over time (17, 18). Assessment scores were taken on an annual basis during initial and annual follow-up visits. GBTM assigns individuals to separate latent subgroups with posterior probabilities over time and regression parameters to define the trajectory of those subgroups. The DodoNA cohort data were used as the training set and the LONG-PD data as the test set. The test set data were entered into the DodoNA model, and the output was compared to the LONG-PD test set for validation. This approach is illustrated in **Figure 1**.

Trajectories were calculated based on the year of the reported initial symptom when the patient is seen for the first time in the movement disorder clinic, thus extending the trajectory duration





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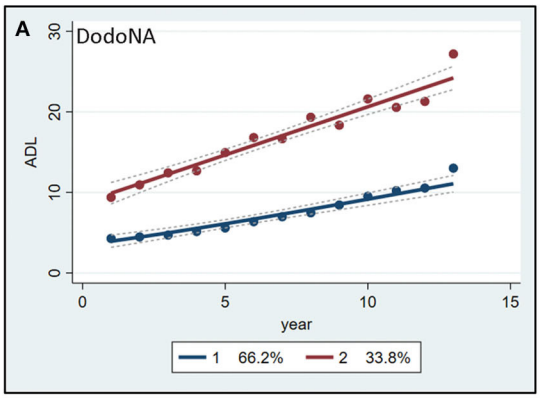
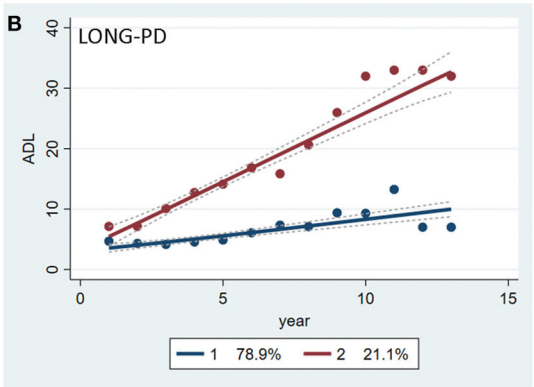
Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	86	134
2	0	126

Accuracy:	0.6127
95% CI:	(0.5592, 0.6643)
Sensitivity:	1.0000
Specificity:	0.4846
Pos Pred Value:	0.3909
Neg Pred Value:	1.0000
Prevalence:	0.2486
Balanced Accuracy:	0.7423

FIGURE 6 | UPDRS-III Bradykinesia sub-score in the DodoNA and LONG-PD cohorts. **(A)** The model trained on DodoNA data (training set). **(B)** The model trained on the LONG-PD data (test set). **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.





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Confusion Matrix and Statistics

	Reference	
Prediction	**1**	**2**
1	276	18
2	3	49

Accuracy:	0.9393
95% CI:	(0.9087, 0.962)
Sensitivity:	0.9892
Specificity:	0.7313
Pos Pred Value:	0.9388
Neg Pred Value:	0.9423
Prevalence:	0.8064
Balanced Accuracy:	0.8603

FIGURE 7 | Activities of Daily Living (ADL) scores in the DodoNA **(A)** and LONG-PD **(B)** cohorts. **(C)** The validation for the LONG-PD prediction trained on NS data against the test set.

to a maximum of 13 years that included at least 5 years of follow-up for the DodoNA cohort and 3 years of annual follow-up for the LONG-PD cohort. This choice to include the interval from the reported initial motor symptom allows a more accurate assessment of the disease course as often the patients come to the clinic at different points in the disease process.

We tested models with one to two subgroups using either constant or linear terms. The best-fitting results were selected using the lowest Bayesian information criterion (BIC) value. We used fixed covariates including patient gender, age of onset of symptoms, positive family history of PD with multiple-member instances, tremor predominance, presence of autonomic symptoms (orthostatism, urinary incontinence, constipation) individually and in combination, levodopa therapy, dopaminergic therapy, and years of education. Each of the fixed covariates was then measured across assigned subgroups to determine group membership totals and statistical significance across subgroups (Wilcoxon rank sum test for continuous variables: age of onset, years of education; Pearson's chi-squared test for count data: all other covariates).

Latent subgroup classes in GBTM cannot be externally validated. However, we attempted to validate whether GBTMs trained on the DodoNA cohort would be predictive of patient subgroup membership in the LONG-PD cohort. To do this, we trained GBTMs on the DodoNA patient cohort (the "training" set), and using their covariate estimates with respect to baseline, predicted subgroup membership when applied to LONG-PD patients for each sub-score. As a validation measure, we separately applied GBTM to the LONG-PD cohort using the same external model parameters (number of subgroups to stratify patients, shape of subgroup trajectories) and assumed these results to be the ground truth "test" set. We validated the overall results of the prediction and test sets using confusion matrices and statistics to assess the GBTM predictive value. The GBTM analysis was also performed in reverse with the LONG PD cohort as the training set and the DoDoNA cohort as the test set.

All data were analyzed using STATA/IC 16.0 using the PROC TRAJ package, and the significance level was set at 0.05.

RESULTS

Statistical Analysis

Assignment of Patients to Different Disease Trajectories Based on Individual Clinical Scores

Individual clinical parameters were assessed for their effect on disease trajectory: With each clinical score with the exception of the tremor sub-score, two separate trajectories are clearly identified: one with a slower and less severe and one with a more rapid and more severe trajectory: for the H&Y stage (UPDRS-V) the group with a slower progression includes 73.2% of the

cohort (**Figure 2A**). This is also observed in the LONG-PD cohort (**Figure 2B**) for 75.8% of the cohort. The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 2C** with a sensitivity of 0.9777 and a specificity of 0.7922.

For the MMSE scores, a similar separation is seen with the larger subset [83.8% in the DodoNA cohort (**Figure 3A**) and 89.8% in the LONG-PD cohort (**Figure 3B**)] having a slower progression. The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 3C** with a lower sensitivity of 0.54286 and a specificity of 0.99678. The apparent improvement of the MMSE scores, **Figure 3B** probably reflects the smaller sample size of the LONG-PD cohort.

For the UPDRS-III score, two groups are identified, with the slower progression group including 62% of the DodoNA cohort (**Figure 4A**) and 57.2% of the LONG-PD cohort (**Figure 4B**). The separation of the two trajectories appears less clear in the LONG-PD cohort, possibly reflecting treatment effects and shorter duration of follow-up. The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 4C** with a sensitivity of 0.8366 and a specificity of 0.9444.

For the tremor sub-score of UPDRS-III, two groups are again identified: the slower progression group of the DodoNA cohort including 47.2% (**Figure 5A**) and the LONG-PD cohort 51% (**Figure 5B**). The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 5C** with a sensitivity of 0.7857 and a specificity of 0.4479. The lower specificity that likely reflects the presence of tremor may not accurately reflect disease severity as it may be more sensitive to treatment effects.

For the bradykinesia sub-score of UPDRS-III, two groups are again identified: the slower progression group of DodoNA cohort including 62.7% (**Figure 6A**) and the LONG-PD cohort including 24.7% (**Figure 6B**). The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 6C** with a sensitivity of 1.000 and a specificity of 0.4648. The lower specificity likely indicated that sub-scores may not accurately reflect disease severity, as they only represent separate cardinal features and do not assess rigidity and postural instability.

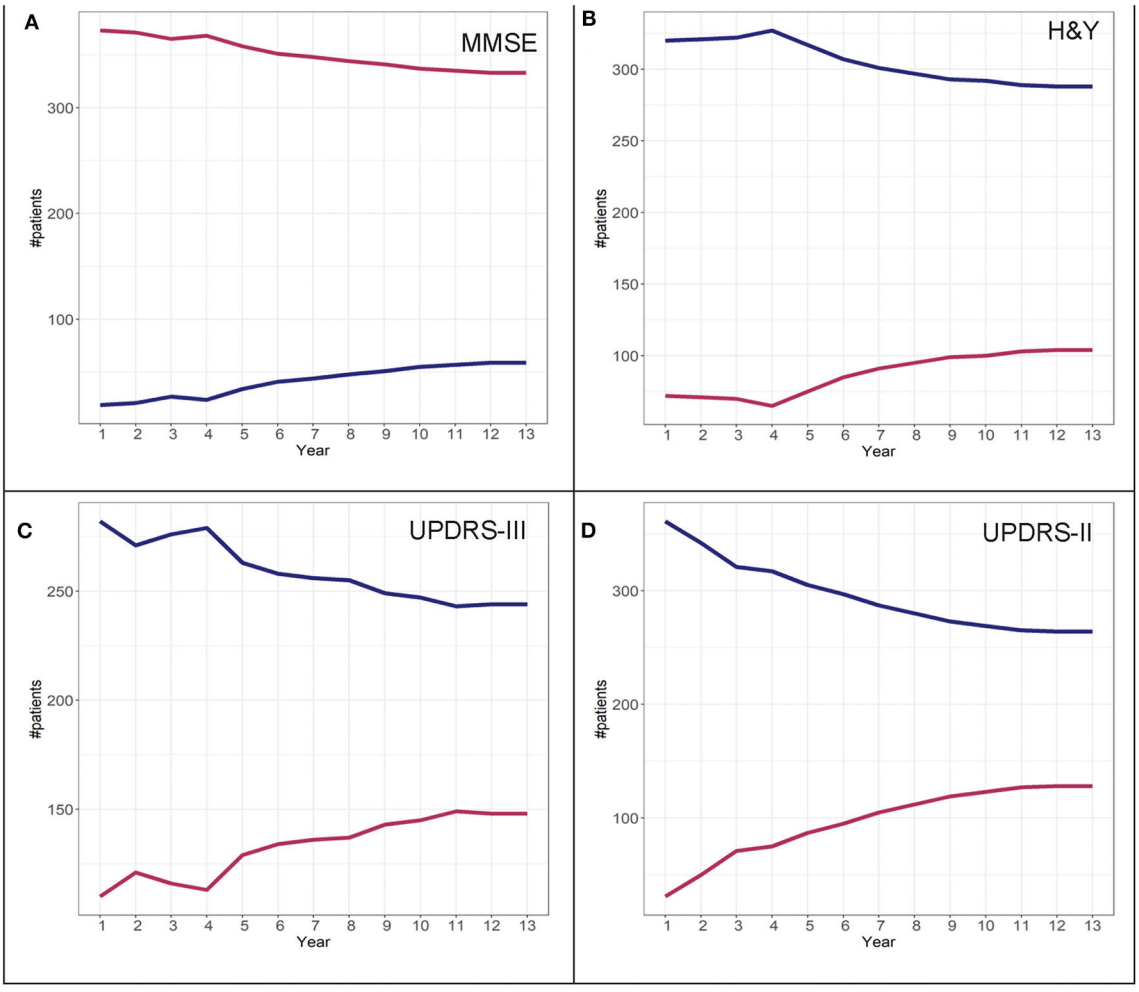

FIGURE 8 | Convergence scores for MMSE **(A)**, H&Y stage **(B)**, UPDRS-III **(C)**, and UPDRS-II **(D)** for the DodoNA cohort.

The UPDRS-II ADL score separates patients in two different trajectories, with 66.2% of the DodoNA cohort (**Figure 7A**) and 78.9% of the LONG-PD cohort (**Figure 7B**) showing a slow trajectory. The validation for the LONG-PD prediction trained on the DodoNA test set is shown in **Figure 7C** with a sensitivity of 0.9892 and a specificity of 0.7313. It is important to note that the UPDRS-II score reflects historical information and subject to a subjective assessment.

To determine adherence to a particular group identified in the GBTM, convergence graphs were generated based on the assumption that the group assignment at year 13 is the "true group." In addition, convergence graphs provide information regarding the time point in the disease course where patients can be reliably classified to their "true group." The time point in which the two trajectories appear to be horizontal reflects the time point when the group assignments "converge" to their "true groups." For H&Y stage for both the DodoNA and LONG-PD cohorts, year 9 represents the time point in which group assignment more closely reflects the "true group" assignment (**Figures 8B**, **9B**). For the MMSE score in the DodoNA cohort, this time point is delayed at year 10 (**Figure 8A**), whereas in the LONG-PD cohort, it occurs earlier in year 8 (**Figure 9A**). For UPDRS-III and II, that time point is later (**Figures 8C,D**, **9C,D**). Taken together, these results point to the H&Y stage and the MMSE as reliable predictors of trajectory group assignment and identify a point relatively early in the disease trajectory in which group assignment can be made.

To further investigate the possibility of the misclassification rate for group assignments, misclassification graphs were generated assuming that the assignment at year 13 is the "true group," complementing the convergence analysis. In the case of H&Y trajectory, convergence was at year 9. At year 9, the misclassification (i.e., 1-accuracy) is 0.05, representing a 5% error rate for group 2 and almost 0% error for group 1 assignment. Based on these graphs, the H&Y stage provides an "acceptable error rate" in both cohorts (**Figures 10**, **11**).

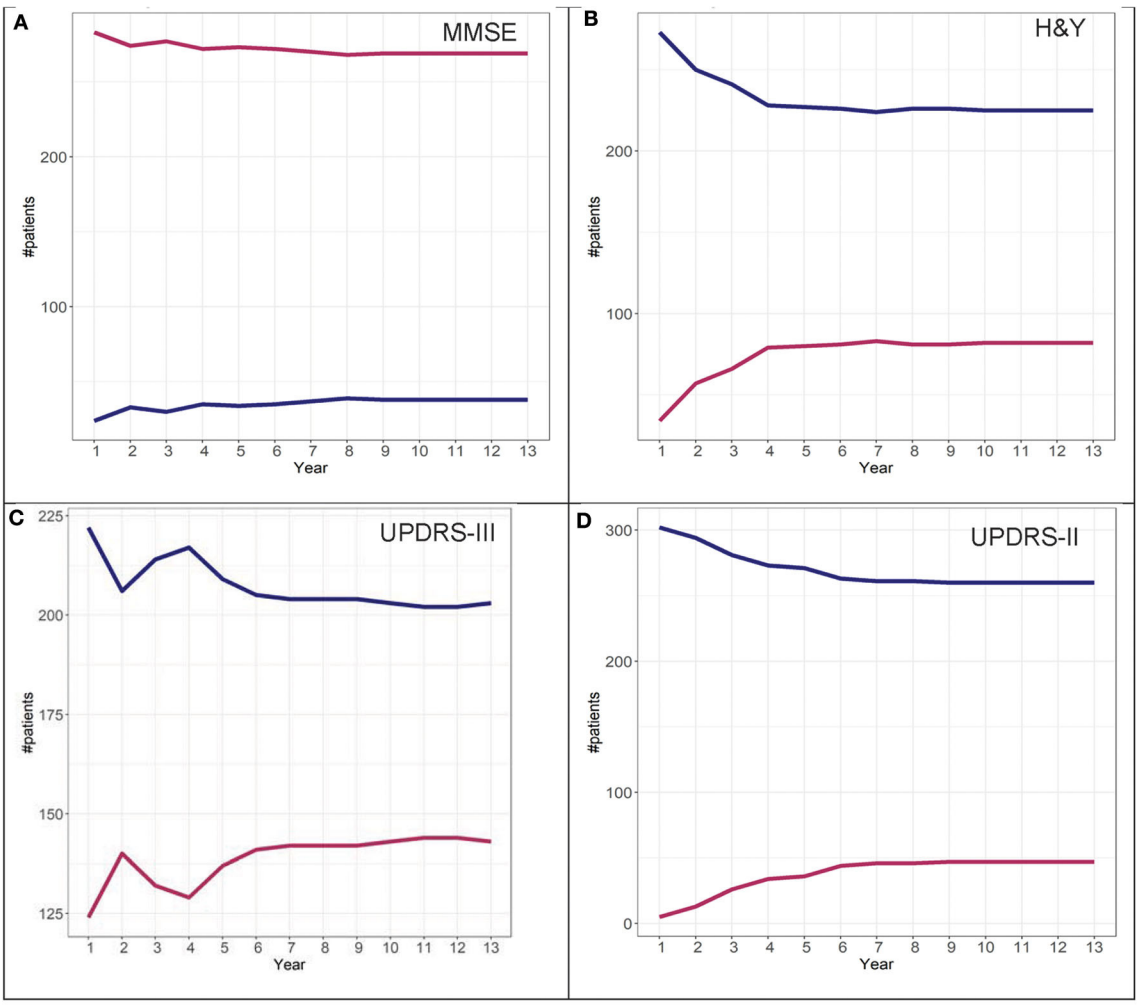

FIGURE 9 | Convergence scores for MMSE **(A)**, H&Y stage **(B)**, UPDRS-III **(C)**, and UPDRS-II **(D)** for the LONG-PD cohort.

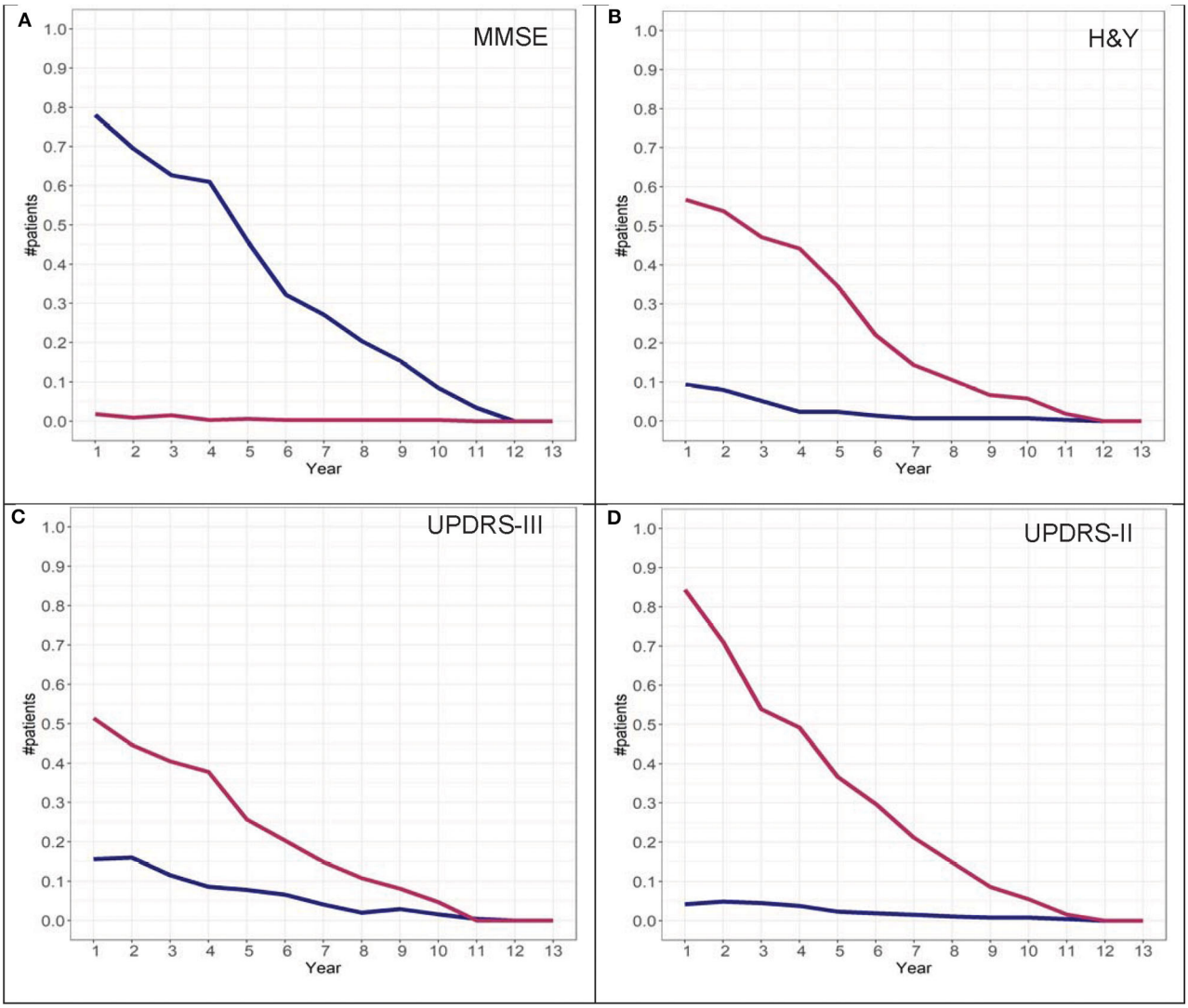

FIGURE 10 | Misclassification scores for MMSE **(A)**, H&Y stage **(B)**, UPDRS-III **(C)**, and UPDRS-II **(D)** for the DodoNA cohort.

To ascertain the reliability of the analyses, the LONG-PD cohort was used as a training set and the DodoNA cohort as the test set. Both types of analyses provided similar results (data not shown).

Covariates Contributing to Trajectory Group Assignment

From the covariates entered into the model, the following contribute to group assignment: older AAO for both cohorts and male sex only for the DodoNA cohort assign patients to the more severe group (group 2) and tremor-predominant disease subtype to the benign group (group 1). Interestingly for tremor scores in the DodoNA cohort only, years of education assigns patients to group 1. Bradykinesia and AAO in the DodoNA cohort only assign patients to group 2. The tremor-predominant subtype in the LONG-PD, but not the DodoNA cohort, assigns patients to group 1. Interestingly, levodopa and dopaminergic therapy are not significant for the DodoNA cohort but are significant for the LONG-PD cohort. Complications of therapy do not contribute to group assignment (data not shown). Cognitive impairment at disease onset likely assigns patients to group 2. The differences noted between the two cohorts may reflect different sample sizes or genetic background effects. Group counts are shown in **Table 1** and the effect of covariates in **Tables 2**, **3**. To assess whether the presence of autonomic symptoms contributes to a more rapid and severe course in PD, we also assessed both cohorts for the presence of autonomic symptoms. Orthostatism, urinary incontinence, and constipation were the most prevalent autonomic symptoms. Therefore, we included these in the analysis, individually and in combination. They did not contribute, either individually or in combination, to a more severe disease course in our cohorts.

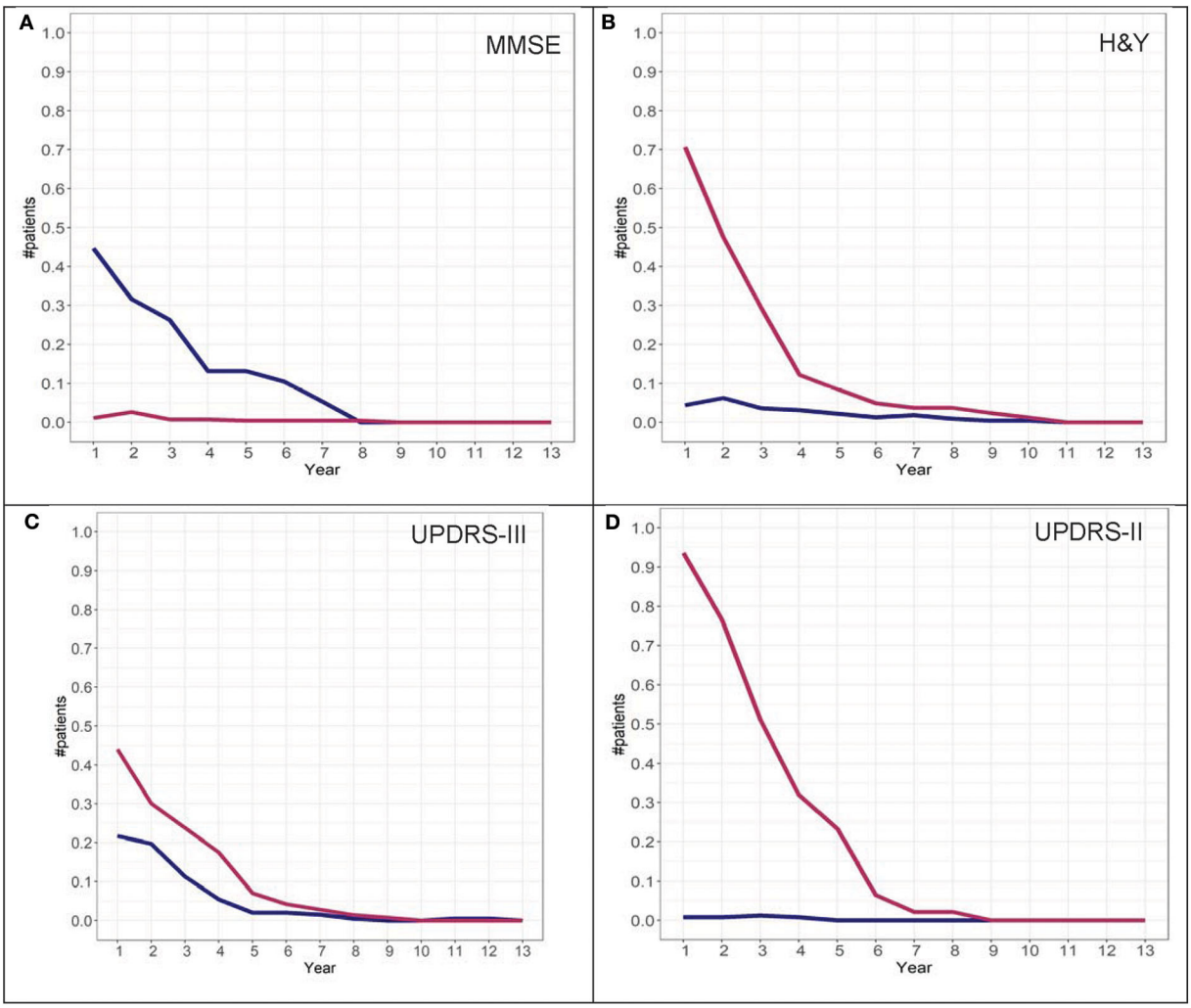

FIGURE 11 | Misclassification scores for MMSE **(A)**, H&Y stage **(B)**, UPDRS-III **(C)**, and UPDRS-II **(D)** for the LONG-PD cohort.

Genotypes were assessed for the presence of *LRRK2* and *GBA* mutations. The prevalence of *LRRK2* and *GBA* pathogenic variants was 1.89%and 2.96%, respectively. The vast majority of these were in the DodoNA, a United States-based cohort. In combination with the lack of significant contribution of family history to the disease trajectory, this suggests that in these two cohorts, at least some genetic factors do not contribute to the disease trajectory.

DISCUSSION

Longitudinal monitoring of PD over long time intervals is essential in order to obtain a more accurate characterization of patterns in the disease course and clinical outcomes, as well as to gain insights into disease etiology. Here, we present a group-based trajectory modeling analysis of five ethnically different PD patient cohorts from the United States (the DodoNA cohort) and from Norway, South Korea, Greece, and Sweden (the LONG-PD cohort) within the GEoPD consortium (https://geopd.lcsb.uni.lu/). The trajectory analysis is based on standardized clinical assessment that takes place at annual intervals in the routine office setting. The choice of clinical assessment parameters reflects a consensus among clinicians with different backgrounds and practice modes and which would facilitate data collection and entry using a web-based format. The analysis of a maximum of 13-year disease course identifies two distinct groups: a slower and more benign course and a faster, more malignant course. Clinical predictors of group assignment include male sex, age at disease onset, presence of tremor as a predominant clinical feature, years of education, and cognitive impairment at onset. Interestingly, levodopa/dopaminergic therapy and family history do not contribute to group assignment. The significance of beneficial effect of years of education for assignment to a particular disease trajectory is supported by the findings of Lee et al., which implicate a passive reserve hypothesis

TABLE 1 | Group counts from the DNA Predictions to Improve Neurological Health (DodoNA) and Longitudinal Clinical and Genetic Study of Parkinson's Disease (LONG-PD) cohorts.

DodoNA cohort	Group 1	Group 2
Hoehn and Yahr (H&Y) stage	291 (73.48%)	105 (26.52%)
Mini Mental State Examination (MMSE)	59 (14.9%)	337 (85.1%)
Unified Parkinson's Disease Rating Scale (UPDRS)-III	251 (63.38%)	145 (36.62%)
UPDRS-II	266 (67.17%)	130 (32.83%)
Tremor sub-score	188 (47.47%)	208 (52.53%)
Bradykinesia sub-score	247 (63.01%)	145 (36.99%)
LONG-PD cohort	**Group 1**	**Group 2**
H&Y	269 (77.75%)	77 (22.25%)
MMSE	35 (10.12%)	311 (89.88%)
UPDRS-III	203 (58.67%)	143 (41.33%)
UPDRS-II	279 (80.64%)	67 (19.36%)
Tremor sub-score	182 (52.6%)	164 (47.4%)
Bradykinesia sub-score	86 (24.86%)	260 (75.14%)

for motor/non-motor symptoms of PD (19). The somewhat unexpected lack of contribution of family history in group assignment may reflect the diverse genetic background of the two cohorts.

Adherence to a particular group occurs at mid-stage disease and remains stable thereafter for the study interval. Interestingly, complications of therapy do not appear to contribute to the assignment to individual trajectories. It is interesting to point out that while there is significant overlap between cohorts for the different covariates, there are covariates in which the two cohorts diverge. This may be explained by the different cohort sizes, but it may also reflect different genetic, environmental, and cultural factors. The prevalence of *LRRK2* disease causing variants in sporadic PD has been reported between 0.5 and 2% (20, 21) and that of *GBA* between 2.3 and 9.4% (22) in the U.S. population, similar to what we find in our cohort. It seems unlikely that the low percentage of *LRRK2* and *GBA* disease-causing variants drives trajectory classification as there is a lack of contribution of family history to trajectory classification. This suggests that genetic factors are not likely to have at best a modest effect.

The GBTM analysis presented here has several strengths: (a) it employs easily assessed standardized clinical parameters that can be assessed at annual intervals and identifies predictable patterns of disease progression; (b) the analysis is performed over a long disease duration (maximum of 13 years); (c) it identifies individual clinical predictors of trajectory patterns; (d) the accurate clinical phenotypic characterization provides an essential background for genotype–phenotype correlations, currently ongoing in our study; (e) it provides an informative template for large-scale clinical and genomic studies.

Our study has also some limitations. Since the intent of this study was to assess measures that could be evaluated in a routine clinical setting, a limitation is its assessment of a

TABLE 2 | Summary statistics, DodoNA cohort: AAO and YOE are continuous covariates and their group values represent within-group means.

H&Y stage	Group 1	Group 2	*P*-value
AAO[a]	66.0	75.0	<0.0001
YOE[b]	16.0	16.0	<0.0001
FH>1[c]	12	5	0.7817
FH	69	22	0.6594
TDS	71	4	<0.0001
LD[d]	254	90	0.8103
DP[e]	94	23	0.0605
Male sex	221	64	0.0050
MMSE			
AAO	75.0	67.0	<0.0001
YOE	15.0	16.0	<0.0001
FH>1	2	15	1.0000
FH	11	80	0.4899
TPS[f]	8	67	0.3354
LD	52	292	0.9176
DP	9	108	0.0141
Male sex	48	237	0.1134
UPDRS-III (Motor)			
AAO	67.0	72.0	<0.0001
YOE	16.0	16.0	<0.0001
FH>1	11	6	1.0000
FH	59	32	0.8388
TPS	60	15	0.0014
LD	212	132	0.0871
DP	80	37	0.2221
Male sex	171	114	0.0337
UPDRS-II (ADL)			
AAO	68.0	70.0	<0.0001
YOE	16.0	16.0	<0.0001
FH>1	12	5	0.9660
FH	63	28	0.7268
TPS	59	16	0.0266
LD	231	113	1.0000
DP	83	34	0.3592
Male sex	188	97	0.4837
UPDRS-III Tremor sub-score			
AAO	69.0000	68.0000	<0.0001
YOE	16.0000	16.0000	<0.0001
FH>1	8	9	1.0000
FH	51	40	0.0809
TDS	0	75	<0.0001
LD	171	173	0.0322
DP	60	57	0.3831
Male sex	130	155	0.2819
UPDRS-III bradykinesia sub-score			
AAO	65.0	74.0	<0.0001
YOE	16.0	16.0	<0.0001
FH>1	9	8	0.5569
FH	60	31	0.5354
TPS	67	8	<0.0001
LD	211	133	0.2263
DP	81	36	0.0999
Male sex	177	108	0.8198

Other covariates are binary, and as such, their values represent within-group sums of positive membership. P-values display statistical significance across groups.
[a] AAO, age at onset; [b] YOE, years of education; [c] FH, family history; [d] LD, levodopa therapy; [e] DP, dopaminergic therapy; [f] TPS, tremor-predominant subtype.

TABLE 3 | Summary statistics from the LONG-PD cohort: AAO and YOE are continuous variables and their group values represent within-group means.

	Group 1	Group 2	*P*-value
H&Y stage			
AAO	61.0	67.0	<0.0001
YOE	12.0	10.0	<0.0001
FH>1	26	6	0.7816
FH	29	11	0.5183
TPS	75	4	<0.0001
LD	248	61	0.0024
DP	142	39	0.8400
Male sex	139	36	0.5273
MMSE			
AAO	69.0	62.0	<0.0001
YOE	6.0	12.0	<0.0001
FH >1	1	31	0.2269
FH	2	38	0.4014
TPS	8	71	1.0000
LD	24	285	0.0003
DP	13	168	0.0860
Male sex	16	159	0.6681
UPDRS-III (Motor)			
AAO	59.0	67.0	<0.0001
YOE	12.0	10.0	<0.0001
FH>1	18	14	0.9176
FH	23	17	1.0000
TPS	56	23	0.0173
LD	186	123	0.1371
DP	115	66	0.0694
Male sex	105	70	1.0000
UPDRS-II (ADL)			
AAO	61.0	67.0	<0.0001
YOE	11.0	10.0	<0.0001
FH>1	26	6	1.0000
FH	32	8	1.0000
TPS	67	12	0.3645
LD	264	45	<0.0001
DP	150	31	0.3337
Male sex	135	40	0.1267
UPDRS-III tremor sub-score			
AAO	62.0	63.0	1.0000
YOE	12.0	11.0	1.0000
FH>1	21	11	0.1729
FH	26	14	0.1332
TPS	39	40	0.5981
LD	152	157	0.0005
DP	80	101	0.0015
Male sex	101	74	0.0689
UPDRS-III bradykinesia sub-score			
AAO	62.0000	62.0000	1.0000
YOE	10.0000	12.0000	<0.0001
FH>1	9	23	0.8146
FH	12	28	0.5445
TPS	31	48	0.0013
LD	60	249	<0.0001
DP	30	151	0.0003
Male sex	46	129	0.6183

Other variables are binary, and as such, their values represent within-group sums of positive membership. P-values display statistical significance across groups.

narrower spectrum of phenotypic characteristics than other comprehensively studied cohorts such as the PPMI, DeNOPA, and LABS-PD cohorts (2–6, 23). Specifically, in our cohorts, CSF analyses, SPECT scans, quantitative olfactory assessment, and polysomnograms were not obtained routinely. Since the study protocols of other longitudinally studied cohorts vary in aims and scope, direct comparisons with our study are challenging. These issues would be better addressed by a meta-analysis.

A second limitation of our study is the lack of autopsy data. However, over a quarter of participants underwent SPECT scans that were abnormal. In the absence of autopsy data, an abnormal SPECT scan in the context of clinically definite PD (Bower criteria) confirms the clinical diagnosis. In that context, it should also be pointed out that the diagnosis of PD in our study was assessed and confirmed at each annual interval.

A strength of this study is that detailed information on comorbidities, head injury, complications of dopaminergic therapy, autonomic dysfunction (orthostatic symptoms, anhidrosis/hyperhidrosis, urinary incontinence), sleep disorders, dysphagia, anxiety, and depression have been, and continue to be, collected at annual intervals. As the study is ongoing, these will continue to be analyzed to inform conclusions regarding the spectrum of factors that contribute to the disease course in intervals longer than 5 years. It is important to point out that the focus of the analysis presented here is to identify individual, clinical parameters that reflect the cardinal features of the disease as well as assess the effect of other covariates on those parameters. Furthermore, it is important to stress that the clinical data collected in the DodoNA and LONG-PD cohorts are pragmatic and can be easily collected within routine clinical practice settings worldwide. Identifying what features in this simplified, reproducible set of clinical parameters can predict disease course complements findings from other longitudinally followed disease cohorts.

In conclusion, the longitudinal study of the DodoNA and the LONG-PD cohorts combines clinically meaningful, easily obtainable information from ethnically different PD cohorts and demonstrates that clinical parameters assessed in the routine office setting can help predict clinical outcomes in PD as well as inform our understanding of the underlying neurodegenerative process. Large international consortia to understand genetic risk factors contributing to PD have been formed where phenotypic information is sketchy and often minimal. This work demonstrates that a detailed phenotypic characterization is essential in informing and interpreting the data from such consortia. The development of the LONG-PD protocol has led to the adoption of a somewhat simplified version of phenotypic information collection by a majority of the GEoPD participating sites and can be easily adapted for genomic information obtained by other international consortia. Ongoing genotype–phenotype analyses will identify molecular predictors of the disease trajectories. Longer longitudinal follow-up of >10 years will help determine whether the adherence to the identified trajectories remains stable or whether splintering occurs as the disease process advances.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee for Central Norway, Ethics committee of the University Hospital of Larissa, Regional Ethics Review Board in Stockholm, Sweden, Institutional Review Board Of Asan Medical Center, Institutional Review Board NorthShore University HealthSystem. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KM, RF, and DM contributed in the conception and design of the study. KM wrote the first draft of the manuscript. KS wrote sections of the manuscript. KM, JA, SC, ED, KW, APP, BS, DM, LG, AP, and NK collected the data. BH and AE built the EMR system. ST and GW performed the statistical analysis. JW performed the genomic analysis. All authors contributed to the manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00548/full#supplementary-material

Supplemental Information: LONG-PD Study Protocol.

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REVIEW
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Perspective: Current Pitfalls in the Search for Future Treatments and Prevention of Parkinson's Disease

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We are gradually becoming aware that there is more to Parkinson's disease (PD) than meets the eye. Accumulating evidence has unveiled a disease complexity that has not (yet) been incorporated into ongoing efforts aimed at slowing, halting or reversing the course of PD, likely underlying their lack of success. There is a substantial latency between the actual onset of PD pathology and our ability to confirm diagnosis, during which accumulating structural and functional damage might be too advanced for effective modification or protection. Identification at the earliest stages of the disease course in the absence of Parkinsonism is crucial if we are to intervene when it matters most. Prognostic and therapeutic inferences can only be successful if we are able to accurately predict who is at risk for developing PD and if we can differentiate amongst the considerable clinicopathologic diversity. Biomarkers can greatly improve our identification and differentiation abilities if we are able to disentangle cause and effect.

Keywords: Parkinson's disease, complex syndrome, pre-diagnostic period, biomarker, disease modification

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PARKINSON'S DISEASE-MODIFICATION AND NEUROPROTECTION ARE NOT YET AVAILABLE

Despite efforts to develop new treatments that can slow, stop or even reverse the trajectory of PD (disease modification) and preserve neural integrity and function (neuroprotection), none have yet been successfully demonstrated (1). The primary reason for this lack of success remains our incomplete understanding of the exact cause(s) of PD, and factors involved in subsequent disease progression (2). Arguably, many of the previous clinical trials aimed at developing new treatments were methodologically and conceptually flawed (2) by assuming that PD can be defined as a single diagnostic entity, without taking into consideration the complexity, diversity and timing of pathogenesis (3–5). Furthermore, past study designs show little or no regard for the state of neuronal degeneration at time of enrollment, or the inter- and intra-individual clinicopathologic heterogeneity (2). This is exemplified by the PD models used to investigate potential new treatments, which have been criticized for their lack of complexity and true representation of the natural course of PD in humans (3). In human trials, the sensitivity and specificity of outcome measures have also received considerable scrutiny, as most are highly subjective, still firmly rooted in the motor domain and unable to accurately assess therapeutic target engagement (6). *In vitro* cellular modeling using person specific stem cells or induced pluripotent stem cells has shown considerable potential as a method to closely reproduce specific pathological circumstances and directly study neurodegenerative processes and mechanisms and the effects of interventions (7–9).

In that regard, *in vitro* cellular modeling has been referred to as "the most robust and phenotypically similar model for PD" (8). The limitation of course is that the complexity of PD is still not fully accounted for, with the requirement to focus on specific aspects of PD while ignoring others (9). Complementary approaches that can mitigate for the unaccounted aspects would be required in order to advance.

We will fail to make progress in the development of new therapeutic strategies until we take into consideration the full natural history of the disease process and associated clinicopathologic diversity under this banner.

In the current perspective we aim to discuss emerging concepts and recent insights into the natural history of PD that will be important to consider before viable disease-modifying therapies can become a reality.

PD IS MORE THAN MEETS THE EYE

To date, clinical and scientific approaches to PD have mainly focused on few primary features, subsequently reducing it to a single diagnostic entity and viewing symptomatology through a dopaminergic lens (**Figure 1**) (10, 11). The characteristic clinical features in PD are observed as a deterioration of motor function expressed as bradykinesia, resting tremor, muscular rigidity and postural instability (12). The underlying pathological characteristics include an ongoing gradual loss of dopaminergic neurons in the nigrostriatal pathway, as well as the presence and proliferation of eosinophilic inclusions called Lewy bodies and Lewy neurites (11, 12). Over time, neural integrity becomes increasingly compromised eventually leading to an unsustainable dopamine deficiency ultimately resulting in functional complications and subsequent disability.

As definitive diagnostic confirmation is still only possible through *post-mortem* histopathological examination, available diagnostic criteria aim to increase the level of clinical diagnostic certainty *in vivo* (13). Current diagnostic criteria require the presence of a combination of cardinal motor symptoms to establish Parkinsonism, a group of neurological disorders with overlapping pathologic and symptomatic expressions (14). A combination of additional supportive features, red flags and exclusion criteria for differential diagnosis, then serve to further strengthen the clinical diagnostic certainty for PD, resulting in either clinically established or clinically probable PD (14). Symptomatic management, by way of compensation for the ensuing dopamine deficiency, remains the gold standard of clinical treatment (15). Although symptomatic management is successful at maintaining quality of life, especially during earlier stages of PD, long-term pharmacotherapy is associated with development of treatment-related motor complications that are difficult to manage (11). Furthermore, treatment options for non-motor symptoms remain limited (16).

Motor symptomatology is still considered the defining characteristic of PD; however, it is now widely recognized that a range of non-motor features (**Table 1**) form an integral part of the symptomatology (17). Although dopaminergic cell loss is considered the predominant pathological hallmark of PD, degeneration is not restricted to the nigrostriatal pathway. Neurotransmitter deficiency due to extranigral degeneration, including the serotonergic, noradrenergic, cholinergic, GABAergic, and glutamatergic systems, underlie numerous neuropsychiatric, autonomic, sensory, and sleep disorders, as well as the non-levodopa responsive motor symptoms of PD (18, 19). Furthermore, neuropathological evidence suggests that the presence of α-synuclein aggregates and Lewy pathology also extends to extranigral structures, including the cerebral cortex, olfactory structures, brainstem, spinal cord and even peripheral tissues (20). Naturally expressed throughout the CNS and many other tissues, α-synuclein is a presynaptic protein (21, 22). The exact function is still unknown, but α-synuclein is thought to play a role in the regulation of neurotransmitter release (23). Increased expression and accumulation of abnormal α-synuclein aggregates is thought to be neurotoxic and associated with pathological processes of PD (22, 23).

TABLE 1 | An overview of the most common non-motor features by category.

Category	Non-motor symptom
Autonomic	Constipation**
	Salivation
	Bladder dysfunction
	Sexual dysfunction
	Respiratory dysfunction
	Cardiovascular dysfunction
	Fatigue
	Excessive sweating
Mood and behavior	Depression**
	Anxiety**
	Panic attacks
	Impulse control disorder
	Visual hallucinations*
	Delusions*
	Dementia
	Apathy
Sensory	Pain
	Olfactory dysfunction**
	Insomnia
Sleep	REM sleep behavior disorder**

**Mostly medication related.*
***Common prodromal symptoms.*

IS IT TIME TO REDEFINE PD?

Not only do these pathological findings provide an explanation for the wide range of non-motor symptomatology, indicating a more complex and systemic nature of PD, they also hint toward possible extranigral origins and earlier disease onset. To that effect, Braak et al. (20) proposed a six-point staging system,

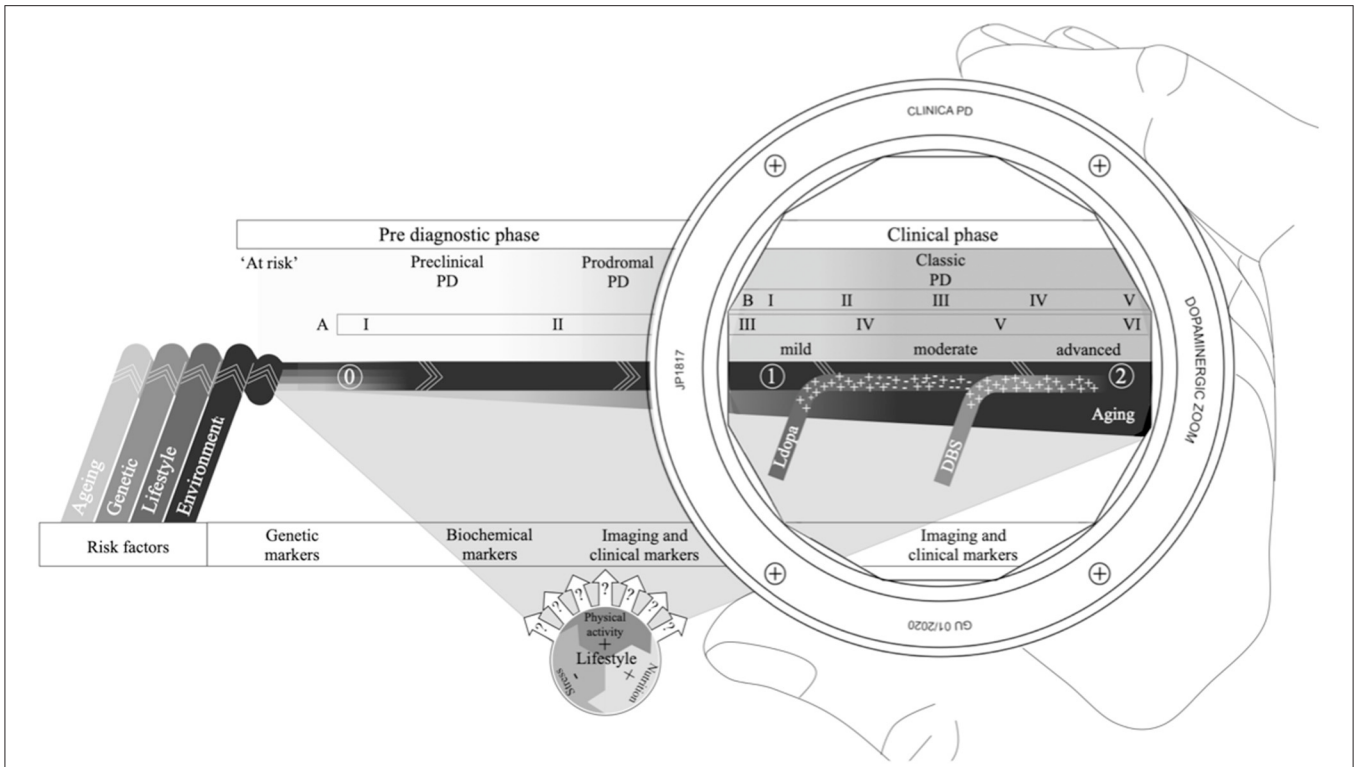

FIGURE 1 | The PD continuum with an extended disease course that includes a combination of risk factors that can pre-dispose or cause PD (0); a preclinical phase, where the pathology has started but no signs are present; a prodromal phase, where non-motor symptoms dominate, but motor symptoms can be present; and the classic or clinical phase often viewed through a dopaminergic lens, delineated by the clinical diagnosis (1) and mortality (2), with Levodopa therapy and/or deep brain stimulation (DBS) forming essential therapies for symptomatic management. Several risk factors (like age and lifestyle) also have the potential to affect disease progression. The Braak six-point staging scheme (A) spans across the entire PD continuum, while the Hoehn & Yahr symptom progression scale, for reference (B), is confined to the classic PD phase. Different biomarkers are better suited at different points along the continuum depending on pathological and clinical evidence.

based on post-mortem histopathological evidence of abnormal α-synuclein accumulation throughout the nervous system of individuals with differing disease durations. They describe a rather systematic propagation of α-synuclein aggregates along interconnected neural networks, starting in the lower brainstem and anterior olfactory system and progressing to cortical areas with advancing disease. The pathology only reaches dopaminergic cells in the *substantia nigra* toward stages three and four, relating to the classic motor symptomatology.

In an effort to explain the extranigral origin, Braak et al. (24) proposed a dual hit hypothesis where an environmental pathogen likely enters the body through the nasal and gastric routes and then spreads via the dorsal motor nucleus of the vagus nerve and olfactory bulb to more central neuronal structures. A prion-like concept of disease progression has since been put forward that proposes a cell-to-cell spread of abnormal α-synuclein (25, 26) The concept was the result of findings of neuronal grafting studies, where, at autopsy after more than a decade of survival, host-to-graft propagation was found in some of the transplanted dopaminergic neurons (25). Since then several studies using animal and cellular models have supported the α-synuclein transmission concept (27–31). This is also problematic for new treatments efforts that focus on regeneration, where patient specific induced pluripotent stem cells are transplanted. Apart from the risks associated with regeneration medicine, such as unwanted biological effects and immune response, toxicity, neoplasm formation, disease transmission, reactivation of latent viruses, to rejection of the cells by the body (7), the transplanted cells would again be susceptible to α-synuclein propagation, compromising their long term health. Further evidence now suggests that different species or strains of α-synuclein can exert different effects depending on their folded state (32). Different oligomeric forms in particular seem to have different pathogenic effects including toxicity, which is suspected to contribute to the clinicopathological diversity of PD (33).

Although there is considerable support for the Braak hypothesis, some studies have shown that not all PD cases follow the systematic pathological progression (34). Higher stage pathology and subsequent symptomatology, such as primary dementia with Lewy bodies, has been found in absence of pathology in lower stage structures (35, 36). The presence of Lewy pathology in otherwise healthy individuals is also well-recognized (37). Furthermore, some genetic variants of PD do not express characteristic Lewy pathology. In addition, the considerable pathological and symptomatic heterogeneity of PD undermines strict systematic progression (20). Different

patterns of pathological progression are most likely underlying the considerable clinical variability seen in PD. Studies have since shown that Lewy pathology and α-synuclein spread can occur in bi-directional manner along interconnected networks (38). This can partly explain some of the discrepancies in the Braak hypothesis, but considerable discussion remains on the topic.

As it stands, the traditional concept of PD as just a movement disorder is gradually making way for a more comprehensive and encompassing definition that recognizes the innate complexity of PD as a syndrome and the multiple affected neuroanatomical structures (nigral and extranigral) that lie at the foundation of the broad symptomatic range. Redefining PD as a multi-system neurodegenerative disorder (39) not only acknowledges the widespread spatial organization of neurodegeneration and possibly a peripheral origin, but also implies earlier temporal progression along a much more extended disease continuum.

THE CONCEPT OF PD WITHOUT PARKINSONISM

It is now widely accepted that the classic PD course actually represents a relatively late stage of a broader process of disease (40). The extended PD course acknowledges a considerable pre-diagnostic phase, during which the underlying pathology has commenced, but symptomatology is either absent, non-specific or too subtle to meet current diagnostic criteria (1) (**Figure 1**). The pre-diagnostic phase is commonly further subdivided into an "at risk" phase, a preclinical or premotor phase and a prodromal phase, depending on clinicopathologic manifestations (40).

The earliest phase in the PD continuum, when the pathology is thought to have commenced, but clinical signs and symptomatology are lacking, is referred to as the preclinical phase (40). As the pathology progresses, compromises to neural integrity and function steadily increase to a point where symptomatology becomes manifest (41). During this prodromal phase, several non-motor symptoms are especially common (**Table 1**), including olfactory dysfunction, constipation, anxiety, depression, sympathetic denervation and REM sleep behavior disorder (40, 42). The non-motor features associated with the prodromal phase are non-specific and are generally easily disregarded as common aspects of normal aging (41). However, most, if not all individuals with PD have indicated the presence of one or more of these features prior to their diagnosis (42). Subtle motor symptoms also start to emerge during the prodromal phase as the underlying pathology slowly progresses (42). It is worthwhile noting that for clinical and scientific purposes subdividing the PD course, whether classic or pre-diagnostic, into different phases can be a meaningful way to deal with the complexity. In reality, definite phases are almost certainly unlikely and the PD course, in all probability, represents a continuum of transient states along which multiple factors continuously interact, with positive or negative impact (43).

If we are to move forward clinically and scientifically, we first need to come to grips that PD can be present in the absence of Parkinsonism. We then need objective and reliable measures to accurately identify those at risk of developing PD or those in the earliest developmental stages when traditional motor symptomatology has not (yet) emerged.

CURRENT TREATMENTS ARE TOO LITTLE, TOO LATE TO AFFECT PROGRESSION

As mentioned in the previous section, PD is now considered much more than just a movement disorder and the pathology extends well-beyond the nigrostriatal neural networks, potentially even originating in sites peripheral to the CNS. This has considerable implications on how we need to consider the timing of key milestones in the disease trajectory. It is now evident, that by the time the cardinal motor features manifest and diagnosis can be made, a vast majority of dopaminergic cells have already been lost (1, 44). The underlying pathology has been able to spread insidiously for years and compensatory mechanisms are no longer able to cope with the steadily increasing dopamine deficiency, resulting in overtly observable motor features (11, 45). In this regard, the cardinal motor features, traditionally used as diagnostic criteria, should instead be considered determinants of clinical progression of PD. Since most clinical trials are designed with PD diagnosis as minimum inclusion criterion, we argue that the compromises to neural integrity and function at this stage are already too advanced for disease modifying or protective therapies to take effect (1, 5). This stark realization is further supported by the fact that, on average, very few dopaminergic terminals remain in the striatum as early as 5 years following a formal clinical diagnosis and the commencement of dopaminergic therapy (46). At the moment, however, these motor symptoms are the only criteria available to guide PD diagnosis and subsequent therapeutic approaches. Any attempt at disease modification would have to commence as early as possible and this will require a reconsideration of how and when the diagnosis is made, what specific disease-related processes need to be targeted and how aggressive these need to be treated.

HOW CAN WE IDENTIFY THOSE AT RISK BEFORE THE EMERGENCE OF SYMPTOMS?

The specific causes of PD remain unknown, and there is no clarity as to when the actual onset of PD occurs (47). Moreover, endophenotypes associated with early stages in the PD continuum are also factors that may pre-dispose for the development of classic movement PD (48) (**Figure 1**). Thus, there is a major challenge in distinguishing between true "symptoms" of a disease process from "risk factors" that are "associated" but neither necessary nor sufficient to result in disease.

Combinations and interactions of risk factors (e.g., lifestyle, environment, genetic, and aging) might differ between individuals, which may explain the considerable clinical and pathological diversity of PD. Although the risk factors can

offer important clues for the pre-disposition of developing PD, even in the presence of certain risk factors, we currently lack the ability to accurately predict if and when pathological conversion will occur in most instances. As mentioned earlier, PD onset most likely does not involve a single triggering event, but is rather the consequence of a sequence of transient aggravating processes that tip the balance and sets the pathological progression in motion further along the disease continuum.

As the contribution of individual risk factors is thought to be relatively small, gene-environment interactions and how they can inform prediction of future PD in neurologically healthy populations have received considerable attention (49). Risk stratification studies, for instance, have started to model incidence scores using a range of known risk and prodromal factors and assigning each a value before calculating their predictive scores using specific algorithms or regression models (50–54). None of these models have yet been incorporated into clinical practice and have only been used for research purposes. Careful consideration of risk stratification attempts and many other investigations that try to elucidate the cause, progression and heterogeneity of PD reveals an ongoing difficulty in our ability to distinguish between cause and effect (55). Included factors are often based on observational associations, which lack essential definitive conclusions to make causal inferences and may be the result of inverse causation (55, 56). As eloquently pointed out by Chen (57), symptoms expressed in the pre-diagnostic phase several years before diagnosis, but at the time not suspected to be part of PD, might have impacted the factors that are now thought of as protective, such as smoking, physical activity, caffeine consumption. For instance, physical activity might be reduced in individuals in the prodromal stages of PD because of their prodromal features and probably not the other way around (57). It is important to realize that etiological factors may play different roles in the cause and/or progression of PD and would have to be monitored over long periods of time before we can make meaningful interpretations about their positive or negative implications.

CAN BIOMARKERS HELP TO USEFULLY STRATIFY CASES ACCORDING TO CAUSALITY?

The closer we get to the beginning of the PD continuum, the greater the reliance on pathogenic evidence and the availability of independent objective markers to identify those at risk, already converted and beyond (**Figure 1**). While the motor features continue to be the primary criteria for identification of PD, the last few years has seen a surge for the development of objective and independent diagnostic and prognostic biological markers for PD, especially for the asymptomatic phases. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" (58).

A growing number of markers have been proposed as effective screening tools for PD, including clinical, imaging, biochemical, and genetic (59). Different types of markers focus on specific features of PD, such as signs and symptoms, structural and functional integrity, accumulation and aggregation of abnormal proteins and other products of molecular processes as well as variations in the genetic make-up. Therefor, some biological markers are more applicable than others in certain phases of PD as they span the entire disease course, from the risk phase to clinical expression.

Although a multitude of biomarkers for PD have been proposed no biomarker can definitively predict PD onset. Some markers are more focused on the earliest phases of PD than others, but each could provide unique information regarding the presence and progression of PD. Crucially, individual biomarkers may lack sensitivity and specificity for accurate diagnosis and combinations of biomarkers implemented at the right time may be needed to achieve this. More importantly, the validation of individual and combinations of biomarkers is required for early diagnostic potential (59).

THE WAY AHEAD

We are making great strides in the efforts to understand the complexity of PD and the subsequent implications for the development of new diagnostic, prognostic and therapeutic methods, but many questions remain. We now know that the motor phenotype of PD is merely a milestone in a far more extended disease trajectory. Although at some point, most cases converge to increasing levels of movement difficulties and functional impairment in the course of the disease, the underlying cause, pathological pathways and molecular mechanisms might be considerably different, which needs to be reflected by future identification, stratification and therapeutic strategies.

Paradoxically, objective diagnostic tools are needed for intervention with new therapies when it matters most, but development of new therapies to effectively change the disease course requires new objective diagnostic tools. One intermediate way to deal with this paradox is to focus on the populations with an above average pre-disposition for developing PD, such as those with a genetic susceptibility or those with disorders like RBD or olfactory dysfunction that are known for a high risk of conversion to the PD phenotype. Prospective studies in these groups could subsequently inform most optimal therapeutic strategies aimed at modification and protection. In turn, these results can then inform new strategies in the treatment of sporadic forms of PD.

In the absence of a cure for PD, the Holy Grail seems the development of new therapies that impact the actual pathological processes. Although disease modification has successfully been shown in PD models, we are not sure if these treatments will ever work in humans. Regardless of whether it is possible, a lot of work can still be done to increase the effectiveness of current symptomatic therapies aimed at maintaining quality of life and wellness. Especially when we learn how to stratify cases

more effectively and use this information to tailor symptomatic approaches to maximize impact on patients' quality of life and wellness. Fundamentally what is needed to move forward in our search for PD solutions is a better understanding of the natural progression of PD and the underlying pathological processes and mechanisms.

AUTHOR CONTRIBUTIONS

PP and GM contributed the conception and design of the manuscript. PP wrote the first draft of the manuscript. PS, KO'M, and GM wrote the sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Exome Sequencing Reveals Signal Transduction Genes Involved in Impulse Control Disorders in Parkinson's Disease

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Introduction: Impulse control disorders (ICDs) frequently complicate dopamine agonist (DA) therapy in Parkinson's disease (PD). There is growing evidence of a high heritability for ICDs in the general population and in PD. Variants on genes belonging to the reward pathway have been shown to account for part of this heritability. We aimed to identify new pathways associated with ICDs in PD.

Methods: Thirty-six Parkinsonian patients on DA therapy with ($n = 18$) and without ICDs ($n = 18$) matched on age at PD's onset, and gender was selected to represent the most extreme phenotypes of their category. Exome sequencing was performed, and variants with a strong functional impact in brain-expressed genes were selected. Allele frequencies and their distribution in genes and pathways were analyzed with single variant and SKAT-O tests. The 10 most associated variants, genes, and pathways were retained for replication in the Parkinson's progression markers initiative (PPMI) cohort.

Results: None of markers tested passed the significance threshold adjusted for multiple comparisons. However, the "Adenylate cyclase activating" pathway, one of the top associated pathways in the discovery data set ($p = 1.6 \times 10^{-3}$) was replicated in the PPMI cohort and was significantly associated with ICDs in a *post hoc* pooled analysis (combined p-value 3.3×10^{-5}). Two of the 10 most associated variants belonged to genes implicated in cAMP and ERK signaling (rs34193571 in *RasGRF2*, $p = 5 \times 10^{-4}$; rs1877652 in *PDE2A*, $p = 8 \times 10^{-4}$) although non-significant after Bonferroni correction.

Conclusion: Our results suggest that genes implicated in the signaling pathways linked to G protein-coupled receptors participate to genetic susceptibility to ICDs in PD.

Keywords: Parkinson's disease (PD), dopamine agonists (DA), impulse control disorders (ICD), pharmacogenetics, exome sequencing

INTRODUCTION

Impulse control disorders (ICDs) and related behaviors are defined by the failure to resist an impulse to perform a self-rewarding act that will cause longer-term harm to oneself or others. Their prevalence is estimated between 1 and 3% in the general population while it raised up to 17% in treated Parkinsonian patients (1, 2). Common manifestations in Parkinson's disease (PD) are pathological gambling (PG), hypersexuality, and compulsive eating and buying. Clinical risk factors associated with ICDs in PD include younger age, younger age of PD onset, unmarried status, current smoking, novelty seeking traits, and family history of gambling (1, 3, 4). Above all, ICDs in PD are strongly associated with the dopamine replacement therapy as demonstrated by a similar prevalence of ICDs in untreated Parkinsonian patients as compared to healthy controls (5) and their independent association with dopaminergic drugs, particularly dopamine agonists (1).

A high heritability has been suggested for ICDs in the general population, estimated to 40–60% in twin studies for pathological gambling (6, 7) and in ICDs in PD patients (8). Genetic association studies have found associations with candidate variants in genes coding monoamine and glutamate receptors, transporters, and metabolism enzymes in both the general population (9, 10) and in PD (11–14). ICDs are also more frequent and more severe in PD patients with *PRKN* mutations (15). The reward pathway—involving ventral areas of basal ganglia and its related cortical structures—is thought to play a central role in the pathophysiology of ICDs as suggested in several studies (16, 17). However, these variants explain only a small part of the phenotype's variance, estimated to be 15–21% for pathological gambling in the general population (9).

Rare genetic variants or combinations of variants may explain this and new strategies that are being developed to explore genetic contribution to complex traits. For instance, significant genes in the course of diabetes mellitus or cystic fibrosis have been discovered by comparing exome data of extreme phenotype groups, reasonably supposed to be enriched in causal variants (18, 19). Moreover, when several genetic variants in a gene or group of genes may contribute to the trait, aggregation tests, by evaluating the cumulative effect of multiple variants, are particularly relevant. The power gained with this method, compared to a single test, allows considering a smaller sample (20).

In this proof-of-concept study, we propose an unbiased approach by comparing exome sequences of two extreme phenotypes of Parkinsonian patients with and without ICDs. Our main objective was to identify new genes and pathways associated to ICDs in PD.

METHODS

Patients and Study Design

Patients were selected from a multicenter, national, case-control study named Behavioral ADdiction and GEnes in Parkinson's disease (BADGE-PD) conducted in France between 2012 and 2015 in 13 centers of the clinical research network for Parkinson's disease (NS-PARK/FCRIN) (21). Three hundred four patients were included with ($n = 172$) or without ($n = 132$) ICDs or related behaviors. Common inclusion criteria were diagnosis of PD according to the UK Parkinson's Disease Society brain bank (22) and age older than 30 years. The study consisted of one visit at the expert center for PD with a neurological and neuropsychological evaluation and a blood sample for DNA extraction. All patients were evaluated by a face-to-face interview with a neuropsychologist with the French PD behavioral scale, validated for the assessment of ICDs and related behaviors in PD (23). This scale explores and quantifies the different components of hyper- and hypo-dopaminergic behaviors in PD, each item being rated on a 5-point scale (severe disorder: 4, marked disorder: 3, moderate disorder: 2, mild disorder: 1, absence of disorder: 0). Patients with ICDs were defined as having two items with a score of ≥ 2 or at least one item with a score of ≥ 2 on the following items: eating behavior, creativity, hobbying, risk-taking behavior, compulsive shopping, punding, pathological gambling, and hypersexuality. Control PD patients were selected to have no more than one of the previous item with a score of 1. All patients had to be of European Caucasian ancestry for at least two generations, and patients with and without ICDs were matched for age and sex. In addition, patients in the control group had more than 5 years since PD diagnosis and were exposed to a dopamine agonist of at least 300 mg of levodopa equivalent daily dose (LEDD). LEDD were calculated as previously described (24). The study was conducted according to international good clinical practice guidelines and submitted to local regulatory and ethical committees. All patients signed an informed consent form prior any procedure. The study was sponsored by the Assistance Publique Hôpitaux de Paris.

For this ancillary study, we selected an extreme phenotype (EP) population corresponding to 36 cases: 18 PD patients with ICDs (cases) and 18 PD patients without ICDs (controls). Cases were selected in descending order of behavioral scale in PD's scores. The five following items were considered: eating behavior, creativity, compulsive shopping, pathological gambling, and hypersexuality. Controls were selected considering the same items if scoring 0 in all of them. Cases and controls were matched for gender and age at PD onset and had been treated with ropinirole or pramipexole, the two major dopamine agonists used in this cohort. When several controls were available for matching, the patient exposed to the highest dose of DA was selected. Finally, as mutations or risk genetic variants have been shown to be associated with neuropsychiatric symptoms in PD patients (15, 25), patients were screened for the G2019S variant on *LRRK2* gene and for pathogenic mutations on *PRKN* gene.

Replication

For replication, we used the Parkinson's progression markers initiative (PPMI) data set (https://www.michaeljfox.org/ppmi-clinical-study, data downloaded on April 20, 2016), selecting cases and controls with similar criteria than for the hypothesis-generation cohort. In this cohort, ICD behaviors were screened by using the Questionnaire for Impulsive-Compulsive Disorders in Parkinson's disease (QUIP) (26) at each visit. Among the 431 *de novo* Parkinsonian patients included in the cohort, we selected as cases the patients who developed ICD (QUIP>0)

during follow-up ($n = 52$) while treating with pramipexole or ropinirole ($n = 45$) with a QUIP $= 0$ at baseline ($n = 30$) and available exome sequencing data ($n = 27$). For controls, we selected patients with QUIP scores remaining null throughout the follow-up ($n = 127$) treated with ropinirole or pramipexole ($n = 107$) with a dose > 290 mg LEDD for more than 3 months ($n = 29$) and exome sequencing data available ($n = 28$). The 10 most associated genetic variants, genes, and pathways were selected for replication using the whole exome sequencing data.

Sequencing, Variant Calling, and Annotation

Exomes from the 36 selected patients were sequenced on an Illumina NextSeq 500 with the MedExome kit, and the resulting reads were aligned to the hg19 reference genome with BWA applying duplicate removal with Picard Tools MarkedDuplicates. We performed GATK base quality score recalibration, indel realignment, and SNP and INDEL discovery and genotyping across all samples simultaneously using HaplotypeCaller, followed by variant quality score recalibration according to the GATK Best Practices recommendation: GATK-HaplotypeCaller primary QC was applied at the variant level, excluding any variants that were not flagged as "PASS" by the VQSR algorithm, thus excluding variants outside the 95% sensitivity tranches (i.e., the lowest 5% of recalibrated quality scores). Finally, variants were annotated using the SnpEff tool and GENome MINing software (GEMINI, version 0.18.0) (27).

For all 36 samples, mean coverage on target was above 84, and 90% of targeted regions were covered at least at 30X.

Data Analysis

Genetic variants were filtered to keep variants likely to have a functional impact based on the Combined Annotation Dependent Depletion (CADD) score (28) as made available in GEMINI (27). Expression values of genes were obtained from the Brain eQTL Almanac (http://www.braineac.org). Variants with a CADD score $\geq$ 12.37 were kept for further analysis, according to published recommendations (29), if also lying in genes expressed in at least one region among putamen, substantia nigra, thalamus, frontal cortex, and temporal cortex. For a pathway to be considered expressed in these brain regions and kept for analysis, 95% or more of its genes should be expressed in at least one of the latter brain areas. Furthermore, pathways were kept for analysis if at least 80% of their genes were represented by at least one variant in the experiment while a minimum of two variants per gene was required for gene-wise test and a minimum allele count of 4 for single-variant testing. For population description, paired sample *t*-test, McNemar, and χ^2 or Fisher's exact tests were used for group comparisons of quantitative and qualitative variables, using Statistica (version 9.1) software.

Association of single variants with the case/control status was tested using the likelihood ratio test as made available in the Efficient and Parallelizable Association Container Toolbox, version 3.2.6 (EPACTS). Group-wise tests were conducted using SKAT-O tests as made available in EPACTS on both genes and Reactome pathways (30, 31). The significance threshold was adjusted with Bonferroni's method regarding the number of tests ran: 6,953 for variants, 4,769 for genes, and 123 for pathways leading to a threshold for significance of 7.2×10^{-6}, 1×10^{-5}, and 4.1×10^{-4}, respectively.

For the replication stage, combined *p*-values were obtained using the Fisher test when SKAT-O *p*-values were found to be nominally significant in both EP and PPMI cohorts. *P*-values were considered as significant when passing the Bonferroni-corrected significance threshold considering the number of tests run during the discovery and replication phases (6,963 for variants, 4,779 for genes, and 133 for pathways, resulting in a threshold of significance of 7.2×10^{-6}, 1×10^{-5}, and 3.8×10^{-4}). Tests were adjusted on age at PD diagnosis and gender.

TABLE 1 | Clinical features of the study population.

Features	ICDs + ($n = 18$)	ICDs – ($n = 18$)	*p*-value
PD age onset, years[m]	49.4 (7.3)	49.8 (7.4)	0.88
Males, n, (%)[m]	11 (61)	11 (61)	1
Disease duration, years[a]	7.5 (4.6)	9.3 (6.2)	0.33
MDS-UPDRS			
Part I	2.4 (1.9)	0.8 (1.3)	0.005
Part II	10.4 (3.0)	7.9 (4.2)	0.05
Part III	15.0 (10.3)	18.0 (10.3)	0.38
Part IV	4.5 (3.3)	3 (1.9)	0.13
Hoehn & Yahr stage $\leq$ 2, n (%)	16 (88.9)	12 (66.7)	0.13
Family history of addiction (alcohol or PG), n (%)	9 (50)	6 (33)	0.25
MMSE	28.8 (1.2)	28.5 (1.7)	0.57
Treatment			
Prami/Ropi, n/n (%/%)	9/9 (50/50)	13/5 (72.2/27.8)	0.29
DA LEDD, mg/day	258.7 (96.6)	370.3 (65.9)	0.0003
Total LEDD, mg/day	707.6 (541)	940.3 (379.9)	0.13

Results are given as mean (SD), otherwise specified. [m]referred to matching criteria. [a]at onset of behavioral addictions for cases and at inclusion for control subjects. PG, pathological gambling; MMSE, Mini Mental State Evaluation; Prami, Pramipexole; Ropi, Rominirole. Student t-test for paired samples and the McNemar test were run for quantitative and qualitative variables, respectively.

RESULTS

Population

The demographic and clinical characteristics of the 36 selected patients with extreme phenotypes (cases, $n = 18$; controls, $n = 18$) are described in **Table 1**. Cases and controls were similar in terms of PD age onset, disease duration, motor severity (MDS-UPDRS 3 and Hoehn & Yahr stage), and cognitive assessment (MMSE). Cases had significantly higher scores than controls at the MDS-UPDRS part I, which was related to a higher score at item I.6 assessing addictive behaviors (respectively, 2.4 vs. 0.8, $p = 5 \times 10^{-3}$). DA doses were significantly higher in control subjects (370.3 vs. 258.7 mg/day, $p = 3 \times 10^{-4}$) due to the study design (controls were recruited based on the absence of ICD behaviors despite high doses of DA). Among the cases, all had at least two different ICDs, five patients had a maximum score of 4

for at least one item, and eight patients accumulated at least two types of ICDs with a score equal to 3. There was no difference in ropinirole/pramipexole or gender distributions regarding to the type of ICDs except for hypersexuality, which was exclusively present in males (see **Supplementary Data 1**).

Single-Variant Analysis

After quality controls, 354,174 variants were available for further analyses. Among the 34,981 with a CADD-score $\geq$ 12.37, 26,418 satisfied the condition of a minimal occurrence of 4 in the cohort for the single test. After selection upon brain expression of genes, 6,953 variants remained for further analyses. The 10 most associated variants are shown in **Table 2**. Five variants were exonic missense—in *PDCD6IP*, *RasGRF2*, *ACAN*, *COL12A1,* and *OMA1*—and 4 variants were intronic—in *GDA*, *PGK1*, *ITGA6,* and *PDE2A*. The last variant was nonsense in the *IL17RB* gene. Two variants (rs34193571 *RasGRF2,* rs1877652 *PDE2A*) were involved in intracellular signal transduction, related to ERK, and cAMP signaling pathways. Three variants were laid on genes belonging to the pathway of focal adhesion (rs2293647, rs3743398, and rs970547 on *ITGA6*, *ACAN,* and *COL12A1*). No mutation was found in the *LRRK2* or *PRKN* genes.

Genes Analysis

Of the 19,962 genes covered by the MedExome kit, 6,250 were represented by at least two variants with a CADD score $\geq$ 12.37. After selection upon brain expression, 4,769 genes remained for association testing. The 10 most associated genes upon SKAT-O test are shown in **Table 3**. Four of them, *DOCK4*, *RasGRF2*, *ITGA6,* and *ITGA11,* were linked to the ERK pathway. *PDE2A* belongs to cAMP signaling. For some genes, such as *PDE2A* and *RasGRF2*, association was led by only one frequent variant, which was strongly associated in the single test. For others, as for *DOCK4* and *MYH14*, association resulted from different rare variants laying in this gene in different individuals of the cohort (see **Supplementary Data 2**).

Pathways Analysis

Of the 1,816 pathways extracted from the Reactome database, 1,130 were represented by at least two variants with a CADD score $\geq$ 12.37. Among them, only 312 had more than 95% of their genes expressed in the brain's regions of interest, and the 123 that had more than 80% of their genes represented by at least one variant were considered for further analysis. The five most associated pathways using SKAT-O are shown in **Table 4**. The most associated one (R-HSA-446343, "Localization of the PINCH-ILK-PARVIN complex to focal adhesions" from Reactome database, p-value 1.52×10^{-3}) was related to focal adhesions with a total of eight variants (two of which were unique, i.e., found only once in the cohort) tested. The second most associated pathway was the adenylate activating pathway (R-HSA-170660, "Adenylate cyclase activating pathway", $p = 1.56 \times 10^{-3}$) with a total of 29 (10 of which were unique) variants tested distributed on eight genes.

TABLE 3 | SKAT-O on genes results (top 10 genes).

	EP		PPMI	
Gene	**tested variants**	***p*-value**	**tested variants**	***p*-value**
ANAPC5	3 (0)	0.0005	6 (2)	NS
PDE2A	2 (0)	0.0009	10 (2)	NS
FANCA	4 (1)	0.0009	*0*	*NA*
SGK494	2 (0)	0.0013	6 (2)	NS
DOCK4	9 (4)	0.0016	*NA*	*NA*
APOL5	2 (0)	0.0018	4 (1)	NS
RasGRF2	2 (1)	0.0019	*NA*	*NA*
ITGA6	3 (0)	0.0019	18 (1)	NS
ITGA11	7 (4)	0.0020	21 (4)	NS
MYH14	7 (4)	0.0031	26 (3)	NS

Tested variants are reported as number of tested variants (number of unique variants, i.e., found only once in the cohort).

TABLE 2 | Single test association study results (top 10 variants).

		Variant ID			Variant distribution in EP cohort				*p*-value	
ID	**Gene**	**Annotation**	**MAF (EXAC)**	**MAF (EP)**	**AAC**	**AAC Case**	**AAC Control**	**RR**	**EP**	**PPMI**
rs3203777	PDCD6IP	V383I	0.44	0.50	36	11	25	0.4	0.0001	NS
rs3802506	GDA	intronic	0.16	0.17	12	11	1	11.0	0.0002	*NA*
rs2007039	PGK1	intronic	0.22	0.18	13	0	13	0.0	0.0002	*NA*
rs34193571	RASGRF2	S753P	0.08	0.10	7	7	0	14.0	0.0005	NS
rs2293647	ITGA6	intronic	0.05	0.10	7	7	0	14.0	0.0007	*NA*
rs3743398	ACAN	P864L	0.21	0.22	16	3	13	0.2	0.0008	NS
rs1877652	PDE2A	intronic	0.28	0.31	22	17	5	3.4	0.0008	*NA*
rs970547	COL12A1	G3058S	0.22	0.28	20	4	16	0.3	0.0008	NS
rs1043261	IL17RB	Stop	0.08	0.08	6	0	6	0.0	0.0011	NS
rs17117678	OMA1	I329L	0.10	0.14	10	1	9	0.1	0.0012	NS

Minor Allele Frequency (MAF) is given according to EXAC data for Caucasian population and in extreme phenotype (EP) cohort. AAC, Alternative Allele Count in all population and in case and control groups; NS, non-significant. RR referred to the ratio of allele frequency in case and in control groups. If the allele count was equal to zero in control group, the value was replaced by 0.5 to enable the division.

TABLE 4 | SKAT-O on pathways results (top 5 pathways).

Pathway		EP		PPMI		combined *p*-value
Name		Tested variants	*p*-value	Tested variants	*p*-value	
R-HSA-446343	Localization of the PINCH-ILK-PARVIN complex to focal adhesions	8 (2)	0.0015	32 (6)	0.6072	NA
R-HSA-170660	Adenylate cyclase activating pathway	29 (10)	0.0016	29 (10)	0.0210	$3.3.10^{-5*}$
R-HSA-5467333	APC truncation mutants are not K63 polyubiquitinated	7 (4)	0.0156	27 (4)	0.925	NA
R-HSA-1483152	Hydrolysis of LPE	5 (4)	0.0231	29 (4)	0.4554	NA
R-HSA-4411364	Binding of TCF/LEF:CTNNB1 to target gene promoters	20 (9)	0.0233	66 (12)	0.4361	NA

*Tested variants are reported as number of tested variants (number of unique variants). EP, Extreme phenotypes cohort. P-value from SKAT-O tests on pathways in EP and in PPMI cohorts are reported. Combined p-values obtained with a Fisher test are reported in the last column. Combined p-values with a Fisher test were only calculated when both p-values in EP and in PPMI cohorts were found nominally significant (i.e., < 0.05). *Significant p-value after Bonferroni correction.*

None of the variants, genes, or pathways tested passed the significance threshold adjusted with Bonferroni's method (significance threshold of 7.2×10^{-6}, 1×10^{-5}, and 4.1×10^{-4} for a single test, SKAT-O on genes and pathways, respectively), in the discovery phase only.

Replication

The replication data set consisted of 59 patients (27 cases and 28 controls). Mean age at PD diagnosis was 58 years old in both groups. There were seven women in the cases and seven in the controls. Pramipexole was the most frequent DA in each group: 60 and 76% in cases and controls, respectively. Doses of DA were significantly higher in controls than in cases (372 LEDD vs. 193 mg/day LEDD, $p < 0.001$).

We applied the same analysis for the 10 most associated variants, genes, and pathways. Only the "adenylate cyclase activating pathway" replicated in the PPMI cohort (**Table 4**, $p = 2 \times 10^{-2}$ in the PPMI cohort and $p = 1.56 \times 10^{-3}$ in the EP cohort, resulting in a Fisher combined p-value of 3.3×10^{-5}). Twenty-nine variants were tested in the EP and PPMI cohorts (see **Supplementary Data 3 and 4**). In the EP cohort, variants laid on eight different genes: *ADCY 1, 3, 4, 5, 6, 8, 9,* and *GNAL*. In the PPMI cohort, the nine genes encoding the adenylate cyclase from 1 to 9 were represented by at least one variant. Six variants of the 29 tested in each cohort were common to both cohorts, resulting in a total of 52 variants tested for this pathway. For five of them (rs3181385 on *ADCY4*, rs3730071, rs115315671, rs55770045 on *ADCY6,* and rs2228949 on *ADCY8*), the effect was observed in the same direction in both cohorts.

DISCUSSION

In this study comparing whole exome sequences from two extreme phenotypes, we found enrichment of functional variants laying on brain-expressed genes of the "adenylate cyclase activating pathway" in PD patients with ICDs. This result is in accordance with a recent genome-wide association study founding an association between pathological gambling and the cAMP protein kinase signaling (32).

The dopamine replacement therapy that has been strongly associated with ICDs in PD patients activates dopamine receptors, 7-transmembrane domain G protein-coupled receptors (GPCR), regulating adenylate cyclase activity. D2-like receptors inhibit adenylyl cyclase, whereas stimulation of D1-like receptors leads to its activation through Galpha(olf) in the striatum (33). Cyclic-AMP then acts as a second messenger by activating the protein kinase A (PKA), which, in turn, phosphorylates several substrates and activates transcription factors. In the case of concomitant glutamatergic activation, the extracellular regulated kinase (ERK) MAPK pathway is also activated in medium spiny neurons in the striatum (34). These different routes converge to alteration in gene expression (35), which is supposed to underlie long-term neuronal plasticity induced by dopamine in the striatum (36). ERK activation in the ventral striatum has been shown to play a central role in the reward-associated pathway and in addictive behaviors related to alcohol (37) and psychostimulant consumption (38). In mice, activation of the ERK pathway in the dorsal striatum has been shown to underlie abnormal involuntary movements, dyskinesia, developed in response to chronic L-DOPA (39). In our study, the pathway directly related to cAMP signaling the "Adenylate cyclase activating pathway" was found to be associated with ICDs in both EP and PPMI cohorts.

Interestingly, although below the significance threshold, many of the most associated variants or genes tested were linked to the cAMP and ERK-dependent pathways.

The most associated variant in our study was a missense variant (rs3203777) in the *PDCD6IP* gene, also named *DRIP4* for dopamine receptor interacting protein 4. This gene encodes a protein that has been shown to upregulate D1 and D3 receptor expression (40).

In addition, the *rs1877652* variant, enriched in patients with ICDs, is an intronic variant of the *PDE2A* gene encoding the phosphodiesterase 2A controlling cAMP rates and is shown to play a key role in the modulation of the signal induced by dopamine in rodents (41). Seven patients with ICDs and none of the controls had a coding variant on *RasGRF2* (rs34193571), previously associated with alcohol addiction (42), and coding a protein that mediates calcium-dependent activation of the ERK pathway, modulating the presynaptic effect on the activation of the basal ganglia mesolimbic pathway (43).

Several top associated variants, genes, and pathways were related to integrin systems, which also act on ERK signaling via

downstream transducers, including the focal adhesion kinase, FAK. Integrin and its downstream effectors have been shown to be involved in structural changes that occurred in the dendritic tree within the addiction to alcohol (44) or cocaine process (45). A rare variant, *rs2293647*, lying in the integrin subunit α 6 gene *ITGA6*, was found only among cases ($p = 7 \times 10^{-4}$) while the *rs3743398*, a variant lying on the *ACAN* gene belonging to the aggrecan family, was found more frequently in controls, suggesting a protective role. The most associated pathway in the EP cohort was related to the integrin system although it was not replicated in the PPMI cohort.

None of these variants was previously found to be associated with ICDs in PD or in the general population. However, most previous studies focused on candidate gene coding receptors, transporters, or metabolism enzymes of the reward pathway but did not investigate their downstream signaling effectors (9–14).

The main limitation of our study is the small sample sizes of both our hypothesis generation and the replication data sets, which partly resulted from our stringent inclusion criteria (high doses of DA, severe ICDs, or no ICDs at all), and our results deserve further replication in larger cohorts. The underpowered analysis may explain that only one pathway was replicated and that most of the *p*-values obtained were far from reaching the adjustment for the multiple comparison threshold of significance. The lack of replicated results might also be due to differences between the two cohorts in terms of exome sequencing coverage or phenotypic definition of ICDs in the two cohorts. The selection of extreme cases strongly associated with DA-induced ICDs might have biased our results toward genes directly interacting with these drugs, which was the purpose of the design of the study. Our results also suggest that genetic susceptibility to ICDs in PD would rather result from the combination of multigenic variations than unique rare variants with strong effects, which was already suggested for ICDs in the general population (9).

This study highlights the potential important role of enrichment of functional variants in genes coding proteins of intracellular signaling cascades beyond neurotransmitter receptors in the genetic susceptibility of ICDs. Subjects with stronger signal transduction of GPCRs might be at higher risk to develop ICDs when exposed to dopaminergic therapy in PD. How these pathways interfere with drugs or increase the individual susceptibility to ICDs remain to be explored. The combination of clinical, neuroimaging, and exome sequencing data focused on the study of the polymorphism of the different pathways could help in providing some predictability, in the future, for ICDs in PD patients.

DATA AVAILABILITY STATEMENT

Data are available upon request to any interested researcher or for the purpose of replicating our results (direct requests to jean-christophe.corvol@aphp.fr).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP Paris VI. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-CC, FD, and SP contributed to the design of the work, data acquisition, interpretation of the data, and draft the manuscript. AR, HB, JG, and YM participated to data analysis. All authors contributed to the interpretation of the data, revised the manuscript for important intellectual content, provide approval for the publication, and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

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BRIEF RESEARCH REPORT
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Genetic and Phenotypic Basis of Autosomal Dominant Parkinson's Disease in a Large Multi-Center Cohort

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LRRK2, *SNCA,* and *VPS35* are unequivocally associated with autosomal dominant Parkinson's disease (PD). We evaluated the prevalence of *LRRK2*, *SNCA,* and *VPS35* mutations and associated clinical features in a large French multi-center cohort of PD patients. Demographic and clinical data were collected for 1,805 index cases (592 with autosomal dominant inheritance and 1,213 isolated cases) since 1990. All probands were

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screened with TaqMan assays for *LRRK2* Gly2019Ser. In the absence of this mutation, the coding sequences of the three genes were analyzed by Sanger sequencing and/or next-generation sequencing. The data for the three genes were analyzed according to age at onset, family history, ethnic origin and clinical features. We identified 160 index cases (8.9%) with known pathogenic variants: 138 with pathogenic *LRRK2* variants (7.6%), including 136 with the Gly2019Ser mutation, 19 with *SNCA* point mutations or genomic rearrangements (1.1%), and three with the *VPS35* Asp620Asn mutation (0.16%). Mutation frequencies were higher in familial than isolated cases, consistent with autosomal dominant inheritance (12.0 vs. 7.3%; OR 1.7, 95% CI [1.2–2.4], $p = 0.001$). PD patients with *LRRK2* variants were more likely to have higher rates of late-onset PD (>50 years; OR 1.5, 95% CI [1.0–2.1], $p = 0.03$), whereas those with *SNCA* mutations tended to have earlier age at onset disease (≤50 years, $p = 0.06$). The clinical features of *LRRK2* carriers and those without any pathogenic variants in known PD-associated genes were similar. The likelihood of detecting disease-causing mutations was higher in cases compatible with autosomal dominant inheritance.

Keywords: Parkinson's disease, *LRRK2*, G2019S, *SNCA*, *VPS35*, autosomal dominant inheritance, genotype-phenotype correlations

INTRODUCTION

Heterozygous sequence variants of *LRRK2* or *VPS35*, and mutations or genomic rearrangements in *SNCA* cause monogenic Parkinson's disease (PD) with autosomal dominant (AD) inheritance [reviewed in (1)]. Only seven of the hundred or so variants of *LRRK2* reported to date (Asn1437His, Arg1441Gly/Cys/His, Tyr1699Cys, Gly2019Ser, and Ile2020Thr), which appear to be clustered in functionally important regions highly conserved throughout evolution, have been demonstrated to be pathogenic on the basis of co-segregation with the disease and absence or rarity in specific control populations (2). The most common of these mutations, *LRRK2* Gly2019Ser, has a reported frequency of 0% to above 40%, depending on the population considered (3), mostly due to a common founder effect (4). *SNCA* mutations are the second most common cause of autosomal dominant inherited PD; genomic duplications have been detected in ∼1–2% of families with AD PD. Other *SNCA* mutations, such as whole-locus triplications and a few missense mutations (Ala53Thr/Glu/Val, Glu46Lys, Ala30Pro, and Gly51Asp), are extremely rare [reviewed in (1)]. Finally, *VPS35* was the first PD-causing gene to be identified by next-generation sequencing (NGS) in large multi-incident families (5, 6). Subsequent studies in multiple ethnic groups, including a large multi-center study, indicated that Asp620Asn was the only pathogenic variant, with a relative frequency ranging from 0.1 to 1% in familial PD, depending on population background (7). As the phenotype of AD PD closely resembles that of idiopathic PD, we assumed that rare variants of genes causing AD PD might also contribute to the etiology of isolated PD in the French population.

Abbreviations: AAO, age at onset; AD, autosomal dominant; *LRRK2*, leucine-rich repeat kinase 2; MMSE, mini mental state examination; PD, Parkinson's disease; *VPS35*, vacuolar protein sorting 35.

In this study, we aimed at determining the relative frequencies of known mutations of three genes, *LRRK2*, *SNCA*, and *VPS35*, in a large cohort of familial and isolated cases. The high prevalence of the *LRRK2* Gly2019Ser mutation provided us with a unique opportunity to compare in details clinical characteristics between carriers of this mutation and patients with no known mutations of PD-associated genes.

MATERIALS AND METHODS

Patients

In total, 673 PD patients from 592 families and 1,213 isolated cases without known consanguinity were recruited from 1990 onwards, through the French Parkinson Disease Genetics Network (the PDG group, **Supplementary Material**). All participants underwent a detailed medical and family history, and a family tree were drawn. Familial cases compatible with AD inheritance, and referred to here as AD PD cases, were defined as AD cases with at least one other affected relative in a different generation, identified by an examination of secondary cases ($n = 146$) or on the basis of family history ($n = 446$). PD was diagnosed according to the clinical diagnostic criteria of the UK Parkinson Disease Society Brain Bank (PDSBB) (8). Comprehensive standardized interviews and neurological examinations were performed by movement disorder experts. Motor and non-motor symptoms were assessed by evaluating Unified Parkinson Disease Rating Scale (UPDRS) scores, Hoehn and Yahr staging, autonomic dysfunction, sleep, cognitive [Mini Mental State Examination (MMSE)], neuropsychological and behavioral scores. Early onset was defined as the onset of symptoms before the age of 51 years.

Informed consent was obtained from all participants, and genetic studies were approved by local ethics committees.

Most index cases were of European ancestry (n = 1,530; 84.8%), mostly French (n = 1,202; 78.6%); the others were North African (n = 221; 12.2%) or of other origins, including Asian, Sub-Saharan African or "mixed" origins (n = 42; 2.3%); or of unknown origin (n = 12; 0.7%). This study included 226 index cases from families with PD compatible with AD inheritance reported elsewhere (9, 10).

Methods

Genomic DNA was obtained from peripheral blood lymphocyte or saliva samples (Oragene™ DNA Self-Collection Kit, DNA Genotek), by standard protocols. Patients with variants of the *GBA* risk factor (n = 153), or with variants of genes for which the causal role in AD PD was uncertain, such as *GIGYF2* (n = 6), *EIF4G1* (n = 2), and *c9ORF72* (n = 4), were not included in this study. In addition, we excluded 25 (23 with bi-allelic *PRKN* mutations and two with bi-allelic *PINK1* mutations) of the 1,089 PD index cases who have been screened for autosomal recessive (AR)-PD associated genes [814 by gene panel/exome sequencing (see below) and 275 by direct sequencing of the two most frequent AR PD genes, *PRKN* and *PINK1*].

All index cases were genotyped in duplicate for *LRRK2* Gly2019ser, by the TaqMan allelic discrimination Assay-By-Design method, in accordance with the manufacturer's instructions, with 8 ng of DNA mixed with the TaqMan Genotyping Master Mix (Thermo Fisher Scientific Inc.) and custom-produced TaqMan SNP genotyping assays [C_63498123_10 (rs34637584), Thermo Fisher Scientific Inc.] on an Applied Biosystems PRISM 7000 sequence detection system (Thermo Fisher Scientific Inc.) or LightCycler® 480 machine (Roche, Life Technologies SAS). All patients found not to carry *LRRK2* Gly2019ser were then screened for pathogenic variants of the coding sequences of *LRRK2*, *SNCA*, and *VPS35*, by Sanger sequencing (n = 855), targeted sequencing of a customized next-generation sequencing (NGS) gene panel containing the 22 most prevalent PD-associated genes (n = 404; **Supplementary Table 1**), or available whole-exome sequencing (n = 410), as previously described (11, 12). We considered known pathogenic mutations of the three genes.

Sanger sequencing was used to confirm variants and co-segregation analyses were performed, where possible. *SNCA* rearrangements were detected by semi-quantitative multiplex PCR (13) or by the SALSA multiplex ligation-dependent probe amplification method (MLPA, MRC Holland, Amsterdam, the Netherlands; http://www.mlpa.com), according to the manufacturer's instructions.

Statistical Analysis

Demographic and clinical characteristics are expressed as means and standard deviations for continuous variables and as counts and percentages for qualitative variables, separately for each group (i.e., with and without mutation). These characteristics were compared between patients with the *LRRK2* Gly2019ser mutation (*LRRK2*+) and those with no mutations of genes known to be associated with PD (genetically undefined PD), in Welch's t-tests for continuous variables and Fisher's exact tests for qualitative variables.

We used generalized linear models (GLMs) to compare clinical features between patients with the *LRRK2* Gly2019ser mutation and those with genetically undefined PD, with adjustment for sex, age at onset (AAO), and disease duration. Disease duration was not included in models of clinical features at onset. We used GLMs with identity links and normal distributions for continuous clinical features, and GLMs with logit links and Bernoulli distributions for binary clinical features. Fisher type II tests were performed to test each effect, and effect size was estimated with Cohen's f^2. We corrected for multiple testing by the Benjamini-Hochberg method. Residual normality and heteroskedasticity were checked visually. Influencers and outliers were checked by calculating hat values and Cook distances. Only patients with no missing data for the covariables included in the models, such as AAO, sex, and disease duration, were retained for analysis. Statistical analyses were performed with R 3.6.1.

RESULTS

Demographic and Clinical Data

Table 1 shows the baseline demographic and clinical characteristics of the 1,805 PD index cases included in the study. Men (n = 1,106, 61.3%) and early AAO cases (mean 47.4 [SD 12.5]; range 9–86 years; **Table 1**) were overrepresented, particularly among isolated cases (mean 46.1 [*SD* 12.5]; range: 9–79 years) as opposed to familial cases (mean 50.0 [*SD* 12.1]; range 10–86 years).

Summary of the Mutations Identified

We identified seven different known mutations of *LRRK2*, *SNCA*, and *VPS35* in 160 of the 1,805 PD index cases (8.9%, 95% confidence interval (CI): [7.6–10.2]). With the exception of rare cases with the homozygous *LRRK2* Gly2019Ser mutation (n = 6), all the known mutations and gene multiplications identified were heterozygous. We found that 136 of the 138 *LRRK2* mutation carriers (7.5% of index cases, 95% CI [6.4–8.9]) carried the Gly2019Ser mutation; 13 of these patients had already been reported elsewhere (9). Two index cases carried the rare *LRRK2* Arg1441His mutation (9). *SNCA* mutations were found in 19 index cases (1.1%, 95% CI [0.63–1.6]), including 11 families already reported (13–15): one with whole-gene triplications, 14 with duplications, one with the Gly51Asp mutation, and three with the Ala53Thr mutation. The three PD index cases with the only *VPS35* mutation identified, Asp620Asn (0.17%) were described in a previous report (10).

The *LRRK2* Gly2019Ser mutation was more common in PD index cases of North-African origin (100/221, 45.2%; 95% CI [38.7–51.8]) than in Europeans (36/1,530, 2.4%; 95% CI [1.7–3.2]) (Fisher's exact test: odds ratio (OR) = 34.3, 95% CI [22.0–53.8], $p < 0.0001$; **Table 2**). By contrast, *SNCA* point mutations and locus triplications/duplications were found mostly in PD index cases of European ancestry, accounting for 1.2% (18/1,530) of such patients, whereas only one case of North-African ancestry (0.45%, 1/221) carried an *SNCA* duplication. *VPS35*

TABLE 1 | Baseline demographics for our study population.

	All index cases	AD PD index cases	Isolated cases
	***n* = 1,805**	***n* = 592**	***n* = 1,213**
Sex, *n* (%)			
Male	1,106 (61.3)	342 (57.8)	764 (63)
Female	699 (38.7)	250 (42.2)	449 (37)
Ancestry, *n* (%)			
Europeans	1,530 (84.8)	525 (88.6)	1,005 (82.8)
North-Africans	221 (12.2)	55 (9.3)	166 (13.7)
Other/mixed origins	42 (2.3)	11 (1.9)	31 (2.6)
Unknown origins	12 (0.7)	1 (0.2)	11 (0.9)
Age at onset, (*SD*)	47.4 (12.5)	50.0 (12.1)	46.1 (12.5)
Range, years	9–86	10–86	9–79
Age at examination, (*SD*)	55.8 (13.3)	57.9 (12.5)	54.9 (13.5)
Range, years	12–88	16–88	12–85
Early-onset (≤50 years), *n* (%)	1,065 (62.7)	311 (54.6)	754 (66.8)
Late-onset (>50 years), *n* (%)	634 (37.3)	259 (45.4)	375 (33.2)
Disease duration, (*SD*)	8.4 (6.8)	7.8 (6.7)	8.8 (7.4)
Range, years	0–52	0–52	0–46

Frequencies were compared in Fisher's exact tests for qualitative traits and means were compared in Welch's t-tests for continuous variables. Age-at-onset was missing for 106 index cases.

AD, autosomal dominant; PD, Parkinson's disease; SD, standard deviation.

mutations were identified exclusively in patients of European origin, accounting for 0.20% (3/1,530) of these individuals.

Overall, mutations were more frequently identified in familial (71/592, 12.0%; 95% CI [9.5–14.9]) than in isolated cases (89/1,213, 7.3%; 95% CI [5.9–9.0], Fisher's exact test: OR 1.7, 95% CI [1.2–2.4], $p = 0.001$), particularly for *SNCA* (2.4%, 14/592 *vs.* 0.41%, 5/1,213, Fisher's exact test: OR 5.9, 95% CI [2.1–16.3], $p = 0.0003$; **Table 2**). An analysis of PD cases according to age at onset (≤50 years vs. >50 years) showed that *LRRK2* mutations were more frequent among late-onset PD cases (61/636, 9.6%; 95% CI [7.4–12.2] vs. 71/1063, 6.7%; 95% CI [5.3–8.4], Fisher's exact test: OR 1.5, 95% CI [1.0–2.1], p = 0.03; **Table 2**). By contrast, *SNCA* mutation carriers tended to have an earlier AAO (16/1063, 1.5% vs. 3/636, 0.5%, $p = 0.06$).

Clinical Characteristics of Mutation Carriers and Comparison of *LRRK2* Gly2019Ser Mutation Carriers (*LRRK2*+) With Individuals With No Known PD Mutations (Genetically Undefined PD)

Co-segregation analyses identified 193 PD patients as mutation carriers: 151 with *LRRK2* Gly2019Ser and five with Arg1441His mutations, 29 with *SNCA* (see below) and eight with *VPS35* Asp620Asn mutations.

SNCA

The clinical characteristics of the 29 PD patients carrying either *SNCA* rearrangements [triplications ($n = 2$) and duplications ($n = 21$)] or missense mutations [Ala53Thr ($n = 3$) and Gly51Asp ($n = 3$)] are shown in **Table 3**. All but three of the families concerned originated from France. The remaining three families, originating from Italy, Turkey and Morocco, all had *SNCA* duplications. Within this cohort, *SNCA* duplications were the most frequent mutation identified (14/19, 73.7%), followed by the Ala53Thr mutation (3/19, 18.8%). Disease onset occurred earliest in patients with the Ala53Thr mutation (mean 34.7 [*SD* 7.6], range 26–40 years).

Patients carrying the Ala53Thr mutation had an extra-pyramidal parkinsonian syndrome, but with heterogeneity between patients with the same mutation: Patient 1172-001, with both *SNCA* Ala53Thr and a heterozygous *PRKN* Thr240Met variant, had atypical PD, with a poor response to levodopa, early motor fluctuations and cerebellar signs. He rapidly developed impulse control disorders. This patient currently displays no cognitive decline. He had a bilateral subthalamic deep brain stimulation (STN-DBS). He received clozapine treatment for delusions with a beneficial effect. Patient 1219-001 presented a parkinsonian syndrome that responded well to levodopa, but developed severe dysarthria. She underwent unilateral internal globus pallidus (GPi)-DBS. This patient presented no major cognitive and behavioral signs other than an alteration of executive functions. A third PD patient, 196-016 presented early-onset (26 years) typical PD that responded well to levodopa. Detailed clinical data are provided in **Supplementary Table 2**.

Both *SNCA* locus triplications and Gly51Asp mutation were associated with early-onset atypical parkinsonism (mean AAO: 42.0 years [*SD* 8.5], range: 36–48 years and mean AAO: 42.0 [*SD* 15.7], range: 31–60 years, respectively). The patients with *SNCA* triplications were characterized by severe cognitive impairment in one of two carriers, dysautonomia, a poor response to levodopa in both patients. The three Gly51Asp

TABLE 2 | Overall frequencies of *LRRK2*, *SNCA,* and *VPS35* mutations according to index case ethnicity, family history of Parkinson's disease and age at onset.

	Europeans	**North-Africans**	**North-Africans vs. Europeans OR, 95% CI, *p***	**AD PD**	**Isolated cases**	**AD PD vs. isolated cases OR, 95% CI, *p***	**EO-PD**	**LO-PD**	**LO-PD vs. EO-PD OR, 95% CI, *p***
	n = 1,530	*n* = 221		*n* = 592	*n* = 1,213		*n* = 1,063	*n* = 636	
All *LRRK2, n* (%, 95% CI)	38 (2.5%, [1.8–3.4])	100 (45.2%, [38.6–52.0])	*OR* = 32.5, [21.4–49.2], $p < 0.0001$	54 (9.1%, [6.9–11.7])	84 (6.9%, [5.6–8.5])	*OR* = 1.3, [0.9–1.9], $p = 0.11$	71 (6.7%, [5.3–8.4])	61 (9.6%, [7.4–12.2])	*OR* = 1.5, [1.0–2.1], $p = 0.03$
Gly2019Ser *n* (%)	36 (2.4%)	100 (45.2%)		52 (8.8%)	84 (6.9%)		71 (6.7%)	59 (9.3%)	
Arg1441His *n* (%)	2 (0.13%)	0 (0%)		2 (0.34%)	0 (0%)		0 (0%)	2 (0.32%)	
All *SNCA*, *n* (%, 95% CI)	18 (1.2%, [0.7–1.9])	1 (0.45%, [0.01–2.5])	*OR* = 0.38, [0.05–2.9], $p = 0.50$	14 (2.4%, [1.3–3.9])	5 (0.41%, [0.1–1.0])	*OR* = 5.9, [2.1–16.3], $p = 0.0003$	16 (1.5%, [0.9–2.4])	3 (0.47%, [0.1–1.4])	*OR* = 0.31, [0.09–1.1], $p = 0.06$
Triplications *n* (%)	1 (0.065%)	0 (0%)		1 (0.17%)	0 (0%)		1 (0.094%)	0 (0%)	
Duplications *n* (%)	13 (0.85%)	1 (0.5%)		11 (1.9%)	3 (0.25%)		11 (1.0%)	3 (0.47%)	
Ala53Thr *n* (%)	3 (0.20%)	0 (0%)		1 (0.17%)	2 (0.16%)		3 (0.28%)	0 (0%)	
Gly51Asp *n* (%)	1 (0.065%)	0 (0%)		1 (0.17%)	0 (0%)		1 (0.094%)	0 (0%)	
VPS35 Asp620Asn, *n* (%, 95% CI)	3 (0.20%, [0.04–0.57])	0 (0%)	$p = 1$	3 (0.51%, [0.1–1.5])	0 (0%)	$p = 0.04$	2 (0.19%, [0.02–0.7])	1 (0.16%, [0.0–0.9])	*OR* = 0.84, [0.08–9.2], $p = 1$
Total mutations, *n* (%, 95% CI)	59 (3.9%, [2.9–4.9])	101 (45.7%, [39.0–52.5])	*OR* = 21.0, [14.5–30.4], $p < 0.0001$	71 (12.0%, [9.5–14.9]	89 (7.3%, [5.9–9.0])	*OR* = 1.7, [1.2–2.4, $p = 0.001$	89 (8.4%, [6.8–10.2]	65 (10.2%, [8.0–12.8]	*OR* = 1.3, [0.9–1.8], $p = 0.22$

Age at onset was missing for 106 cases, including 6 with the LRRK2 G2019S mutation. We also considered patients with missing AAO data examined at an age ≤50 years (n = 26) to have early-onset PD.
AD, autosomal dominant; CI, confidence intervals; EO, early-onset; LO, late-onset; OR, odds ratio; PD, Parkinson's disease.

mutation carriers had shorter disease duration (mean: 3.7 years [*SD* 3.1], range: 1–7 years), a mild-to-moderate response to levodopa, frequent psychiatric symptoms but no dementia or autonomic dysfunction (**Supplementary Table 2**). By contrast, AAO was highest in patients with *SNCA* duplications (mean AAO: 45.3 years [*SD* 6.3], range: 36–56 years), consistent with typical PD and a good response to levodopa (100%), with more than 50% of these patients reporting levodopa-induced motor complications. Non-motor symptoms, including depression/psychosis and dysautonomia, were present in about one third of the reported cases, but cognitive decline was less frequent (18.8%, 3/16). Detailed clinical characteristics for PD patients with *SNCA* multiplications are provided in **Supplementary Table 3**.

VPS35

Five of the eight PD patients carrying *VPS35* Asp620Asn mutations have been described before (10). The three newly genotyped patients were relatives of patient 838–006 (**Supplementary Table 4**). Briefly, patients carrying *VPS35* mutations had features similar to those with idiopathic PD, with a mean AAO of ∼57 years (range: 38–71 years, **Table 3**): all patients presented the classical triad, with akinesia as the predominant symptom at onset (100%), but a much lower frequency of tremor as an initial symptom (37.5%), a good response to levodopa (100%), with <37% of those treated developing dyskinesias and motor fluctuations, and a low rate of dysautonomia (2/8, 25%), with no cognitive or neuropsychiatric symptoms or atypical signs.

LRRK2

We compared the clinical features of the *LRRK2* G2019S+ PD patient (*LRRK2*+) group with those of PD patients with no mutations of known PD-associated genes, excluding subjects with missing data for the covariables included in the models, such as sex, AAO, and disease duration: 135/151 *LRRK2*+ and 1,552/1,693 PD patients without mutations were included in the final analysis (**Table 4**). The proportion of men was greater in the genetically undefined PD patient group than in the *LRRK2*+ group (61.1 vs. 51.9%, $p = 0.04$). The mean AAO of the *LRRK2* Gly2019Ser carriers was 4 years higher than that of non-carriers ($p < 0.001$). The Gly2019Ser carriers were more likely to be of North-African ancestry ($p < 0.001$) and to report a family history of PD ($p < 0.02$). They had a higher UPDRS III score during the "OFF" state and a higher Hoehn and Yahr score during the "ON" state than non-carriers, but these results were no longer significant after adjustment for AAO and disease duration. The frequencies of signs at onset and at examination, the degree of response to treatment, motor complications and non-motor signs, including cognitive impairment and autonomic dysfunction were similar in both groups.

Clinical comparisons between heterozygous ($n = 144$) and homozygous ($n = 7$) Gly2019Ser mutation carriers revealed no significant differences in sex (men: 52.1 vs. 42.9%, *OR* 1.4, CI [0.31–6,7], $p = 0.71$), AAO (mean 51.4 [*SD* 12.1] vs. mean 53.7 [*SD* 11.2] years, $p = 0.62$), disease duration (mean 8.7 [*SD* 6.7] vs. 9.1 [*SD* 4.5] years, $p = 0.87$) or clinical presentation, but the number of homozygous carriers was small.

Unlike *LRRK2* Gly2019Ser carriers, all patients with the Arg1441His mutation were French and all reported a family history of PD. They had a shorter disease duration (mean: 5.2 years [*SD* 5.1], range: 2–14 years vs. mean 9.0 years [*SD* 8.0], range: 0.5–63 years), were more likely to develop an akinetic-rigid motor phenotype (80 vs. 53%), had a slightly better response to levodopa (100 vs. 90%), and an absence of cognitive and neuropsychiatric symptoms, but a similar mean age at onset (52.6 years [*SD* 9.6], range: 39–64 years vs. 51.4 years [*SD* 12.1], range: 29–86 years).

DISCUSSION

This is one of the largest national multi-center studies to investigate the frequency of variants of the three major genes unequivocally linked to AD PD—*LRRK2*, *SNCA*, and *VPS35*—and their associated phenotypes in a large cohort of >1,800 French and North African index PD cases. We report an overall mutation frequency of 8.9% across both populations, the *LRRK2* G2019S mutation being the most frequently identified variant (7.5%), particularly in familial rather than isolated cases. However, the frequency of mutations differed considerably between populations. We confirm here that the *LRRK2* Gly2019Ser mutation is the principal genetic cause of PD in our cases of North-African ancestry, reaching an overall frequency of 45% (100/221) and 62% (34/55) in familial cases. By contrast, this mutation was present at a much lower rate of 2.4% (36/1,530) in our native French PD cases. Our findings are consistent with those of previous multi-center studies (16). *SNCA* duplications were the second most common type of mutations, identified in 14 index cases (0.78%). *SNCA* duplication carriers were mostly of European ancestry, particularly French (93%), tended to be predominantly females, probably due to random or recruitment bias, and had a higher frequency of a family history of PD. We also identified three unrelated PD patients carrying the *SNCA* Ala53Thr mutation. Although generally rare, this mutation appears to be particularly common in the Italian and Greek populations, due to a founder effect (17, 18). Only four individuals without Greek or Italian ancestry have been reported to carry this mutation, in haplotypes different from those reported in Greek and Italian families (19–22). Additional haplotype analysis would determine the ancestral origin of our three French mutation carriers. Other rare known mutations were also identified in our study: *VPS35* Asp620Asn and *LRRK2* Arg1441His in three and two AD PD families, respectively. At least 25 *LRRK2* Arg1441His carriers, including those described here, have been reported to date [(3); www.mdsgene.org]. Most were Caucasian, and all but one case reported a family history of the disease. This pathogenic variant was not found in more than 6,000 healthy controls tested (23) and is very rare in the Genome Aggregation Database (GnomAD) (1/31,298 alleles); it is therefore very likely to be pathogenic. Consistent with this conclusion, the Arg1441His variant affects the same amino-acid residue as two other recurrent PD-causing mutations

TABLE 3 | Summary of the clinical data for patients carrying *SNCA*, *VPS35,* and *LRRK2* Arg1441His mutations identified in this study.

	***SNCA* rearrangements**		***SNCA* missense mutations**		***VPS35***	***LRRK2***
	Triplications[a]	**Duplications[a]**	**Ala53Thr**	**Gly51Asp[b]**	**Asp620Asn[c]**	**Arg1441His[d]**
No. of carriers (index cases)	2 (1)	21 (14)	3 (3)	3 (1)	8 (3)	5 (2)
No. of index cases with family history of PD	1/1	11/14 (78.6%)	1/3 (33.3%)	1/1	3/3 (100%)	2/2 (100%)
Sex (M:F)	2:0	7:14	1:2	1:2	5:3	2:3
Mean age at onset (*SD*) [range], years	42 (8.5) [36–48]	45.3 (6.3) [36–56]	34.7 (7.6) [26–40]	42.0 (15.7) [31–60]	57.1 (10.7) [38–71]	52.6 (9.6) [39–64]
Mean age at examination (*SD*) [range], years	50 (11.3) [42–58]	52.2 (6.0) [43–64]	42.7 (17.5) [28–62]	45.7 (18.7) [32–67]	65.0 (10.1) [52–80]	57.9 (10.0) [42–66]
Mean disease duration (*SD*) [range], year	8 (2.8) [6–10]	6.3 (3.9) [1–16]	8.2 (12.0) [0.5–22]	3.7 (3.1) [1–7]	7.9 (5.4) [1–17]	5.2 (5.1) [2–14]
Signs at onset						
Akinesia	1/2 (50%)	14/18 (77.8%)	3/3 (100%)	2/2 (100%)	7/7 (100%)	4/5 (80%)
Tremor	2/2 (100%)	6/18 (33.3%)	1/3 (33.3%)	0/2 (0%)	3/8 (37.5%)	1/4 (25%)
Micrographia	0/2 (0%)	8/18 (44.4%)	1/2 (50%)	1/2 (50%)	3/6 (50%)	2/5 (40%)
Dystonia	0/2 (0%)	0/17 (0%)	0/3 (0%)	0/2 (0%)	0/8 (0%)	1/4 (25%)
Clinical signs at examination						
Tremor	2/2 (100%)	11/19 (57.9%)	2/3 (66.7%)	1/3 (33.3%)	6/8 (75%)	4/4 (100%)
Bradykinesia	2/2 (100%)	19/19 (100%)	3/3 (100%)	3/3 (100%)	8/8 (100%)	5/5 (100%)
Rigidity	2/2 (100%)	19/19 (100%)	3/3 (100%)	3/3(100%)	8/8 (100%)	5/5 (100%)
Asymmetry	1/2 (50%)	16/17 (94.1%)	3/3 (100%)	3/3 (100%)	8/8 (100%)	5/5 (100%)
Apraxia	NA	0/16 (0%)	0/3 (0%)	0/2 (0%)	0/8 (0%)	0/5 (0%)
Dysarthria	NA	3/15 (20%)	1/3 (33.3%)	0/2 (0%)	0/8 (0%)	0/3 (0%)
Mean (or value) UPDRS III OFF (/108) (*SD*) [range], year	51 (7.1) [47, 56]	40.7 (23.7) [5–86]	47.7 (20.4) [30–70]	13	25 (9.9) [18–32]	23.8 (23.3) [2–56]
Mean (or value) UPDRS III ON (/108) (*SD*) [range], year	44.5 (2.1) [43–46]	17.7 (13.2) [4–48]	34 (1.4) [33–35]	7	21.9 (8.1) [8–33]	10.5 (9.5) [0–19]
Mean (or value) Hoehn and Yahr ON (/5) (*SD*) [range], year	NA	1.9 (0.64) [1–3]	3	1.5	2.3 (0.5) [2–3]	1.3 (0.3) [1–1.5]
Treatment and its complications						
Levodopa responsiveness[#]	0/2 (0%)	15/15 (100%)	2/3 (66.7%)	Mild to moderate	5/5 (100%)	5/5 (100%)
Dyskinesias	NA	9/15 (60%)	2/3 (66.7%)	2/3 (66.7%)	2/8 (25%)	1/4 (25%)
Motor fluctuations	NA	8/15 (53.3%)	2/3 (66.7%)	2/3 (66.7%)	3/8 (37.5%)	3/4 (75%)
Dystonia	NA	3/15 (20%)	0/3 (0%)	1/3 (33.3%)	0/8 (0%)	2/4 (50%)
Non-motor signs						
Cognitive impairment (MMSE<24/30)	1/2 (50%)	3/16 (18.8%)	0/3 (0%)	0/2 (0%)	0/8 (0%)	0/5 (0%)
Dysautonomia*	2/2 (100%)	6/16 (37.5%)	1/3 (33.3%)	0/2 (0%)	2/8 (25%)	2/5 (40%)
Depression/neuropsychiatric disorders	NA	4/16 (25%)	1/3 (33.3%)	2/3 (66.7%)	0/8 (0%)	0/5 (0%)

Patients previously reported by.
[a] *Ibanez et al. (13) and Books et al. (15).*
[b] *Lesage et al. (14).*
[c] *Lesage et al. (10).*
[d] *Lesage et al. (9).*
[#] *Levodopa responsiveness was defined as a >30% improvement in subjective perceived motor symptoms.*
**Dysautonomia included at least one of the following three signs: orthostatic hypotension, erectile dysfunction, and/or urinary problems.*
AD, autosomal dominant; MMSE, Mini Mental State Examination; NA, not available; PD, Parkinson's disease; UPDRS III, the motor subsection of the Unified Parkinson's Disease Rating Scale.

(Arg1441Cys and Arg1441Gly). Finally, previous haplotype analyses did not support the hypothesis of a common founder for the Arg1441His variant, instead suggesting that there might be a mutational hotspot. Following initial reports of the existence of several *VPS35* variants (5, 6), pathogenicity has been confirmed only for the Asp620Asn variant. Consistent with our findings,

TABLE 4 | Comparison of demographic and clinical characteristics of patients with Parkinson's disease by *LRRK2* mutation status (*LRRK2* carriers vs. patients without mutations in known Parkinson's disease-associated genes).

	***LRRK2* G2019S+ and patients without mutations; unadjusted comparisons**			***LRRK2* G2019S + and patients without mutations; adjusted comparisons**		
	***LRRK2*+ *n* = 135 (8%)**	**Genetically undefined PD *n* = 1,552 (92%)**	***p*-value**	**Coefficient or OR (CIs) (reference: non-mutation carriers) ∫**	***p*-value**	***p*-value adjusted¥**
Demographic characteristics						
Sex (% male)	70/135 (51.9%)	949/1,552 (61.1%)	0.04*			
Age at onset (*SD*), years	51.6 (12.8)	47.3 (12.8)	<0.001*			
Age at examination (*SD*), years	60.6 (13.3)	55.7 (13.4)	<0.001*			
Disease duration (*SD*), years	9.0 (8.0)	8.4 (7.0)	0.32			
Ancestry			<0.001*			
European	43/135 (31.9%)	1,390/1,543 (90.0%)				
North-African	92/135 (68.1%)	110/1,543 (7.1%)				
Other/Mixed origins	0/135 (0.00%)	43/1,543 (2.8%)				
Family history of PD			0.02*			
AD PD	61/135 (45.2%)	536/1,552 (34.5%)				
Isolated cases	74/135 (54.8%)	1,016/1,552 (65.5%)				
Clinical characteristics						
Levodopa responsiveness#	69/77 (89.6%)	788/958 (82.3%)	0.12	1.76 [0.81;3.80]	0.13	0.44
Symptoms at onset						
Dystonia	8/101 (7.9%)	125/1,306 (9.6%)	0.72	0.88 [0.41;1.87]	0.74	0.78
Akinesia	57/108 (52.8%)	805/1,332 (60.4%)	0.13	0.75 [0.50;1.11]	0.15	0.44
Tremor	72/107 (67.3%)	809/1,350 (59.9%)	0.15	1.32 [0.87;2.01]	0.19	0.44
Micrographia	22/102 (21.6%)	454/1,314 (34.6%)	0.009*	0.50 [0.31;0.81]	0.003*	0.06
Symptoms at examination						
Bradykinesia	109/111 (98.2%)	1,369/1,411 (97.0%)	0.77	1.73 [0.41;7.26]	0.42	0.63
Rigidity	106/111 (95.5%)	1,333/1,406 (94.8%)	1.00	1.12 [0.44;2.85]	0.80	0.80
Tremor	89/111 (80.2%)	1,040/1,397 (74.4%)	0.21	1.32 [0.81;2.15]	0.25	0.50
Asymmetry	105/108 (97.2%)	1,313/1,366 (96.1%)	0.79	1.64 [0.50;5.40]	0.38	0.62
Motor features						
UPDRS III ON (/108) (*SD*)	19.5 (13.4)	18.7 (13.2)	0.57	−0.73 [−3.62;2.16]	0.62	0.76
UPDRS III OFF (/108) (*SD*)	38.9 (18.2)	32.5 (17.6)	0.02*	4.91 [−0.33;10.14]	0.07	0.44
Hoehn and Yahr ON (/5) (*SD*)	2.2 (1.0)	2.0 (0.9)	0.03*	0.13 [−0.06;0.31]	0.17	0.44
Hoehn and Yahr OFF (/5) (*SD*)	2.7 (1.1)	2.4 (1.0)	0.21	0.18 [−0.18;0.54]	0.33	0.59
Motor complications						
Dyskinesias	52/98 (53.1%)	537/1,164 (46.1%)	0.21	1.42 [0.90;2.25]	0.13	0.44
Motor fluctuations	58/98 (59.18%)	640/1,164 (55.0%)	0.46	1.18 [0.75;1.86]	0.48	0.66
Dystonia	27/98 (27.6%)	303/1,164 (26.0%)	0.72	1.11 [0.68;1.80]	0.68	0.77
Non-motor features						
Dysautonomia*	9/94 (9.6%)	111/1,214 (9.1%)	0.85	0.84 [0.40;1.75]	0.64	0.76
MMSE score (/30) (*SD*)	27.4 (3.7)	28.2 (3.2)	0.07	−0.56 [−1.41;0.28]	0.19	0.44

Data are given as the mean ± standard deviation for continuous variables and as counts (percentages) for qualitative variables.

Welch's t-test was used to compare the groups for continuous variables and Fisher's exact test was used for binary variables. Coefficients for continuous clinical features and odds ratios (ORs) for binary clinical features, confidence intervals (CIs) and P-values were calculated from GLMs with mutation status, sex, age, and disease duration, for all variables except for onset variables, for which only mutation status, sex, and age at onset were added. Linear models were used for continuous variables; GLMs with logit link and Bernoulli distributions were used for binary variables.

#Levodopa responsiveness was defined as a >30% improvement in subjective perceived motor symptoms.

**Dysautonomia included at least one of the following three signs: orthostatic hypotension, erectile dysfunction, and/or urinary problems.*

AD, autosomal dominant; MMSE, CI, Confidence Intervals; Mini Mental State Examination; OR, Odds Ratio; PD, Parkinson's disease; UPDRS III, the motor subsection of the Unified Parkinson's Disease Rating Scale.

¥ P corrected for multiple testing by the Benjamini-Hochberg procedure.

**p < 0.05.*

this recurrent mutation has been identified predominantly in families of Caucasian descent affected by AD PD. A meta-analysis of 21,824 PD patients from 15 case-control studies performed worldwide from 2011 to 2016 identified an overall mutation frequency of 0.12% (0.29% in familial cases and 0.023% in isolated cases) [reviewed in (24)], and an absence of this mutation from healthy controls and the GnomAD public database.

The clinical features of our *LRRK2* mutation carriers, whether heterozygous or homozygous, were indistinguishable from those of patients with no mutations in known PD-associated genes. These features overlapped those of typical, idiopathic PD. In our study, patients with the G2019S mutation had a mean AAO of ∼52 years, a high proportion of patients with late AAO (>50 years), a good response to levodopa, a predominance of tremor as a first symptom of PD, about a quarter had cognitive impairment, about 10% had dysautonomia, but no other atypical signs after a mean disease duration of ∼10 years. Although the clinical features of the *LRRK2* Gly2019Ser carriers compared with patients with idiopathic PD in literature are conflicting [meta-analysis in (25)], even for the same ethnic PD population [i.e., of North-African origin; (26–31)], our data are consistent with those of 724 *LRRK2* mutation carriers listed in the MDSGene database. Like *LRRK2* mutation carriers, *VPS35* Asp620Asn carriers had a phenotype very similar overall to that of idiopathic PD: absence of atypical signs, excellent levodopa response, normal cognition, and absence of neuropsychiatric features. However, the mean AAO of our patients appeared to be later (57 years) than that reported in a recent meta-analysis (51 years) (32), due to the presence of multiple affected relatives with a late onset of disease within the same family (see **Supplementary Table 4**). Lastly, *SNCA* mutation carriers had motor features similar to those of idiopathic PD, but an overall earlier AAO, a shorter disease course, a higher frequency of motor complications, a higher frequency of non-motor signs and symptoms (cognitive decline in 17%, autonomic dysfunction in 39%, and psychotic symptoms, and depression in 32%). Atypical signs have also been observed in rare PD patients carrying the *SNCA* Gly51Asp mutation (14). In this study, we also identified a rare known variant of *SNCA*, His50Gln, in the homozygous state. This variant has been described as a causal variant associated with late-onset PD, dementia, and dystonia (33, 34), but a revaluation in larger datasets of PD patients and controls, including the GnomAD database (23/282,808 alleles), provided no evidence of pathogenicity for this variant (35). However, interestingly, the 42 year-old female patient carrying the His50Gln variant in our cohort had clinical features similar to those observed in carriers of other types of *SNCA* mutation carriers. She presented an early AAO (32 years), an excellent response to levodopa, motor complications, akinetic-rigid parkinsonism, and dystonia, an absence of cognitive decline and neuropsychiatric symptoms, but the presence of autonomic dysfunction and atypical neurological signs, such as postural instability, REM sleep behavior disorder (RBD) and impulse control disorders (see **Supplementary Table 2**). However, in this study, the *SNCA* His50Gln was found using our customized gene panel and in absence of whole exome/genome sequencing to detect other possible pathogenic mutations, its pathogenicity remains inconclusive.

The principal strength of this national multi-center study is the large group of well-phenotyped and genotyped patients and family members recruited at the 16 different PDG centers, and the use of a standardized protocol, ensuring comparable, and consistent clinical data reporting and diagnoses at each center. This enabled us to refine the estimated prevalence of mutations in genes causing AD PD in France. We show that our population, although mixed, has a relatively high frequency of *SNCA*, *LRRK2,* and *VPS35* mutations. However, the clinical data were cross-sectional, most patients were European or North African, and our populations were biased toward EO cases.

In most instances, the phenotypes of cases due to AD PD mutations are indistinguishable from those of cases without mutations, demonstrating the need for genetic analysis for their identification. Gene-specific disease-modifying therapies are currently being developed and tested. More generalized genetic testing is therefore required in PD patients, to identify those most likely to benefit from personalized care (36).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by INSERM, CCPPRB (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) du Groupe Hospitalier Pitié-Salpêtrière, Paris, France. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SL conceived, designed and organized the study, wrote the first draft, reviewed, and critically revised the manuscript. J-CC and AB conceived the project, reviewed, and critically revised the manuscript. MH contributed to the statistical analysis and critically revised the manuscript. GM, CT, HB, SF, MA, CB-C, EB, ST, PD, FD, ER, FT, DG, FO-M, BD, FV, FC-D, A-MO-H, MV, EL, and AS contributed to the execution of the research project and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Bidirectional Relation Between Parkinson's Disease and Glioblastoma Multiforme

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Cancer and Parkinson's disease (PD) define two disease entities that include opposite concepts. Indeed, the involved mechanisms are at different ends of a spectrum related to cell survival - one due to enhanced cellular proliferation and the other due to premature cell death. There is increasing evidence indicating that patients with neurodegenerative diseases like PD have a reduced incidence for most cancers. In support, epidemiological studies demonstrate an inverse association between PD and cancer. Both conditions apparently can involve the same set of genes, however, in affected tissues the expression was inversely regulated: genes that are down-regulated in PD were found to be up-regulated in cancer and vice versa, for example p53 or *PARK7*. When comparing glioblastoma multiforme (GBM), a malignant brain tumor with poor overall survival, with PD, astrocytes are dysregulated in both diseases in opposite ways. In addition, common genes, that are involved in both diseases and share common key pathways of cell proliferation and metabolism, were shown to be oppositely deregulated in PD and GBM. Here, we provide an overview of the involvement of PD- and GBM-associated genes in common pathways that are dysregulated in both conditions. Moreover, we illustrate why the simultaneous study of PD and GBM regarding the role of common pathways may lead to a deeper understanding of these still incurable conditions. Eventually, considering the inverse regulation of certain genes in PD and GBM will help to understand their mechanistic basis, and thus to define novel target-based strategies for causative treatments.

Keywords: Parkinson's disease, glioblastoma multiforme, pleiotropy, cancer, neurodegeneration

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CANCER AND NEURODEGENERATION

The Inverse Association of Parkinson's Disease and Cancer

There is now accumulating evidence for an inverse association between Parkinson's Disease (PD) and cancer (1–3). Studies suggest that people affected by a neurodegenerative disorder have a reduced incidence for most cancers (4, 5). Molecular studies showed that there is an inverse correlation of the expression of shared genes in PD and cancer: genes down-regulated in PD can be up-regulated in cancer and vice versa (6, 7). These inversely correlated gene expression may affect the same pathways in opposite ways, either involving genetic or environmental factors

(5, 8, 9). Shared genetic pathways deregulated in opposite ways are a major focus, particularly those favoring apoptosis and cell proliferation, influencing cell cycle control, DNA repair, and kinase signaling (4). Common mechanisms such as chronic inflammation (10) and immunosenescence, and common risk factors like diabetes and obesity, have been implicated in both conditions (11, 12).

Parkinson's Disease

PD is a neurodegenerative disease characterized by three cardinal motor symptoms: tremor, rigidity and bradykinesia resulting from loss of dopaminergic neurons in the *substantia nigra pars compacta* (13). PD affects 1–2% of the population over 60 years (14). Age of onset before the age of 40 is seen in <5% of the cases in population-based cohorts, which is typical of familial cases of PD with underlying genetic cause like mutations in *SNCA*, *Parkin*, *PINK1, DJ-1*, *LRRK2*, *ATP13A* (**Table 1**). Monogenic forms of PD are rare. In general, genetic factors are claimed to be involved in 5–10% of the cases (14). Histopathological hallmarks of PD are proteolytic inclusions called Lewy bodies (LB) and Lewy neurites containing α-synuclein (47). Cellular hallmarks of PD are an impairment of proper functioning of molecular and organelle degradation pathways like the ubiquitin–proteasome system and autophagy (48). In particular, the process of removing defective mitochondria from the cells is known to be impaired in PD (49). This process is a special form of autophagy, called mitophagy (50), and is regulated by the PD-linked proteins PINK1 and Parkin (51). The impairment of autophagy, lysosomal and mitochondrial function in PD can lead to the accumulation of α-synuclein and defective mitochondria (52) and, ultimately, to neurodegeneration. The diagnostic of PD is mostly a clinical diagnosis as it is based on neurological tests when the PD patients already show motor symptoms. Due to the complexity and heterogeneity of PD, the etiology is not yet fully understood. Therefore, there is no cure for PD and no treatment that will stop the progress of the disease and treatment is only symptomatic, e.g., levodopa therapy. This is why it is important to investigate underlying mechanisms of PD to stratify causative treatments.

Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most malignant tumor of the central nervous system. GBM tumors are most likely developing from astrocytes (53). Based on their histological and clinical features, astrocytomas are classified into four different subtypes according to the WHO classification: Pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, and GBM. Pilocytic and diffuse astrocytoma are characterized by a rather low growth rate, while anaplastic astrocytoma and GBM show common uncontrolled proliferation and diffuse tissue penetration (54). GBM is characterized by poor prognosis, low survival rates, and extremely limited opportunities for therapy. Symptoms of GBM are rather unspecific like increased intracranial pressure, including headache and focal or progressive neurologic deficits. Seizures are the presenting symptom in 25% of patients and can occur at a later stage of the disease in 50% of patients (55). Malignant gliomas are the third leading cause of cancer death for people aged between 15 and 34, accounting for 2.5% of the global cancer death toll. GBM has a maximum incidence in patients aged more than 65 years, and is mainly affecting the cerebral hemispheres (54). A cellular hallmark of GBM and all cancers is the so-called Warburg effect which describes the phenomenon that cancer cells use aerobic glycolysis to produce ATP (56). GBM cells are characterized by increased glucose uptake and lactate production (57). GBM cells also use oxidative phosphorylation (OXPHOS) (57). The hypoxic GBM tumor environment allows the constant expression of hypoxia inducible factors 1 alpha and 2 alpha (HIF-1α, HIF-2α). Hypoxia and hypoxia-stabilized HIFs regulate GBM metabolism by stabilizing genes involved in metabolism like the glucose transporters GLUT1 and GLUT3, thereby sustaining an increased glucose uptake of the GBM cells (57). Also, the enzyme catalyzing the first step in glycolysis, hexokinase, is hypoxia/HIF regulated (57). As for PD, the diagnosis of GBM is typically made when first symptoms occur and rely on clinical examination and neuroimaging methods. However, mostly both diseases are diagnosed at an advanced stage of tumor growth or neurodegeneration, respectively. Treatment strategies of GBM are based on a multidisciplinary approach. Current standard therapy is a combination of maximal safe surgical resection of the tumor and subsequent radiation and chemotherapy with temozolomide (Temodar®), an oral alkylating agent. However, even with advances in surgical resection, the prognosis for GBM patients remains poor, with a median survival of 15 months (55).

COMMON GENES IN PD AND GBM

A common set of genes like the tumor suppressor p53, epidermal growth factor and its receptor EGF(R), the glyoxalase and deglycase DJ-1 and biological processes are deregulated in opposite directions in PD and GBM (6). Particularly, there is evidence that PD-associated genes are involved in GBM pathogenesis (**Table 1**). A summary of publications examining and exhibiting the involvement of PD-associated genes in GBM is shown in **Table 1**. Consistent with PD-associated genes being involved in GBM, it is important to note that mutations in the same gene can behave differently if they are germline or somatic mutations. For example, mutations in *PARK2* affecting the Parkin protein can cause neuronal cell death in PD if they are present in the germline, or increased cell survival in GBM if they are present in somatic cells like astrocytes (**Figure 1**). (25). Pathways that are affected in PD and GBM are overlapping but are regulated inversely by alternatively regulated genes. These pathways are regulating cell proliferation and cell metabolism as well as mitochondrial clearance (1). In the following, examples for inversely regulated pathways in PD and GBM are illustrated and the role of commonly involved genes in both diseases in the regulation of these pathways will be outlined.

Pro-Survival Signaling

Pro-survival signaling is one of the most important pathways regulating and sustaining cell proliferation. Once dysregulated, uncontrolled cell proliferation can lead to tumorigenesis. This is why cell proliferation and apoptosis need to be in a tight equilibrium, which is well controlled by many mediators.

TABLE 1 | Overview PD-genes in GBM.

PD-associated gene	GBM	Function	Involvement in disease
PARK1 (SNCA)	(15–23)	Important role in maintaining an adequate supply of synaptic vesicles in presynaptic terminals	Meningioma: (24) PARK1 was shown to contribute to malignant progression of tumors
PARK2 (Parkin)	(25–33)	Regulation of autophagy, important for mitochondrial maintenance	Autophagy pathway
PARK5 (UCHL1)	(21, 34)	Hydrolase activity, removes and recycles ubiquitin molecules from degraded proteins Ligase activity, links together ubiquitin molecules for use in tagging proteins for disposal	Degrades not needed proteins UCHL1 acts as a colorectal cancer oncogene via activation of the β-catenin/TCF pathway through its deubiquitinating activity (35)
PARK6 (PINK1)	(23, 36, 37)	Regulation of autophagy, important for mitochondrial maintenance	PINK1 is a Negative Regulator of Growth and the Warburg Effect in Glioblastoma
PARK7 (DJ-1)	(38–41)	ROS scavenger, antioxidative role, cyto-protective	Pro-tumor survival, mitochondrial dysfunction
PARK8 (LRRK2)	Somatic mutations [The Cancer Genome Atlas (TCGA)] (42)	GTPase and kinase function LRRK2 has been associated with a diverse set of cellular functions and signaling pathways including mitochondrial function, vesicle trafficking together with endocytosis, retromer complex modulation and autophagy	LRRK2 mutation carriers have a pos. correlation with cancer incidence (43)
PARK9 (ATP13A2)	Somatic mutations [The Cancer Genome Atlas (TCGA)]	P5 subfamily of ATPases which transports inorganic cations as well as other substrates	ATPase that plays a role in intracellular cation homeostasis and the maintenance of neuronal integrity
PARK15 (FBXO7)	(44)	F-box protein Phosphorylation-dependent ubiquitination	Oncogenic properties of FBXL10, but also tumor suppression by FBXL10 has been reported (45, 46)

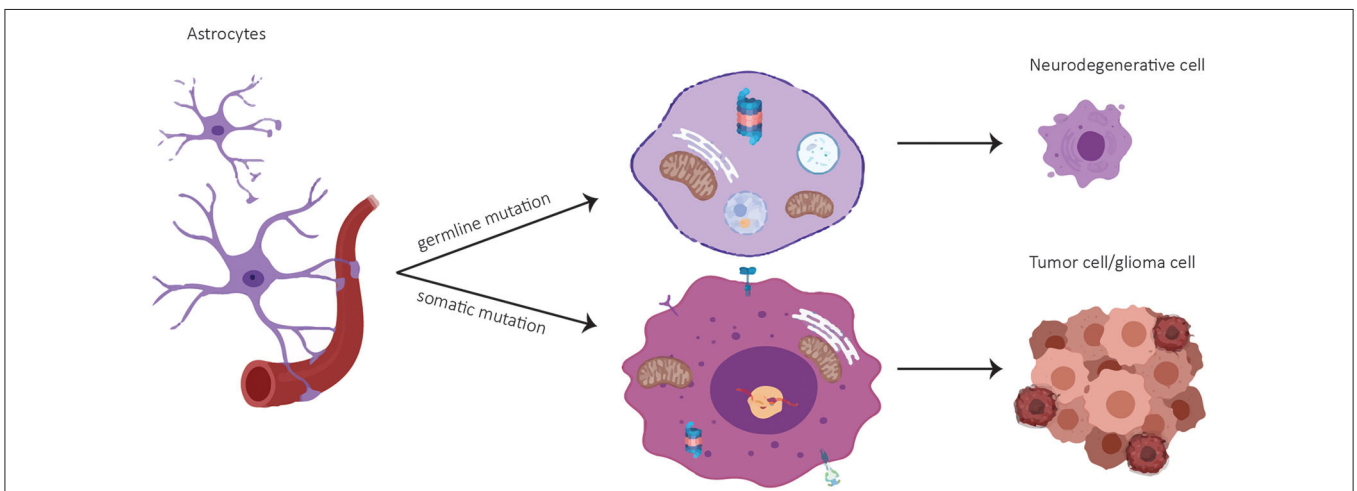

FIGURE 1 | Cell fate of astrocytes depending on mutational status. A germline mutation in a PD-associated gene might result in a neurodegenerative cell whereas a somatic mutation can lead to a tumor cell.

P53—The Master Controller of Cell Proliferation and Its Regulation in PD and GBM

One key player in the regulation of cell proliferation is the tumor suppressor p53. p53 is upregulated in PD, but downregulated in GBM (**Figure 2A**) (58–60).

p53 inhibits cell proliferation by both blocking cell cycle progression and promoting apoptotic cell death (**Figure 2A**). This way, p53 provides a clear prevention from stem cell tumor growth and thereby GBM development. p53 itself is also regulated via several stress signals occurring during malignant progression like genotoxic damage, oncogene activation, loss of normal cell contacts, and hypoxia (**Figure 2A**). This leads to a model where growth inhibitory functions of p53 are normally held dormant, to be unleashed only in nascent cancer cells (61). In PD, the level of p53 and its activity in neurons can increase not only as a result of oxidative stress and DNA damage, but

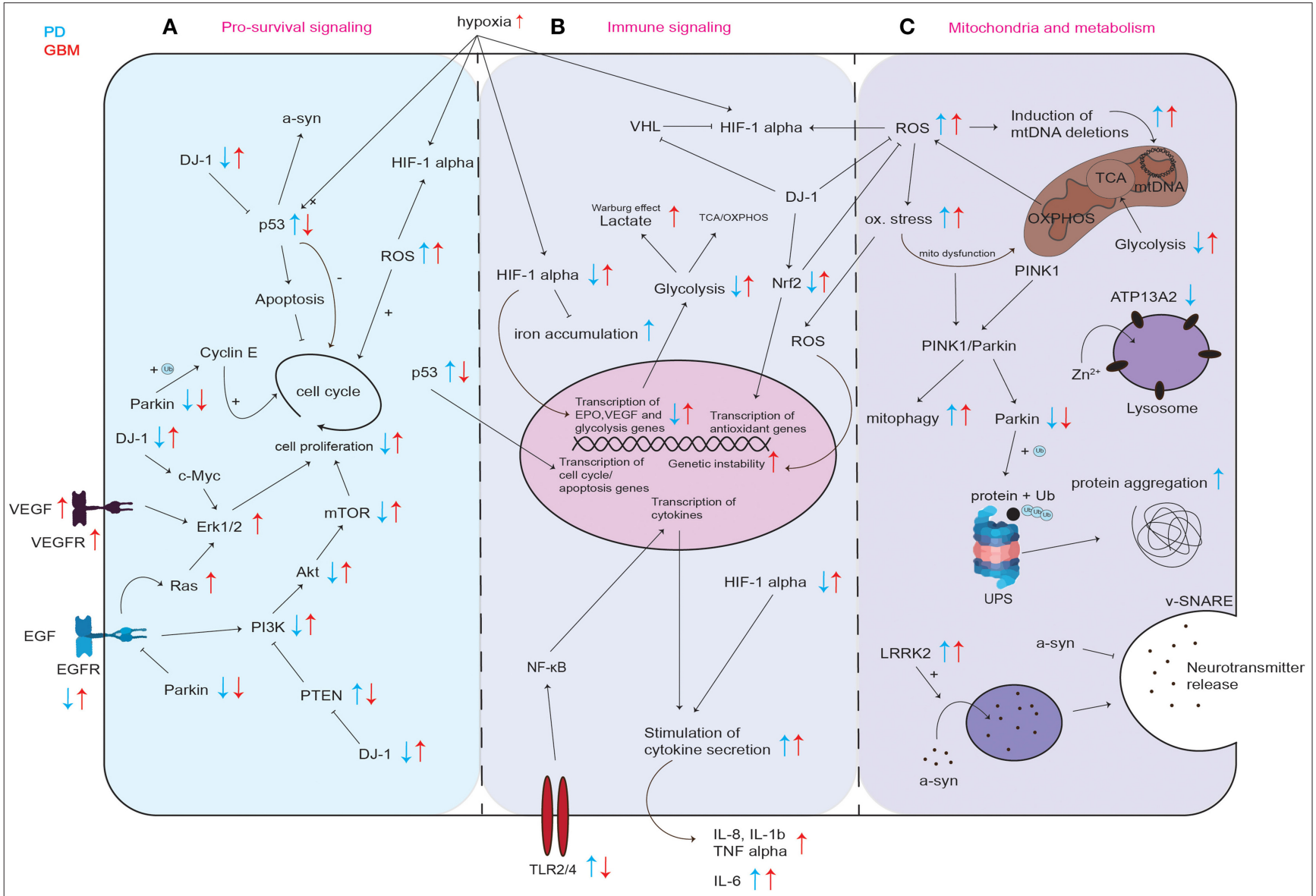

FIGURE 2 | Graphical representation of common cellular pathways described in literature to be dysregulated in PD and GBM. Dysregulation (up- or downregulation) of mediators and proteins of commonly involved mediators and proteins in PD and GBM is illustrated with blue and red arrows, while blue arrows correspond to the situation in PD, red arrows indicate the regulation in GBM. Differential regulation of discussed mediators regarding pro-survival signaling **(A)** immune signaling **(B)** and their involvement in mitochondria and metabolism **(C)**. UPS, ubiquitin proteasome system; ox. stress, oxidative stress; mito dysfunction, mitochondrial dysfunction.

also due to aberrant regulation of its expression for example by mutated or incorrectly cleaved proteins involved in the process of neurodegeneration (58). An increase in p53 expression and its activation results in enhanced expression of genes that are responsible for apoptosis and/or cell cycle arrest and may trigger neuronal cell death (58). In line, Mogi et al. found increased levels of p53 protein in the nigrostriatal dopaminergic region in PD patients compared to controls (62). It was shown that p53 regulates α-synuclein expression since the α-synuclein promoter harbors a p53 responsive element (63). Therefore, an increase in p53 in PD could not only lead to increased apoptosis induction but also to an increase in expression of potentially dysfunctional α-synuclein and to its subsequent aggregation (63). Kato et al. found that DJ-1 inhibits the transcriptional activity of p53 (**Figure 2A**) (64). Loss of DJ-1 protein in PD could thereby lead to increased expression of p53 target genes leading to cell death. In GBM, p53 is frequently downregulated or inactivated by mutations leading to a reduction in apoptosis induction (**Figure 2A**) (65) and p53 inactivation positively correlates with GBM tumor invasiveness (66). Zheng et al. showed that central nervous system (CNS)-specific deletion of p53 and Phosphatase And Tensin Homolog (PTEN) in the CNS of mice leads to a high-grade malignant glioma phenotype resembling human GBM (67). These results are in line with the data found at The Cancer Genome Atlas in the exploration mode when looking at the TCGA-GBM data set, which reports PTEN, p53 and EGFR as the most frequently mutated tumor suppressor genes in GBM (https://portal.gdc.cancer.gov).

EGFR Signaling in PD and GBM

EGFR is downregulated in PD and upregulated in GBM (**Figure 2A**). EGFR activates the phosphoinositide 3-kinase (PI3K)-Akt pathway (**Figure 2A**). The PI3K/Akt signaling pathway is known as one of the most important kinase cascades that mediates crucial cellular functions such as survival, proliferation, migration, and differentiation (68). Activated receptor tyrosine kinases (RTKs) like EGFR activate PI3K through direct binding or through tyrosine phosphorylation of scaffolding adaptors, which can then bind and thereby activate PI3K (**Figure 2A**). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), in a reaction that can be reversed by the PIP3 phosphatase PTEN. AKT can then activate its downstream targets like mTOR, eventually leading to cell proliferation (**Figure 2A**). It was shown that EGFR endocytosis and degradation are accelerated in Parkin-knockout cells from mouse brain, and EGFR signaling via the PI3K/Akt pathway is reduced (69). Fallon et al. propose that Parkin delays EGFR internalization and degradation, thereby promoting PI3K/Akt signaling (69). Therefore, by decreasing the efficiency of EGFR-mediated Akt signaling in neurons, the loss of Parkin leads to neuronal degeneration (69). In post-mortem brains of idiopathic PD patients, protein levels of EGF and EGFR were shown to be decreased in the prefrontal cortex and the striatum (70). Mutations in *EGFR* are commonly occurring in GBM (71). These mutations result in EGFR gene amplification and intrinsic alterations of the EGFR structure (71). Brennan et al. showed that gene amplification and mutation of EGFR results in enhanced EGFR activation and is found in about 60% of GBM (72). The most common EGFR mutation in GBM is EGFRvIII, which is caused by the deletion of exon 2–7 leading to constitutively activated EGFR (71, 73, 74). It was shown that EGFR is overexpressed in most of primary GBM and some of the secondary GBM and that EGFR overexpression is associated with more aggressive GBM (75).

PTEN/PI3K/Akt Signaling in PD and GBM

In PD, PTEN/PI3K/Akt signaling is down-regulated and therefore causes decreased pro-survival signaling (76). In GBM, PTEN/PI3K/Akt signaling is upregulated (77–79). PTEN negatively regulates PI3K (**Figure 2A**), thereby inhibiting PI3K/Akt mediated proliferation and cell survival. In PD patient-derived post mortem brains, Sekar et al. found an increase in PTEN levels (80). Absence of PTEN protected dopaminergic neurons in PTEN knockout mice from neuronal death after neurotoxin treatment (81). In another mouse model, depletion of PTEN attenuated the loss of tyrosine hydroxylase-positive (dopaminergic) cells after neurotoxin treatment (82). An increase in PTEN in PD results in decreased pro-survival signaling leading to increased neuronal cell death. In line, it was shown that the ratio of phospho-Akt/total-Akt decreases in dopaminergic neurons indicating a decrease in activation of the pro-survival signaling mediated by Akt upon phosphorylation (83). Overall, an impaired PTEN/PI3K/Akt signaling in PD leading to neuronal cell death can be due to mutations in PD-associated genes regulating Akt signaling [e.g., DJ-1 (84), (**Figure 2A**)], excessive Akt dephosphorylation, inhibition of Akt activation or oxidative stress (85). In GBM, PTEN/PI3K/Akt signaling is upregulated due to EGFR overexpression or loss of PTEN (78). Mutations or homozygous deletions of PTEN were shown in 36% of the GBM cases that were studied by McLendon et al. and 86% of the GBM harbored at least one genetic event in the receptor tyrosine kinase PI3K pathway (86). High level of phosphorylated Akt was shown to correlate with a poor prognosis for patients with GBM (87). Mutations in the phosphatidylinositol-4,5-bisphosphcxate 3-kinase catalytic subunit alpha (PIK3CA), which is one subunit of PI3K, were shown to induce gliomagenesis (77).

The PD-Associated Oncogene DJ-1 and Regulation of Cell Proliferation in PD and GBM

The protein DJ-1 was shown to be inversely regulated in PD and GBM. (**Figure 2A**). Homozygous mutations in *PARK7* (DJ-1) resulting in loss of protein lead to PD (88). DJ-1 expression was shown to be increased in GBM (38, 89, 90). Wang et al. found that high DJ-1 and high β-catenin expression in GBM were significantly associated with high grade and poor prognosis in glioma patients, suggesting DJ-1 levels in GBM as a strong independent prognostic factor (89). DJ-1 also accelerates transformation of tumor cells by c-Myc activating the Erk pathway (91). Hinkle et al. found that GBM tumor tissue expressed DJ-1 protein at significant levels, and

typically in a cytoplasmic, non-nuclear manner. They found that immunostaining intensity of DJ-1 varied directly with strong nuclear p53 expression and inversely with EGFR amplification (38). In addition to the fact that DJ-1 negatively regulates pro-apoptotic p53 (**Figure 2A**) (92), and EGFR signaling is crucial for gliomagenesis (72), these observations suggest that DJ-1 might be involved in tumorigenesis of GBM (38). Toda et al. found that in a serial transplantation study, DJ-1 knockdown resulted in a prolonged survival of mice in secondary transplantation (39). DJ-1 is known to counteract ROS, among others via Nrf2 stabilization leading to the expression of endogenous antioxidant synthesis and ROS-eliminating enzymes like glutathione (**Figure 2A**) (93, 94). It was shown that a reduction in DJ-1 protein is associated with reduced Nrf2 transcriptional activity and that in PD patients, Nrf2 activation is associated with dysregulated downstream gene expression (93, 95). In contrast, it was found that Nrf2 overexpression accelerates proliferation and oncogenic transformation of glioma cells and that GBM patients have reduced overall survival when Nrf2 levels are upregulated (**Figure 2A**) (96).

Immune-Signaling

The innate immune system obtains various functions in health and disease. It represents the first line of defense against infection and it is involved in many different processes like tissue repair, wound healing and the clearance of apoptotic cells and cellular debris. An excessive or non-resolving activation of the innate immune system can result in systemic or local inflammatory complications and cause or contribute to the development of neurodegeneration and cancer. In the brain, the innate immune cells are represented by microglia, which regulate brain development, brain maturation, and homeostasis. An impairment of functional microglia through abnormal activation or decreased functionality can occur during aging and during neurodegeneration and the resulting inflammation was shown to be involved in neurodegenerative diseases and cancer (97).

Hypoxia and HIF-1α in PD and GBM

It is well known that hypoxia-inducible factor-1α (HIF-1α) plays an important role in gliomagenesis due to its angiogenesis-promoting effects (98). While HIF-1α is upregulated in GBM, it was shown that HIF-1α is impaired in PD (**Figure 2B**) (99, 100).

Treatment with MPTP, a prodrug to the neurotoxin MPP+, which causes Parkinsonism symptoms by destroying the dopaminergic neurons, was shown to inhibit HIF-1α accumulation in mice and in dopaminergic cell lines (99). Moreover, Milosevic et al. found that a conditional knock-down of HIF-1α in mice resulted in a 40% decrease in expression of tyrosine hydroxylase, a known marker for dopaminergic neurons, in the *substantia nigra* of mice (101). In healthy individuals, HIF-1α mediates protection of dopaminergic neurons by regulation of iron homeostasis, improved defense against oxidative stress by upregulation in response to reactive oxygen species (ROS) (**Figure 2B**) and mitochondrial dysfunction (100). PD is characterized by an accumulation of iron in dopaminergic neurons of the *substantia nigra* (102). Free cytosolic iron can lead to oxidative stress and trigger α-synuclein aggregation (102). HIF-1α influences iron homeostasis by expression of its target genes ferroportin and heme oxygenase in the *substantia nigra* which are known to be involved in the attenuation of iron accumulation (100). This way, HIF-1α can counteract iron accumulation (**Figure 2B**). However, in PD, downregulation of HIF-1α can lead to a dysregulation in iron homeostasis eventually leading to iron accumulation (**Figure 2B**). In turn, iron accumulation decreases HIF-1α activity, because iron is a necessary cofactor for prolyl hydroxylases that inactivate HIF-1α via subsequent ubiquitinylation through von Hippel-Lindau factor (VHL) (**Figure 2B**) (102, 103). HIF-1α target genes Erythropoietin (EPO) and vascular endothelial growth factor (VEGF) (**Figure 2B**) have been shown to contribute to the protection of neurons from PD pathogenesis (100). EPO was shown to be neuroprotective against dopaminergic neurotoxins (104). In rat explants of the ventral mesencephalon, VEGF treatment was shown to be mitogenic for endothelial cells, astrocytes, and could promote growth and survival of neurons and specifically dopaminergic neurons (105). There are accumulating data which suggest that the activation of HIF-1α can exert neuroprotective effects through the induction of intrinsic adaptive mechanisms in neuronal and non-neuronal cells (106). Lee et al. showed that stabilization of HIF-1α leads to the upregulation of several proteins involved in iron efflux and mitochondrial integrity and bioenergetics, cell components that are compromised in PD. This is why Lee's data emphasize the concept that the pharmacological induction of HIF-1α could have neuroprotective effects in PD cells and mice models, with a beneficial impact on dopamine synthesis, iron homeostasis, antioxidant defenses and mitochondrial dysfunction (107).

In contrast to these observations in PD, in GBM, HIF-1α levels are increased (**Figure 2B**) (108). Liu et al. found that HIF-1α expression was associated with high grade glioma and the overall survival of glioma patients, which indicates that HIF-1α could predict prognosis and provide clinical insights into the therapeutic strategy for GBM patients (109). The lack of oxygen in the GBM microenvironment results from inappropriate neovascularization, irregular blood flow, and excessive consumption of oxygen from the uncontrolled proliferating GBM cells (110). The hypoxia in the GBM tumor induces the expression of genes involved in tumor cell growth and angiogenesis like the signal transducer and activator of transcription 3 (STAT3), which triggers the synthesis of HIF-1α that subsequently induces activation of T-regulatory cells (Tregs) and the production of VEGF (111). Tregs are important modulators of the immune response, and VEGF has known immunosuppressive effects. Moreover, the hypoxic microenvironment causes the transformation of CNS macrophages into tumor-associated macrophages (TAMs), which are capable of adopting immunosuppressive and tumor-supportive phenotypes. Via the STAT3 pathway, this transformation triggers TAMs to enhance angiogenesis and tumor cell invasion (26, 112). Furthermore, HIFs are critical for the upregulation of glycolysis (**Figure 2B**) (113). Hypoxia is also a known regulator of many other innate immunological functions like cell migration, apoptosis, phagocytosis of pathogens,

antigen presentation and production of cytokines, chemokines, and angiogenic and antimicrobial factors (113). In summary, HIF is an important factor in the regulation of the tumor microenvironment due to its central role in promoting proangiogenic and invasive properties. Since HIF activation results in angiogenesis and the emerging vasculature is often abnormal, this leads to a vicious cycle that causes further hypoxia and HIF upregulation in GBM (98).

Interleukins and Immune Escape

In PD, increased cytokine levels in response to cellular stress can lead to neuronal cell death whereas in GBM, cytokines like interleukins IL-1β, IL-6, and IL-8 released by the tumor cells, inhibit the immune response and allow the tumor cells to escape the eradication by the immune system (**Figure 2B**).

IL-6 was found to be increased in the nigrostriatal region and in the cerebrospinal fluid of patients with PD (114). Further, Hofmann et al. found that patients with more severe PD had higher IL-6 levels compared to patients with a milder phenotype (114). In addition, a study from Chen et al. found that patients with PD had elevated levels of transforming growth factor-beta 1 (TGF-β1), IL-6, and IL-1β in cerebrospinal fluid compared to controls (115). In line, it is described that, in autopsy brains of PD, the number of activated microglia, which were among others TNF- α, and IL-6-positive, increased in the *substantia nigra* and putamen during the progress of PD (116). The activated microglia in PD was observed in various brain regions like the nigro-striatal region, the hippocampus and the cerebral cortex. The levels of IL-6 and TNF- α mRNAs increased in the hippocampus of PD patients (116). It is postulated that cytokines (IL-1β, TNF-α, IL-6) from activated microglia (117) in the *substantia nigra* and putamen may be initially neuroprotective, but may later turn to be neurotoxic during PD pathogenesis (116).

In contrast to PD, in GBM, the cells can profit from the cytoprotective effects of specific cytokines like IL-1β, IL-6, and IL-8 leading to increased robustness regarding cellular stress (118). As already mentioned, GBM arises from glial cells with surrounding brain parenchyma that contains CNS cells like astrocytes, neurons and microglia, as well as a distinctive extracellular matrix composition. GBM induces a tumor microenvironment characterized by immunosuppressive cytokines secreted by tumor cells, microglia and tumor macrophages. IL-6, IL-10, and TGF-β, and prostaglandin-E collectively inhibit both the innate and adaptive immune systems leading among others to the suppression of natural killer cell activity, T-cell activation and proliferation and induction of T-cell apoptosis (119). IL-1β is a known master pro-inflammatory cytokine that triggers various malignant processes driving oncogenic events such as proliferation and invasiveness (118, 120). Elevated levels of IL-1β were observed in many different GBM cell lines (121) and in human GBM tumor specimens (122). IL-6 was shown to be overexpressed in GBM clinical samples and cell lines and IL-6 gene expression seems to correlate with the aggressiveness of the tumor (123). It was shown that IL-6 is secreted by GBM cells and sustains the cell proliferation by activation of STAT3 pro-survival pathway (124). IL-6 is produced by GBM cells in response to external stimuli or intrinsic factors, for example oncogenic mutations (118). IL-1β and TNF-α induce stabilization of IL-6 mRNA and increase IL-6 biosynthesis (125). Like IL-6, IL-8 is highly expressed and secreted from GBM cell lines, tumor stem cells and human specimens (118). It was shown that the expression of the constitutively active mutant EGFRvIII is associated with significantly higher expression of IL-8 induced by nuclear factor kappa B (NF-κB) (**Figure 2B**) in human GBM specimens and GBM cell lines (126). In a similar manner as the regulation of IL-6, IL-8 expression can be enhanced by TNF-α, IL-1β or macrophage infiltration (127). Thus, elevated levels of one cytokine like TNF-α for example can lead to an increase in other cytokines. These findings of elevated cytokines and their associated roles in GBM underline the importance of specific cytokines for immune escape mechanisms and tumor proliferation and invasiveness observed in GBM pathogenesis.

Toll-Like Receptors in PD and GBM

Toll-like-receptors (TLRs) are receptors that recognize distinct molecular patterns like lipopolysaccharides, single and double stranded RNAs, hemagglutinin, viral proteins etc. (128), and allow an appropriate immune response to be initiated. The TLR family consists of 10 members (TLR1-10) in humans with different expression profiles and ligands (129). TLR2 is essential for the recognition of peptidoglycans and lipoproteins, whereas TLR4 recognizes bacterial lipopolysaccharide (LPS) (130). TLR2 and TLR4 are both the most important TLRs with regard to innate immune response as they are both implicated in the recognition of endogenous ligands involved in the inflammatory response regardless of the source of infection (131). This is why the implication of TLR2 and TLR4 in PD and GBM will be discussed in the following.

TLR2 and TLR4 are frequently upregulated in PD and downregulated in GBM allowing the tumor cells to escape clearance by the innate immune system. TLR2 and TLR4 were shown to be upregulated in many α-synuclein-overexpressing or toxin-induced animal models (132–135), and accumulating evidence from human studies further implicates these receptors in the pathogenesis of PD (136). Clinical studies revealed that TLR2 expression is increased in PD (137). It was shown that microglial TLR2 is increased in the *substantia nigra* and the hippocampus in the early stages of PD, but not during the late stages (138), while another study found that TLR2 is increased in the striatum of advanced PD patients (135).

In contrast, GBM cancer stem cells downregulate TLR4 to evade immune suppression (139). Alvarado et al. showed that in GBM, cancer stem cells have low TLR4 expression which enables cell survival by avoiding inhibitory innate immune signaling (e.g., clearance by dendritic cells, cytotoxic T cells, and natural killer cells) that aims to suppress self-renewal of the GBM stem cells (140). This is why TLR agonists that trigger antitumoral immune signaling are being discussed as therapy for GBM (141).

Mitochondria and Metabolism

Mitochondria and cellular metabolism are closely linked. Mitochondria host many enzymatic reactions of cellular metabolism like the tricarboxylic acid (TCA) cycle and oxidative

phosphorylation (OXPHOS) which generate ATP from pyruvate in the presence of oxygen (**Figure 2C**). In age-related disease, like PD and GBM, damaged mitochondria lead to impaired cellular metabolism (142).

Cellular Metabolism in PD and GBM

The human brain, even though constituting only 2% of the total body weight, uses ∼20% of the body's total oxygen consumption and 60% of our daily glucose intake (143). Furthermore, the brain needs a constant supply of glucose since it lacks fuel stores and cannot store glycogen. This is why cellular changes in glucose metabolism can have high impact on brain cell homeostasis, proliferation and viability.

It was shown that glycolysis and mitochondrial function like respiration are decreased in individuals with PD (**Figure 2C**) (144–146). In GBM, increased glycolytic activity results from certain oncogenic alterations like c-Myc amplification, PTEN deletion or mutations in p53 (**Figure 2C**) (147, 148).

While mitochondrial dysfunction in PD can cause increased generation of ROS and subsequent oxidative damage (**Figure 2C**), it can also result in failing neuronal compensation of their insufficient ATP generation (149). Activation of glycolysis in neurons leads to excessive oxidative stress and apoptosis, suggesting that neurons are predominantly restricted to OXPHOS (150). In line, Hall et al. showed that the majority of ATP used by neurons is produced by OXPHOS (151). Powers et al. found that overexpression of α-synuclein in N27 dopaminergic cells resulted in an impairment in glycolysis, a reduction in glycolytic capacity and mitochondrial respiration (152). This is why an increase in glycolysis as counteract mechanism to neuronal energy failure induced by mitochondrial dysfunction in PD eventually leads to neuronal cell death (153–155). Neurons also metabolize glucose via the pentose phosphate pathway (PPP) to maintain their antioxidant status (156). It was shown that inhibition of the PPP in neuronal cell models causes cell death (157). In rodents, PPP inhibition caused dopaminergic cell death causing motor deficits that resemble Parkinsonism (158). Using postmortem human brain tissue, Dunn et al. characterized glucose metabolism via the PPP in early sporadic PD and controls and observed a down-regulation of PPP enzymes in patients compared to controls (156). This observation suggests that the impairment of the PPP is an early event in sporadic PD (156).

In the absence of oxygen, pyruvate can be metabolized into lactate, a process known as glucose fermentation or anaerobic glycolysis. Rapidly proliferating cells, such as cancer cells, also have the ability to ferment glucose into lactate, even in the presence of abundant oxygen; this process is called aerobic glycolysis. It has been observed already decades ago, that cancer cells, even in aerobic conditions, tend to favor metabolism via glycolysis rather than OXPHOS, which is preferred by most other cells. This phenomenon is called the Warburg effect (56, 159). This is why, in contrast to PD neurons, GBM cells ferment glucose into lactate, even in the presence of abundant oxygen (**Figure 2B**). Even though ATP production is less efficient in aerobic glycolysis when compared to ATP production via complete oxidative metabolism of glucose, it is being hypothesized that GBM cells use aerobic glycolysis to generate precursors for anabolism to grow and are able to generate enough ATP to sustain their cellular function (160). By modulating glycolysis and altering mitochondrial metabolism, GBM cells generate biomass, namely nucleotides, lipids, proteins, and NADPH by using glycolytic/TCA intermediates (160). Knockdown of glycolytic genes strongly inhibits GBM growth further emphasizing that glycolytic enzymes are essential for GBM growth (148). GBM cells also generate large amounts of lactate for several pro-tumor growth functions (161). Li et al. found that EGFR activation in GBM cells promotes the translocation of phosphoglycerate kinase (PGK1) into mitochondria (162, 163). In the mitochondria, PGK1 phosphorylates and activates pyruvate dehydrogenase kinase that phosphorylates and thereby inhibits pyruvate dehydrogenase and thus mitochondrial pyruvate consumption which eventually leads to enhanced lactate production (162, 163). In addition to the aerobic glycolysis, GBM cells also utilize TCA and OXPHOS (160).

The differential expression of metabolic genes in neurons and astrocytes might explain the differences in glycolysis and OXPHOS rates. For example, neurons lack 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) since it is continuously degraded by the ubiquitin-proteasome pathway. PFKFB3 regulates the biogenesis and degradation of fructose-2,6-bisphosphate, a known glycolytic activator. In contrast, in astrocytes, PFKFB3 is activated by adenosine monophosphate-activated protein kinase (AMPK) and promotes glycolysis (149). In line, it was shown that the expression of PFKFB3 is higher in mouse astrocytes than in murine neurons due to proteasomal degradation in the neurons (164). In neurons, the activation of PFKFB3 results in enhanced glycolysis but eventually leads to cell death since neurons lose their ability to generate glutathione, an essential antioxidant involved in the management of oxidative stress. This means that unlike astrocytes, neurons use glucose to maintain their antioxidant status and not for bioenergetic purposes (164). These findings might help to explain why PD neurons fail to increase their glycolysis rates and why increased glycolysis leads to sustained cell proliferation in astrocyte-originating GBM cells.

EPIDEMIOLOGY OF PD AND CANCER

Epidemiological evidence suggests that patients with PD have a reduced incidence of primary CNS tumors (165, 166). In contrast, there are a few epidemiological studies that show a positive association of PD with benign and malignant brain tumors, but not specifically with GBM (167–169). However, the problem with these studies is that they do not distinguish between the types of brain cancer, e.g., meningioma or astrocytoma. The described increased risk of all types of brain cancers in PD might be caused by diagnostic misclassification and detection bias. Increased incidence of meningioma in PD patients for example might result from the fact that the symptoms can be wrongly diagnosed as a sign of PD, if the intracranial tumor leads for example to a compression of the basal ganglia resulting in PD

symptoms (170–173). Moreover, a positive association of brain tumors and PD can be caused by detection bias as brain tumors can be diagnosed during the clinical work-up for PD (174). Since patients diagnosed with parkinsonism are more likely to have a Magnetic Resonance Imaging at the time of diagnosis, this may explain a higher risk of detecting silent brain tumors (173, 175). The close temporal association between diagnosis of PD and the incidence of brain tumors further leads to the suggestion that brain tumors might be misdiagnosed as PD or *vice versa* (176). Specifically, for GBM, as it is lethal, it is difficult to study PD in individuals who survived GBM. This is why future studies should focus on evaluating the risk of GBM in PD patients.

Interestingly, there is an increased risk of melanoma in PD patients compared to controls (177–179). In 1985, Dr. Rampen reported a 55-year-old male with PD who developed a local recurrence of a primary melanoma and multiple primary melanomas 4 years after primary excision and 4 months after starting levodopa (180). An increased risk of malignant melanoma in PD patients has been confirmed since in many studies (8, 176, 181, 182). Several hypotheses could account for this association. Since levodopa is a metabolite in the biosynthesis of dopamine and melanin which involves the enzyme tyrosinase, and increased tyrosinase activity is found in melanoma, it was initially hypothesized that levodopa could enhance and stimulate growth on any residual melanoma tissue (183). However, recent studies have refuted a causal association for several reasons (178, 184). In particular, the observation that the risk of melanoma is increased in PD patients before diagnosis argues against an effect of levodopa. Additional explanations may be the existence of shared genetic or environmental factors, or the common embryonic origin of melanocytes and neurons from neural crest cells (178, 185). In addition, mechanistic links caused by common mutations or other alterations in a number of genes or proteins in PD and melanoma could explain the co-occurrence of PD and melanoma (184). Common mechanisms that are dysregulated in PD and melanoma are for example cellular detoxification, melanin biosynthesis or oxidative stress response (184).

Future studies should investigate underlying mechanisms of decreased risk of some cancers and increased risk of other cancers like melanoma in PD patients.

CONCLUSION

PD and GBM are two highly complex disease entities characterized by multiple cellular changes. Similar mutations within the same gene, for example Parkin (25), can have inverse effects, depending on whether they are germline or somatic mutations and depending on the type of cell in which they occur: a dividing cell in GBM or a post-mitotic neuron in PD. One could hypothesize that neurons are primarily unaffected in GBM due to their postmitotic state. On the contrary, somatic mutations causing tumorigenesis can spread through proliferative astrocytes.

Another inverse association of PD and GBM that requires future causal investigation is the time frame of the pathophysiology of both diseases. While PD is a chronic, generally slowly progressing neurodegenerative disease characterized by gradual neuronal loss, GBM is a rapidly progressing disease with rapid proliferation of glial cells in a much shorter time frame. Possible explanations for these observations are that in PD, the neuronal loss can be compensated for a long time whereas the aggressiveness of GBM due to highly infiltrative growing and metastasizing cells that also display a vast cell heterogeneity leads to a rapid disease progression.

In this review, we showed that there are common pathogenic mechanisms involved in PD and GBM including inversely deregulated pro-survival and immune signaling, mitochondrial dysfunction and metabolic alterations. There is an inverse regulation for p53, EGF(R), PTEN/PI3K/Akt, DJ-1, HIF-1α in PD and GBM. Due to the complexity of both PD and GBM etiology and pathogenesis, future studies need to unveil so far unknown mechanisms of both diseases that will help to better understand and to compare both diseases and to explain why common inverse dysregulated cellular pathways can lead to two such different diseases. Eventually, a deeper understanding of the pathological mechanisms underlying PD and GBM will guide the identification of possibly shared drug targets that need to be modulated inversely for causative treatment of both diseases.

AUTHOR CONTRIBUTIONS

PM wrote the review. ZH, IB, AE, P-ES and RK advised, structured, and reviewed. All authors contributed to the article and approved the submitted version.

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Mitochondrial Mechanisms of LRRK2 G2019S Penetrance

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Several mutations in leucine-rich repeat kinase-2 (LRRK2) have been associated with Parkinson's disease (PD). The most common substitution, G2019S, interferes with LRRK2 kinase activity, which is regulated by autophosphorylation. Yet, the penetrance of this gain-of-function mutation is incomplete, and thus far, few factors have been correlated with disease status in carriers. This includes (i) LRRK2 autophosphorylation in urinary exosomes, (ii) serum levels of the antioxidant urate, and (iii) abundance of mitochondrial DNA (mtDNA) transcription-associated 7S DNA. In light of a mechanistic link between LRRK2 kinase activity and mtDNA lesion formation, we previously investigated mtDNA integrity in fibroblasts from manifesting (LRRK2+/PD+) and non-manifesting carriers (LRRK2+/PD−) of the G2019S mutation as well as from aged-matched controls. In our published study, mtDNA major arc deletions correlated with PD status, with manifesting carriers presenting the highest levels. In keeping with these findings, we now further explored mitochondrial features in fibroblasts derived from LRRK2+/PD+ ($n = 10$), LRRK2+/PD− ($n = 21$), and control ($n = 10$) individuals. In agreement with an accumulation of mtDNA major arc deletions, we also detected reduced NADH dehydrogenase activity in the LRRK2+/PD+ group. Moreover, in affected G2019S carriers, we observed elevated mitochondrial mass and mtDNA copy numbers as well as increased expression of the transcription factor *nuclear factor erythroid 2-related factor 2* (*Nrf2*), which regulates antioxidant signaling. Taken together, these results implicate mtDNA dyshomeostasis—possibly as a consequence of impaired mitophagy—in the penetrance of LRRK2-associated PD. Our findings are a step forward in the pursuit of unveiling markers that will allow monitoring of disease progression of LRRK2 mutation carriers.

Keywords: leucine-rich repeat kinase-2 (LRRK2), G2019S, Parkinson's disease, penetrance, mitochondria, mitochondrial DNA (mtDNA), fibroblasts

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder with a prevalence of 1% over the age of 60 years old (1). The majority of cases are sporadic, and only 5–10% suffer from a familial form (2). PD is characterized by a progressive loss of dopaminergic neurons within the *substantia nigra*. The ensuing lack of neurotransmitter dopamine in the basal ganglia results in

motor symptoms such as tremors, rigidity, bradykinesia, and postural instability (2).

To date, at least 12 genes have been unequivocally associated with the development of familial PD (3), including the gene coding for leucine-rich repeat kinase 2 (LRRK2) (4). There are seven definitely pathogenic mutations in LRRK2 (5). The most frequent genetic cause of PD, the G2019S substitution, is found in 4–5% of familial cases and ∼1% of sporadic cases in the general population. Among Ashkenazi Jews and North African Arab Berbers, the mutation explains 15–40% of PD cases, respectively (1). By contrast, in Europe, only 1–7% of PD patients harbor this nucleotide change (6), and it is even rarer in Asian populations (2). The LRRK2 G2019S mutation is inherited with reduced penetrance (7). Thereby, the risk to develop motor symptoms increases with age, ranging from 28% at 59 years to 74% at 79 years (8). However, the molecular determinants of LRRK2-G2019S penetrance are largely unknown.

LRRK2 is a large protein (268 kDa) composed of 51 exons. Its enzymatic core comprises two main structures: a GTPase and a serine-threonine kinase domain. The G2019S mutation is situated in the kinase domain and increases LRRK2 kinase activity, which in turn causes hyperphosphorylation of LRRK2 targets (6). The cellular function of LRRK2 has not been fully elucidated, but there is evidence for an involvement of the protein in endocytosis, retromer complex modulation, autophagy, and mitochondrial homeostasis (2). Specifically, mutations in LRRK2 have been shown to interfere with the removal of mitochondria from microtubules during the initiation phase of mitophagy (9). In addition, altered respiratory chain function coinciding with increased oxidative stress and morphological changes has been observed in cellular models of LRRK2-PD (10). In the presence of the G2019S mutation, mitochondrial DNA (mtDNA) lesions accumulate in patient-derived neurons (11)—a process that can be reversed by kinase inhibitor treatment (12).

To monitor the movement disorder not only at the clinical but also at the molecular level, there are increasing scientific efforts to identify biological markers of LRRK2-PD onset and progression. However, thus far, few candidates have been described that distinguish PD patients with LRRK2 mutations and such carriers who do not (yet) show the typical hallmarks of the motor disorder. First, the assessment of LRRK2 phosphorylation rates in urinary exosomes revealed higher levels in manifesting (LRRK2+/PD+) compared to non-manifesting (LRRK2+/PD−) individuals harboring the common G2019S mutation in LRRK2 (13). Second, a linkage analysis in Tunisian Arab-Berbers identified a polymorphism in dynamin 3 *(DNM3)* as a penetrance modifier (14). Third, serum levels of the antioxidant urate were shown to be reduced in LRRK2+/PD+ compared to LRRK2+/PD− individuals (15). Fourth, mtDNA transcription was altered in LRRK2+/PD+ cases (16). Lastly, our own research previously demonstrated an increase in the mitochondrial reactive oxygen species (ROS) scavenger superoxide dismutase (SOD)2 (17) and an accumulation of somatic mtDNA major arc deletions in fibroblasts from LRRK2+/PD+ compared to LRRK2+/PD− individuals (18).

To further elucidate the role of the mitochondria in defining the penetrance of *LRRK2*-associated PD, we built on our published research in fibroblasts from controls, LRRK2+/PD−, and LRRK2+/PD+ cases. Extending the mtDNA integrity and oxidative stress analyses, we now assessed mtDNA abundance as well as functional parameters such as transcriptional and respiratory chain complex activities.

MATERIALS AND METHODS

Study Cohort

Study participants were recruited at movement disorder clinics in Lübeck (Germany) and Trondheim (Norway). All participants gave written informed consent, and the study was approved by the local ethics committees. Genetic testing was performed as previously described (19). Individuals with the G2019S mutation in LRRK2 were examined by movement disorder specialists for clinical signs of PD. Mutation carriers diagnosed with PD according to the MDS Clinical Diagnostic Criteria were included in the LRRK2+/PD+ group. By contrast, non-manifesting carriers who did not fulfill these criteria were classified as LRRK2+/PD−. Demographic data of the cohort are summarized in **Table 1**. All individuals were of Caucasian descent.

Cell Culture

Dermal fibroblasts from 10 LRRK2+/PD+ cases, 21 LRRK2+/PD− individuals, and 10 age-matched healthy controls were phenotyped. Fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) high glucose without pyruvate (Life Technologies, 41965-039), supplemented with 12% fetal bovine serum (Life Technologies, 10500064) and 1% penicillin/streptomycin (Life Technologies, 15140163), and were incubated at 37°C and 5% CO_2. Cells were split with Trypsin-EDTA (Life Technologies, 25300-096) when sub-confluent.

Mitochondrial DNA Copy Number and 7S DNA Analysis

DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, 51306) following the manufacturer's instruction. Transcription-associated 7S DNA and copy number were assessed using a

TABLE 1 | Demographics of the study cohort.

	Controls	LRRK2+/PD−	LRRK2+/PD+
N	10	21	10
Number of men (%)	4 (40%)	7 (33.33%)	4 (40%)
Mean age (*SD*), years	60.8 (13.11)	58.52 (15.35)	66.00 (12.45)
Median age (IQR), years	65 (54.75–69.00)	55 (52.5–66.5)	66 (57.25–77.5)
Mean age at onset (*SD*), years	-	-	55.89 (9.87)
Median age at onset (IQR), years	-	-	57.00 (48.00–64.00)

LRRK2, leucine-rich repeat kinase-2; PD, Parkinson's disease; LRRK2+/PD−, non-manifesting LRRK2 G2019S mutation carriers; LRRK2+/PD+, manifesting LRRK2 G2019S mutation carriers; IQR, interquartile range; SD, standard deviation.

real-time PCR approach based on TaqMan probes. A probe targeting the mtDNA gene mitochondrial NADH-dehydrogenase 1 (*MT-ND1*), located in the minor arc and typically spared from deletions, was measured relative to the nuclear-encoded single-copy gene beta-2-microglobulin (*B2M*) to quantify the amount of wild-type mtDNA copies (*MT-ND1:B2M*). In addition, with a probe targeting the non-coding region (NCR) of the mitochondrial genome, the proportion of transcriptionally active mtDNA molecules was assessed. During transcription, 7S DNA is incorporated in the NCR forming a triple-stranded displacement loop (D-loop) (20). By measuring the NCR relative to *MT-ND1*, the abundance of 7S DNA per mitochondrial genome can be determined.

Quantification was achieved using a dilution series of an internal standard. Multiplex real-time PCR was performed using genomic DNA, LightCycler 480 Probes Master reaction mix (Roche, 04707494001), TaqMan probes, and primers (**Supplementary Table 1**) as specified in the manufacturer's guidelines. The PCR reaction was run on a LightCycler 480 (Roche, 05015243001). The samples were denatured for 10 min at 95°C. Amplification ran over 45 cycles with a denaturation step of 10 s at 95°C, primer annealing of 30 s at 60°C, and elongation of 3 s at 72°C.

Nuclear Factor Erythroid 2-Related Factor 2 and Mitochondrial DNA Gene Expression

RNA was extracted using the RNeasy Mini Kit (Qiagen, 74106) following the manufacturer's instructions. cDNA was synthesized using the SuperScript^{TM} III Reverse Transcriptase (Invitrogen, 18080044) using 400 ng of RNA as starting material. PCR was performed using iQ SYBR Green (Biorad, 170-8885). Primer sequences are shown in **Supplementary Table 2**. The expression of NADH dehydrogenase 1 (*MT-ND1*), NADH dehydrogenase 4 (*MT-ND4*), cytochrome b (*MT-CYTB*), cytochrome c oxidase (*MT-CO1*), and nuclear factor erythroid 2-related factor 2 (*Nrf2)* was normalized to β-actin (*ACTB*) expression. The PCR reaction was run on a LightCycler 480. The samples were denatured for 5 min at 95°C. Amplification ran over 45 cycles with a denaturation step of 10 s at 95°C, primer annealing of 10 s at 60°C, and elongation of 10 s at 75°C.

Mitochondrial Function Assessment

Mitochondrial Isolation

Mitochondria were isolated from frozen fibroblast pellets from three LRRK2+/PD+ (mean age ± SD: 59.7 ± 5.7 years) and three LRRK2+/PD− (mean age ± SD: 65.3 ± 20.6 years) individuals. Briefly, pellets were washed and mechanically lysed with a pestle in homogenization buffer [10 mM Tris pH 7.4 (T1503, Sigma)], 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma, E5134), and 250 mM sucrose (Sigma, 84100) with protease and phosphatase inhibitors (Thermo Fisher Scientific, 78440). Pure mitochondrial pellets were obtained after serial centrifugation steps and used in subsequent enzymatic assays.

Mitochondrial Enzyme Kinetics

NADH:ubiquinone oxidoreductase and cytochrome c oxidase activities were evaluated adapting well-established protocols, which measure the kinetics of NADH to NAD^+ oxidation by complex I (21) or the oxidation of reduced cytochrome c by complex IV (22), respectively. We downsized the assays to 96-well plate format and used a microplate reader (BioTek Cytation 5) to follow absorbance. The detection of respiratory chain enzyme activities requires exposure of those mitochondrial enzymes, which was achieved by three cycles of snap freezing (liquid nitrogen) and thawing of the samples. Finally, complex I and IV activities were normalized to mitochondrial mass, which was determined by citrate synthase kinetic analysis as previously reported (23). Three (citrate synthase and complex IV) to six (complex I) independent replicates per sample were performed.

Statistics

For statistical analyses, GraphPad Prism software (version 8.3.0) was used. The ROUT test was used to evaluate the presence of outliers. Datasets were then independently tested for the assumptions of parametric data. More precisely, normality (Shapiro–Wilk, D'Agostino, and Pearson tests) and homoscedasticity (Brown–Forsythe test) were evaluated. As parametric assumptions were not met, Mann–Whitney and Kruskal–Wallis (followed by Dunn's *post-hoc* test) tests were therefore utilized. Differences were considered significant (*) when *p*-values were below 0.05. Moreover, to assess the impact of age on the different outcomes, we estimated regression models with age as a covariate. We also tested the interaction of each outcome and age. However, these analyses indicated no impact or interaction of age on the reported results.

RESULTS

Decreased Complex I Activity in Manifesting Carriers of the G2019S Mutation

We have recently reported that the accumulation of mtDNA deletions serves as a discriminator between affected and unaffected LRRK2 mutation carriers (18). The mtDNA deletions studied encompass *MT-ND4*, which codes for a subunit of complex I. To understand if those mtDNA deletions have an impact on respiratory chain function in LRRK2+/PD+ patients, we assessed the activity of complexes I and IV in a subset of the previously investigated samples. The quantification of NADH:ubiquinone oxidoreductase activity relative to citrate synthase activity showed significantly reduced complex I function (Mann–Whitney test: $p = 0.003$) in the LRRK2+/PD+ group [median: 0.26, interquartile range (IQR): 0.16–0.37] compared to the LRRK2+/PD− group (median: 0.49, IQR: 0.26–0.70) (**Figure 1A**). By contrast, analyzing cytochrome c oxidase activity relative to citrate synthase activity did not reveal differences between the two groups (LRRK2+/PD−, median: 0.035, IQR: 0.027–0.040; LRRK2+/PD+, median: 0.023, IQR: 0.014–0.037; Mann–Whitney test: $p = 0.114$) (**Figure 1B**).

Altered Antioxidant Signaling in Manifesting Carriers of the G2019S Mutation

A recent study measuring serum levels of the antioxidant urate in carriers of LRRK2 mutations reported reduced levels in the LRRK2+/PD+ group (15). Bakshi et al. (15) speculated that the resulting increase in ROS levels may induce the NF-E2-related factor 2–antioxidant responsive element (Nrf2–ARE) pathway. Considering this study and literature indicating that ROS induces nicks and subsequent somatic mutations in the mitochondrial genome (24), we decided to test whether our previously observed penetrance-associated mtDNA deletion phenotype (18) could be due to impaired antioxidant signaling.

Quantifying the expression of the transcription factor *Nrf2* in our cohort, we observed significantly increased mRNA levels in LRRK2+/PD+ individuals (median: 1.18, IQR: 0.92–1.38) compared to controls (median: 0.79, IQR: 0.65–1.03; Kruskal–Wallis followed by Dunn's tests: $p = 0.033$). By contrast, LRRK2+/PD− individuals (median: 0.92, IQR: 0.78–1.10) showed no upregulation (**Figure 2A**, **Supplementary Table 3**).

Increased Mitochondrial Mass in Manifesting Carriers of the G2019S Mutation

In a groundbreaking penetrance study, Fraser et al. (13) observed enhanced LRRK2 autophosphorylation in urinary

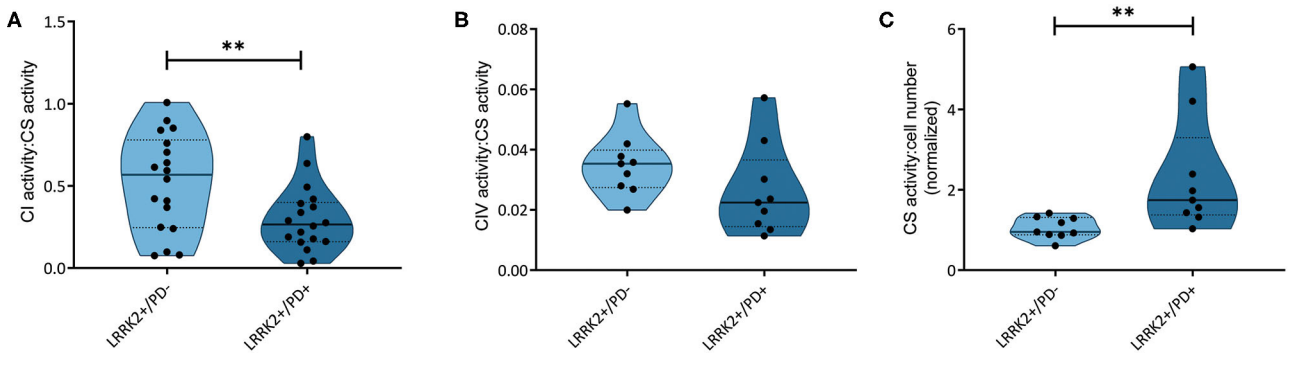

FIGURE 1 | Respiratory chain complex and citrate synthase activities. **(A)** NADH dehydrogenase activity (complex I, CI; Mann–Whitney test: $p = 0.003$) and **(B)** cytochrome c oxidase (complex IV, CIV; Mann–Whitney test: $p = 0.114$) normalized to citrate synthase (CS) activity in non-manifesting [leucine-rich repeat kinase-2 (LRRK2)+/PD−, $n = 3$] and manifesting carriers (LRRK2+/PD+, $n = 3$) of the G2019S mutation in LRRK2. **(C)** CS activity relative to cell number, with one sample serving as an internal standard (Mann–Whitney test: $p = 0.001$). Experiments performed on six (CI) or three (CIV and CS) independent replicates. $^{**}p < 0.01$.

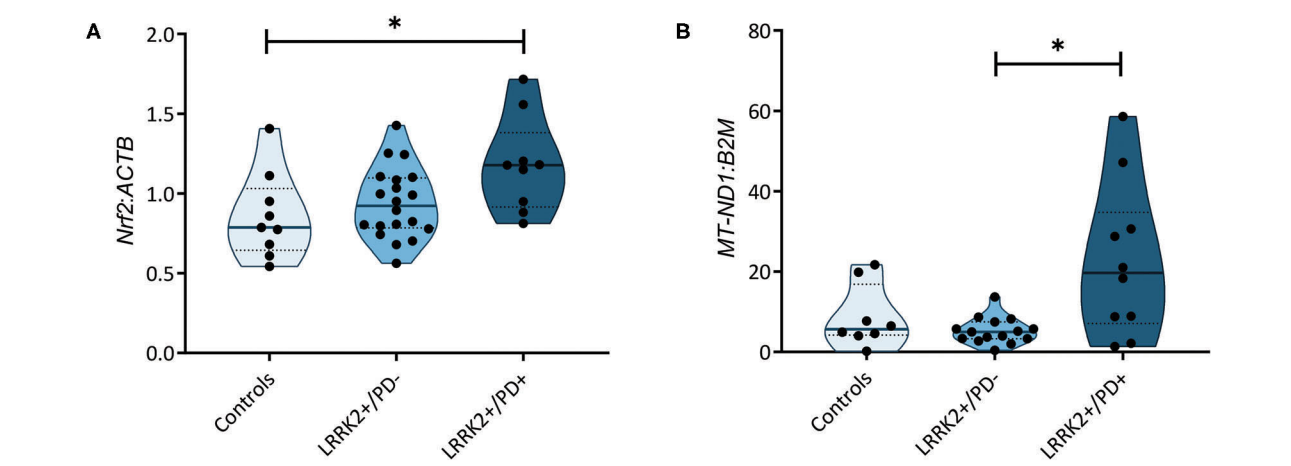

FIGURE 2 | Nuclear factor erythroid 2-related factor 2 *(Nrf2)*-mediated antioxidant signaling and mitochondrial DNA (mtDNA) copy number. **(A)** *Nrf2* gene expression normalized to β-actin (*ACTB*) in controls ($n = 9$) as well as non-manifesting [leucine-rich repeat kinase-2 (LRRK2)+/PD−, $n = 20$] and manifesting carriers (LRRK2+/PD+, $n = 10$) of the G2019S mutation in LRRK2. Kruskal–Wallis test and Dunn's *post-hoc* test: controls vs. LRRK2+/PD−, $p > 0.99$; controls vs. LRRK2+/PD+, $p = 0.03$; LRRK2+/PD− vs. LRRK2+/PD+, $p = 0.12$. **(B)** MtDNA copy number in control ($n = 8$), LRRK2+/PD− ($n = 19$), and LRRK2+/PD+ ($n = 10$) individuals. Kruskal–Wallis test and Dunn's *post-hoc* test: controls vs. LRRK2+/PD−, $p > 0.99$; controls vs. LRRK2+/PD+, $p = 0.36$; LRRK2+/PD− vs. LRRK2+/PD+, $p = 0.02$. Experiments performed on three independent replicates. $^{*}p < 0.05$.

exosomes from LRRK2+/PD+ compared to LRRK2+/PD− cases. In light of an established mechanistic link between LRRK2 kinase activity and lysosomal dysfunction (25), we speculated that mtDNA disintegration and elevated oxidative stress might be the consequence of impaired mitochondrial clearance in LRRK2+/PD+ individuals. To test this hypothesis, we determined the citrate synthase activity to cell number ratios as an indicator of mitochondrial mass in our fibroblast samples. We observed a significant increase (Mann–Whitney test: $p = 0.001$) in the LRRK2+/PD+ group (median: 1.74, IQR: 1.38–3.30) compared to the LRRK2+/PD− group (median: 0.95, IQR: 0.88–1.31) (**Figure 1C**). Moreover, mtDNA copy number analysis also revealed increased levels in LRRK2+/PD+ (median: 19.68, IQR: 7.11–34.77) compared to LRRK2+/PD− fibroblasts (median: 4.98, IQR: 3.32–7.46; Kruskal–Wallis followed by Dunn's tests: $p = 0.024$) (**Figure 2B**, **Supplementary Table 3**).

Impaired Mitochondrial DNA Transcription Initiation in Carriers of the G2019S Mutation Independent of Disease Status

Further highlighting the role of mtDNA maintenance in determining LRRK2-PD penetrance, an increase in mtDNA transcription-associated 7S DNA was recently detected in LRRK2+/PD+ compared to LRRK2+/PD− fibroblasts (16). By measuring the abundance of 7S DNA per mtDNA molecule (*MT-ND1*), we observed a significant decrease (Mann–Whitney test: $p = 0.003$) in all LRRK2 G2019S carriers (median: 0.80, IQR: 0.75–0.87) compared to controls (median: 0.93, IQR: 0.82–0.97) (**Figure 3A**). However, contrary to the abovementioned study, we detected no difference in the 7S DNA:*MT-ND1* ratios between manifesting (median: 0.84, IQR: 0.74–0.87) and non-manifesting (median: 0.79, IQR: 0.76–0.85; Kruskal–Wallis followed by Dunn's tests: $p > 0.99$) carriers (**Figure 3B**, **Supplementary Table 3**).

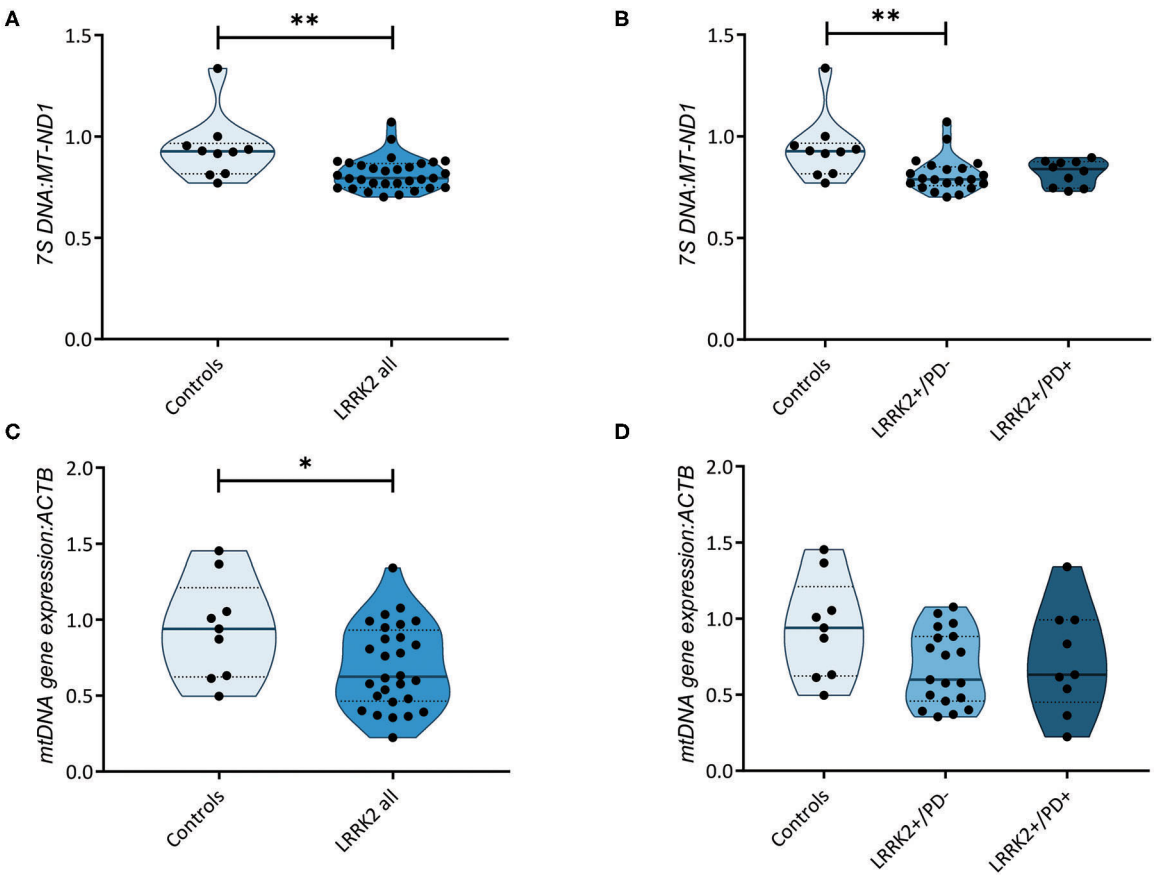

FIGURE 3 | Transcription of the mitochondrial genome. **(A)** Mitochondrial DNA (mtDNA) transcription-associated 7S DNA normalized to *MT-ND1* in controls ($n = 10$) and leucine-rich repeat kinase-2 (LRRK2) G2019S mutation carriers ($n = 31$). Mann–Whitney test: $p = 0.003$. **(B)** 7S DNA:*MT-ND1* ratios in controls ($n = 10$) and non-manifesting (LRRK2+/PD−, $n = 21$) and manifesting carriers (LRRK2+/PD+, $n = 10$) of the G2019S mutation in LRRK2. Kruskal–Wallis test and Dunn's *post-hoc* test: controls vs. LRRK2+/PD−, $p = 0.009$; controls vs. LRRK2+/PD+, $p = 0.14$; LRRK2+/PD− vs. LRRK2+/PD+, $p > 0.99$. **(C)** Mitochondrial gene expression derived from averaging the mRNA levels of *MT-ND1*, *MT-ND4*, *MT-CO1*, and *MT-CYTB* in controls ($n = 9$) and LRRK2 G2019S mutation carriers ($n = 28$). Mann–Whitney test: $p = 0.04$. **(D)** MtDNA gene expression in control ($n = 9$), LRRK2+/PD− ($n = 19$), and LRRK2+/PD+ individuals ($n = 9$). Kruskal–Wallis test and Dunn's *post-hoc* test: controls vs. LRRK2+/PD−, $p = 0.12$; controls vs. LRRK2+/PD+, $p = 0.49$; LRRK2+/PD− vs. LRRK2+/PD+, $p > 0.99$. Experiments were performed using three independent replicates. $^{**}p < 0.01$; $^{*}p < 0.05$.

We then tested whether mtDNA-encoded genes were differentially expressed in the LRRK2+/PD+ and LRRK2+/PD− groups. We averaged the expression of the polycistronic transcripts of *MT-ND1*, *MT-ND4*, *MT-CYTB*, and *MT-CO1*. In line with our results for the 7S DNA:*MT-ND1* ratios, we observed a significant decrease in mtDNA gene expression in all G2019S mutation carriers, independent of the disease status (median: 0.62, IQR: 0.46–0.93) compared to controls (median: 0.94, IQR: 0.62–1.21; Mann–Whitney test: $p = 0.044$) (**Figures 3C,D**, **Supplementary Table 3**).

DISCUSSION

There is a myriad of biological pathways implicated in LRRK2-dependent neurodegeneration including cytoskeletal dynamics, autophagy, trafficking, and mitochondrial dysfunction (1). However, the pathophysiology for penetrance of mutations in LRRK2 still remains an enigma.

Elevated phosphorylation of Ser-1292 in urinary exosomes has been shown to predict LRRK2 mutation status and risk for PD (13). Additionally, LRRK2 kinase activity has an impact on mtDNA integrity, where the LRRK2 G2019S mutants present with increased mtDNA lesions compared to kinase-dead (D1994A) mutants in rat cortical neurons (12). These mtDNA findings in model organisms seem to be translatable to patient-derived cell lines. Our group recently reported an accumulation of mtDNA deletions in LRRK2 G2019S patient-derived fibroblasts. The mtDNA deletions correlated with disease status: the manifesting carriers presented a higher load of mtDNA deletions than non-manifesting carriers and controls (18).

In this study, we further investigated mitochondrial function and related factors. In LRRK2+/PD+ compared to LRRK2+/PD− fibroblasts, we found reduced complex I activity but no changes in complex IV activity. NADH dehydrogenase deficiency is a hallmark of PD pathology (10) and has been proposed to play a role in the penetrance of LRRK2 mutations (26). Our data do not exclude the dysfunction of other mitochondrial respiratory complexes. Indeed, besides complex I, the G2019S mutation in LRRK2 also compromised the function of respiratory chain complexes III and IV in previous studies (27). Furthermore, reduced cellular ATP levels and a loss in mitochondrial membrane potential were observed in fibroblasts from PD patients harboring the G2019S LRRK2 mutation (28). However, in the context of LRRK2-PD penetrance, somatic mtDNA deletion accumulation appears to primarily affect complex I function.

Somatic mtDNA major arc deletions may be a result of impaired antioxidant signaling in LRRK2+/PD+. Situated in close vicinity to the respiratory chain, the mitochondrial genome is permanently exposed to free radicals, which can cause single- and double-strand DNA breaks. If such nicks remain unrepaired, somatic mtDNA mutations arise (24). Recently, a blood screen in ∼1,500 *LRRK2*+/PD− and *LRRK2*+/PD+ individuals revealed reduced urate serum concentrations in the latter group (15). Urate can modulate antioxidant signaling, including the Nrf2–ARE pathway (15). When quantifying *Nrf2* gene expression in our samples, we found increased mRNA levels in LRRK2+/PD+ compared to control fibroblasts, suggesting a compensatory upregulation in affected individuals.

Via nuclear respiratory factor 1 (NRF-1), Nrf2 can act on the mitochondrial transcription factor A (TFAM), thereby interfering with mtDNA gene expression (29). During the initiation phase of mtDNA transcription, a small DNA fragment is incorporated in the D-loop region of the mitochondrial genome. This, so-called 7S DNA, can serve as a marker of mtDNA molecules undergoing transcription. A study quantifying 7S DNA in fibroblasts from four LRRK2+/PD+ and five LRRK2+/PD− cases showed elevated 7S DNA levels and an increase in mtDNA heavy-strand transcription in manifesting carriers (16). Moreover, contrary to what was previously observed in postmortem nigral neurons from idiopathic PD patients (30), the authors found increased 7S DNA:mtDNA ratios in sporadic patients compared to controls (16). When testing the abundance of 7S DNA in a larger number of controls ($n = 10$) and LRRK2+/PD− ($n = 21$) and LRRK2+/PD+ ($n = 10$) cases, we did not observe a penetrance-specific phenotype. By contrast, 7S DNA:mtDNA ratios were reduced in all individuals with LRRK2 G2019S independent of affection status. In line with these results, we detected a reduction in mtDNA gene expression in all mutation carriers. Thus, whether impaired mtDNA gene expression contributes to penetrance-associated mitochondrial dysfunction in LRRK2-associated PD warrants further studies in replication cohorts.

Overall, LRRK2 mutations cause mitochondrial dysfunction in multiple model systems including patient-derived cell lines. Mutant LRRK2 can interfere with mtDNA maintenance, mitochondrial dynamics, trafficking, and mitophagy (10, 11, 28, 31). LRRK2 pathogenic point mutations have been shown to impair mitophagy in patient induced pluripotent stem cell (iPSC)-derived neurons in a kinase-dependent manner. Wild-type LRRK2 forms a complex with Miro1, which connects mitochondria with kinesin motor proteins that transport cargo along microtubules. During the initial phase of mitophagy, LRRK2 mediates the removal of Miro1 from depolarized mitochondria, thereby reducing mitochondrial motility. In the presence of the G2019S mutation, the LRRK2-Miro1 complex is disrupted, causing delays in the induction of mitophagy (9). In light of these findings, mtDNA disintegration, respiratory chain dysfunction, and increased mitochondrial mass and mtDNA copy numbers in LRRK2+/PD+ cases may be different signs of impaired turnover of damaged mitochondria.

In conclusion, we showed that mitochondrial phenotypes such as somatic mtDNA deletions or respiratory chain complex I activity can serve as markers of LRRK2 G2019S penetrance in peripheral tissues. Further experiments are required to understand whether, unlike LRRK2+/PD− individuals, LRRK2+/PD+ cases present a faulty mitophagy system. Moreover, despite the recognized value of patient fibroblasts for PD research, inherent model limitations warrant further investigations in iPSC-derived neurons. The latter model will allow the investigation of the contribution of mtDNA disintegration to the selective death of dopaminergic neurons.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are provided in the **Supplementary Material**. Raw data files are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Board of the University of Lübeck, Germany and the Luxembourg Comité National d'Éthique de Recherche (CNER). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SD, JG, NO, LG, and CD collected the data. SD, JG, NO, and SP performed the analysis. SD, JT, SP, and AG wrote the manuscript, which was reviewed by all authors. KW, JT, JA, CK, SP, and AG conceived the study. PS, CK, and AG acquired funding for the study. AG was in charge of direction and planning of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00881/full#supplementary-material

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Association Between a Variable Number Tandem Repeat Polymorphism Within the DAT1 Gene and the Mesolimbic Pathway in Parkinson's Disease

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The loss of ventral striatal dopaminergic neurons in Parkinson's disease (PD) predicts an impact on the reward system. The ventrostriatal system is involved in motivational processing and its dysfunction may be related to non-motor symptoms such as depression and apathy. We previously documented that patients with PD had blunted Blood Oxygen Level Dependent functional magnetic resonance imaging (BOLD fMRI) reward task related activity during both reward anticipation (i.e., in the ventral striatum) and reward outcome (i.e., in the orbitofrontal cortex). Evidence for the modulation of brain function by dopaminergic genes in PD is limited. Genes implicated in dopamine transmission, such as the dopamine transporter gene (DAT1) may influence the clinical heterogeneity seen in PD, including reward processing. This study therefore sought to determine whether genetic differences in the DAT gene are associated with brain activity associated with response to reward in PD patients and controls. A sample of PD cases on treatment ($n = 15$) and non-PD controls ($n = 30$) from an ethnic group unique to South Africa were genotyped. We found a three-way interaction between GENOTYPE × BOLD fMRI REWARD × DIAGNOSIS [$F_{(1, 40)} = 4.666$, $p = 0.037$, partial $\eta^2 = 0.104$]. PD patients with the DAT1 homozygous 10/10 repeat genotype showed a relative decrease in orbitofrontal cortex reward outcome related activity compared to the patient group who did not have this repeat. PD patients with other genotypes showed an expected increase in orbitofrontal cortex reward outcome related activity compared to controls. Given the small sample size of the PD group with the 10/10 repeat, these results should be considered preliminary. Nevertheless, these preliminary findings highlight the potential modulation of dopamine transporter polymorphisms on orbitofrontal reward system activity in PD and highlight the need for further studies.

Keywords: DAT1, SLC6A3, Parkinson's disease, monetary incentive delay, orbitofrontal cortex, ventral striatum, functional magnetic resonance imaging

INTRODUCTION

Parkinson's disease (PD) is characterized by decreased dopaminergic availability in the brain, especially in the striatum (1). Changes in striatal dopaminergic tone in PD have been related to cognitive as well as reward processing abnormalities (2). Further, differences in fronto-striatal reward processing have been demonstrated in both medicated and unmedicated patients (2, 3). Specifically, blunted neural activity related to performance on a reward task was found to be a distinguishing factor in PD patients when compared to similarly aged controls (3). Brain activity patterns associated with reward may be linked to the non-motor symptoms of PD such as apathy (4) and impulsivity (5), and warrant investigation.

There is a growing body of literature documenting an association between candidate genes involved in the dopamine system and reward related brain activity (6–8). This includes the commonly occurring 10-repeat allele of the 40 bp variable number tandem repeat (VNTR) polymorphism within the Dopamine Active Transporter (DAT1) gene, also referred to as solute carrier family 6 member 3 (SLC6A3). Although the individual frequencies of the DAT1 repeats differ between ethnic groups, the 10-repeat allele is still the most common with frequencies ranging from 37 to 93% across several ethnic groups (9). The majority of *in vitro* studies have shown that the 10-repeat allele is associated with increased DAT1 expression in comparison to a commonly occurring 9-repeat (10–12). Increased DAT1 expression leads to increased activity of DAT1 and increased dopamine uptake, which results in a decrease of dopamine levels in the synapse, and this could potentially be related to poorer reward related activity in the ventral-striatal system. Using single photon emission computed tomography (SPECT), subjects homozygous for the DAT 10-repeat allele had a 22% relative increase in DAT protein availability in homozygous DAT 10-repeat homozygotes compared to those with the 9-repeat/10-repeat genotype (10). Using Quantitative real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR), it was shown that increased levels of DAT1 expression were associated with the number of 10-repeat alleles (11). Radioligand binding and immunoblotting techniques also revealed statistically significant differences in DAT expression attributable to the DAT1 genotype, with lower the DAT1 density for the 9- and 10-repeat variants (12). Not all studies have found increased expression of DAT1 associated with this allele, with some reporting the opposite (13). Blood oxygen level dependent functional MRI (fMRI) nevertheless demonstrated relatively decreased reward anticipatory activity in the ventral striatum as well as decreased reward outcome related activity in the orbitofrontal cortex during a Monetary Incentive Delay (MID) reward processing task in individuals with the DAT1 10/10 repeat, compared to those with the 9/9 repeat (6). The MID is known to robustly activate the ventral striatum during reward anticipation and orbitofrontal cortex during positive reward outcome (14). This finding was replicated in another reward processing study examining orbitofrontal and ventral striatal activity in DAT1 10/10-repeat vs. 9/9 repeat carriers, showing greater responses to smoking vs. non-smoking cues (15). However, there were contrasting findings in a study examining the impact of genotype on reward processing thought to underlie long-term memory formation where the DAT1 10-repeat homozygotes demonstrated increased striatal activity compared with 9/10-repeat heterozygotes (8).

As the relationship between DAT1 genotypes and reward processing is further clarified, it is important to examine the impact that the DAT1 genotype could have on the course of illnesses such as PD, where disease related alterations in dopamine tone are particularly evident (16). To our knowledge, there are no studies that have examined the relationship between the DAT1 genotype and abnormalities in brain activation associated with reward processing in PD.

Previously, we identified a relative decrease in both anticipatory activity seen in the ventral striatum and reward outcome related activity in the orbitofrontal cortex in PD (3). Here, we aim to investigate the potential modulating effects of the DAT1 10/10 genotype on these brain changes in a subsample of 15 PD patients and 30 matched healthy controls, drawn from a larger cohort, who were genotyped and underwent fMRI whilst performing a monetary incentive delay task (17, 18). Given the aforementioned findings in the literature, we predicted that PD patients relative to controls, with the DAT1 10/10 repeat genotype, compared with DAT1 heterozygotes, would have the lowest levels of reward related activity in both the ventral striatum during anticipation and in the orbitofrontal cortex during reward outcome.

MATERIALS AND METHODS

The study participants form part of a larger cohort examining the genomic and environmental signatures that are common to PD, Posttraumatic Stress Disorder, Schizophrenia and metabolic syndrome (named the "Shared Roots" study, MRC-RFA-UFSP-01-2013). The study has been approved by Health Research Ethics Committee (HREC N13/08/115) of Stellenbosch University, Tygerberg Hospital, Cape Town, South Africa, with annual renewal.

All participants were recruited from the same geographical region in Cape Town, South Africa, were unrelated and matched to socioeconomic status (lower to middle income status). All self-identified as "mixed ancestry" which refers to an ethnic population unique to South Africa and resulting from an admixture of individuals of African, European and Asian ancestral origins (19). This is the first published report on *DAT1* genotypes in a South African Mixed Ancestry population. A diagnosis of PD was clinically confirmed by a neurologist according to MDS diagnostic criteria (20). A healthy (non-PD) control group was recruited and matched for ethnicity. Controls did not have current significant psychopathology or other significant confounding medical conditions.

Clinical Assessments

All participants received a full clinical examination. They were screened for any confounding psychopathology using the Mini-International Neuropsychiatric Interview (MINI version 6.0.0).

The Unified Parkinson's Rating Scale (UPDRS) (Version 3.0) was completed for the PD patients (21). Handedness was determined by the Edinburgh Handedness Inventory (22). All participants were asked to take their PD medication as normal, prior to scanning. All participants received a urine drug screen immediately before their MRI scan. Participants with severe head injury, confounding intra-cranial pathology, current severe psychopathology and/or drug abuse and other medical conditions that could confound behavioral as well as fMRI measures were excluded.

Genotyping of 40 bp DAT1 VNTR Polymorphism

Venous whole blood was collected from all study participants for the genetic analyses. Genomic DNA was isolated with the use of an in-house phenol/chloroform method prior to 2016 and a salting-out precipitation method (Gentra Puregene Blood Kit), for samples collected post 2016. Polymerase chain reaction (PCR) amplification was performed using primers DAT1 forward: 5′-ATGGGGGTCCTGGTATGTCT-3′ and reverse: 5′-GGCACGCACCTGAGAGAAAT-3′; that were designed using OligoAnalyzer (www.idtdna.com/oligoanalyzer), BLAST (www.ncbi.nlm.nih.gov/BLAST/) and primerBLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) for optimal binding to the region of interest. PCR was performed in 25 μl reactions which contained 0.4 μM DAT1 forward and reverse primers (Inqaba biotec™, South Africa), 0.075 μM of each dNTP, 0.25 U GoTaq® G2 Flexi DNA Polymerase, 1x Colorless GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$ solution (Promega, Madison, WI, USA) and 30 ng genomic DNA. The PCR conditions comprised of: initial denaturation at 95°C for 10 min; followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 58°C for 30 s and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min using a thermocycler (Applied Biosystems, GeneAmp® PCR System 2700, Singapore). The PCR product was visualized using electrophoresis on a 1% SeaKem® LE Agarose gel (Consort Electrophoresis Power Supply, 800 Series, E844, Belgium). Genotyping was carried out by comparing the size of the PCR product, visualized on the agarose gel, to the expected product size determined based on the reference DNA sequence (NM 001044.5) in Ensembl (www.ensembl.org/index.html).

Monetary Incentive Delay (MID) fMRI Paradigm

All participants performed a modified version of the MID task (23). To enhance task comprehension, as well as keep the number of scan acquisitions to a minimum, only reward and neutral cues were used in this task. The task is described in detail elsewhere (23). Briefly, during each scan trial participants were required to respond as rapidly as possible when a target cue was presented. A smiling face immediately preceded the target, to indicate a potentially rewarding trial, and a neutral face was presented prior to neutral trials. After seeing the face cue, a blue star was shown for a short pseudo random interval immediately followed by the target cue (i.e., reward anticipation). If a participant responded in time to the target cue, a screen with green lettering appeared indicating the total reward won (i.e., reward outcome). If a participant did not respond in time, red letters appeared. During reward trials, the monetary reward was incrementally increased (fixed increments of ZAR10) (see **Figure 1**).

The reward anticipation period as well as the inter-trial interval were "jittered" to reduce collinearity between reward anticipation and reward outcome (mean duration 3,286 ms, range 779–6,729 ms; mean duration 3,535 ms, range 1,029–6,979 ms, respectively). The reward outcome period was 2,000 ms per trial. The entire task therefore consisted of 60 trials, with a mean duration of 9,571 ms (range 4,946–16,107 ms), resulting in a total task duration of 9 min 35 s.

To ensure an equal number of rewarded and unrewarded trials, the duration of the target cue was adapted to the fastest response time of the participant during a training session. By matching task performance across subjects in this way, we controlled for differing levels of performance across the groups. The target score was set to approximately ZAR150 (∼10 USD) for each group.

Behavioral Data Analysis

Neutral correct trials and rewarded trials were compared between case-control (diagnostic) and DAT1 genotype groups using a repeated measures analysis of variance (RMANOVA), modeling for REWARD (i.e., neutral correct vs. rewarded trials) × DIAGNOSIS (i.e., PD and controls) × GENOTYPE (i.e., DAT1 10/10 repeat vs. other genotypes) interaction effects. Monetary reward across diagnostic and genotype groups was compared with a standard *t*-test. If the 3-way modeling for REWARD × DIAGNOSIS × GENOTYPE interaction was significant, *post-hoc* testing was performed to identify whether the 3-way interaction was driven by disease status or genotype.

Image Acquisition

Scans were acquired on a 3T Siemens Allegra at the Combined Universities Brain Imaging Center (CUBIC). A total of 360 whole-brain 2D-EPI images (TR = 1,600 ms, TE = 23 ms, flip-angle: 72.5 degrees, FOV: 256×256, 30 slices, 4 mm isotropic voxels) were acquired in 9 min 35 s. For image registration, a T1 ME-MPRAGE weighted structural scan was acquired (TR = 2,530 ms; TE1 = 1.53 ms TE2 = 3.21, ms, TE3 = 4.89 ms, TE4 = 6.57 ms, flip-angle: 7 degrees, FoV: 256 mm, 128 slices, 1 isotropic voxel size) (24).

Image Pre-processing

Images were analyzed using SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). Pre-processing and first-level statistical analysis was undertaken as previously described (3). In brief, pre-processing involved correction for slice timing differences, re-alignment to correct for head motion, spatial normalization to the Montreal Neurological Institute template brain, and spatial smoothing to accommodate inter-individual differences in neuro-anatomy. Head motion parameters were analyzed to ensure that the maximum motion did not exceed a predefined threshold (scan-to-scan >2 mm).

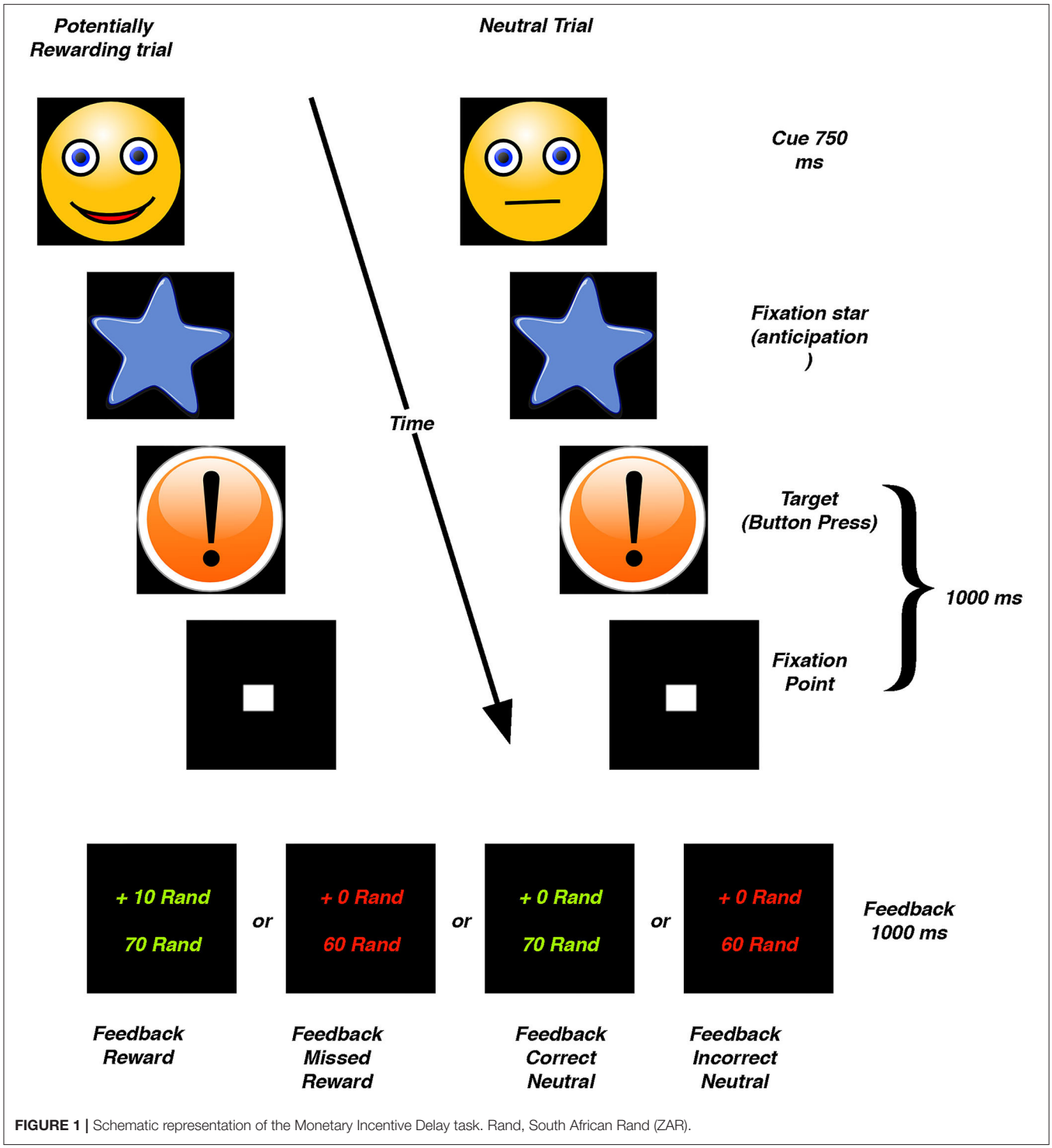

FIGURE 1 | Schematic representation of the Monetary Incentive Delay task. Rand, South African Rand (ZAR).

First Level fMRI Statistical Analysis

The pre-processed time-series data for each participant was analyzed using a standard general linear model (GLM) analysis. The model consisted of six factors of interest, representing haemodynamic changes time-locked to trial periods of (1) anticipation of receiving a potential reward, i.e., during and after the presentation of the reward cue (reward anticipation), (2) the lack of reward anticipation during and after a neutral cue (neutral anticipation), (3) feedback reflecting when money was received for a successful reward trial (reward outcome), (4) feedback when no reward was received, (5) feedback reflecting when the button was pressed in time during a neutral

TABLE 1 | Demographics of the 45 study participants.

	Patients $n = 15$		Controls $n = 30$			
	Mean	**SD**	**Mean**	**SD**	**Test score**	***p*-value**
Age	61.59	9.56	56.56	6.59	$t = 2.070$	0.04*
Sex (M/F)	11/4		12/18		$X^2 = 4.447$	0.04*
Handedness (R/L)	14/1		29/1		$X^2 = 0.262$	0.61
Months since diagnosis	56.6	41.92				
LED (mg/day)	560	307.76				
Hoehn & Yahr staging	2.57					
ADL (best/worst)	73.33/65.33					
Reward Won (ZAR)	118.67		121.67		$t = -0.309$	0.76
fMRI motion parameters						
Mean motion	0.09	0.03	0.10	0.03	$t = -1.141$	0.26
Maximum motion	0.47	0.28	0.52	0.31	$t = -0.517$	0.61
Total number of movements	124.87	80.20	138.27	64.75	$t = -0.604$	0.55
Mean rotation	0.001	0.0003	0.001	0.0004	$t = -0.867$	0.39
Maximum rotation	0.006	0.0025	0.008	0.0070	$t = -0.803$	0.43

**Significant at $p < 0.05$ level.*

trial (neutral correct outcome), and (6) feedback reflecting an incorrect response in a neutral trial, i.e., when the target was missed when no reward was offered (**Figure 1**). The onset of the factors modeling anticipation (duration range 1,529–7,479 ms) was at the presentation of the cue, while the onset of the factors modeling feedback (duration: 2,000 ms) was at the presentation of the target, including the button press to the target and subsequent feedback (see **Figure 1**). Motion parameters from the realignment procedure were included as factors of no interest. Low frequency drifts were removed from the signal by applying a high-pass filter with a cut-off frequency of 128 Hz.

Region of Interest Analyses

Primary analyses were performed in one region of interest (ROI): the combined bilateral ventral striatum for anticipation, and combined bilateral orbitofrontal cortex for reward outcome, based on previous findings (23). These regions were defined using the Automated Anatomical Labeling (AAL)-atlas (25) and the Oxford-GSK-Imanova Striatal Connectivity Atlas for the ventral striatum (26). For each participant, the mean activation level (expressed as percent signal change) during the contrasts of interest specific to reward anticipation and reward outcome (reward anticipation, neutral anticipation, reward outcome, and neutral correct outcome) was averaged over all the voxels of each ROI using SPM12 and custom MATLAB R2019a scripts.

Similar to the behavioral data analysis, these values were used in a RMANOVA, testing for main and group effects in activation levels between neutral vs. potentially rewarding trials, reward anticipation vs. reward outcome, and correct neutral trials vs. positive reward outcome. As in the behavioral analysis, we modeled for a REWARD × DIAGNOSIS × GENOTYPE interaction effect.

RESULTS

The Shared Roots cohort comprised 81 PD patients and 79 controls. All participants were genotyped for the 40 bp DAT1 VNTR. Data was originally collected from 2 separate scan sites. Due to the low number of controls relative to patients available at the second site ($n = 6$ with DAT1 10/10 genotype, $n = 2$ with other genotypes) which resulted in an unbalanced sample for the second scan site, we chose only to include data from the first scan site. Of these, 18 patients and 39 controls had fMRI, T1 structural scan and genotype data. Three PD patients and seven controls were excluded due to the presence of motion or other scanner related artifacts. Two controls were excluded due to poor task performance. This resulted in a final sample of 15 patients and 30 controls. The demographics of the 45 study participants are reflected in **Table 1**. There was a small but significant difference in age between the cases and controls. We therefore included age as a covariate in all analyses. Although there were significantly fewer females present in the patient group than in the control group, we chose not to correct for this in our final model, as we found no sex-based differences on the MID in our larger sample (3, 18). Patient and control groups were also matched in terms of several important motion parameters (**Table 1**) (27).

Similar to European cohorts, the DAT1 10/10 repeat was the most commonly occurring repeat in the sample (45.8%), followed by the 10/9 repeat. Interestingly, our sample contained few DAT1 9/9 repeats (4.2%, see **Figure 2**) unlike cohorts of European ancestry where it is often frequent. Importantly, there was no significant difference in the frequencies of the genotype subgroups, which were balanced between patients and controls ($X^2 = 2.179$, $p = 0.14$). As we had an *a priori* hypothesis for the DAT1 10/10 repeat and had observed a relatively low frequency of the other repeats, we divided the sample into two groups: a "10/10 repeat" genotype group compared to all the other genotypes.

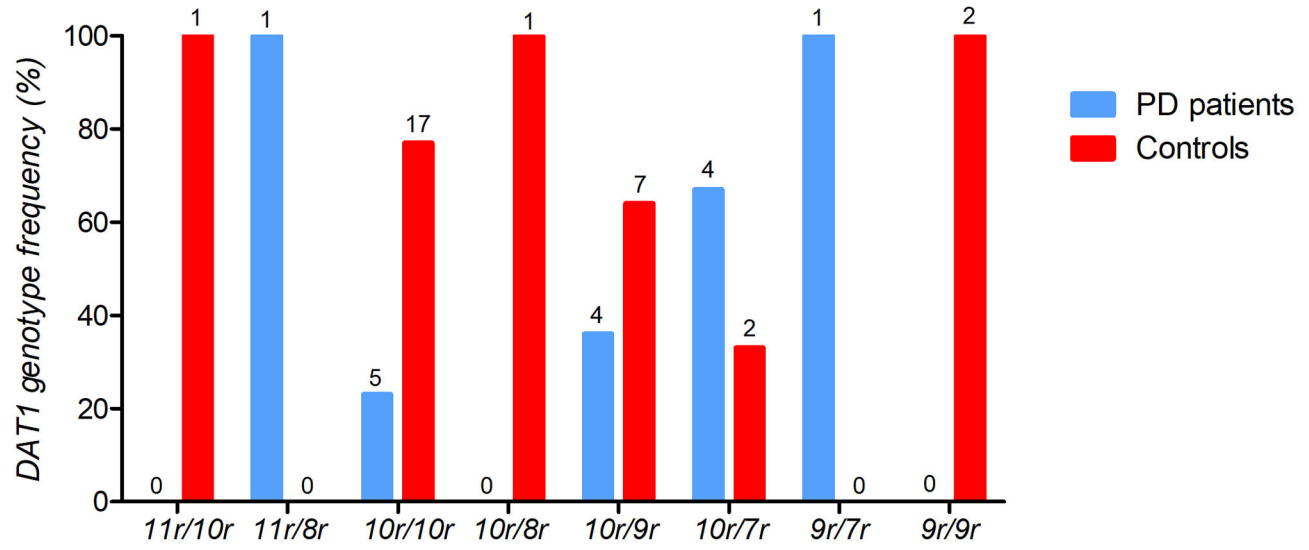

FIGURE 2 | The frequencies of *DAT1* variable number tandem repeat (VNTR) for PD patients ($n = 15$) and healthy controls ($n = 30$). The percentage of *DAT1* VNTR frequencies are depicted with the individual counts (n) displayed above each genotype bar.

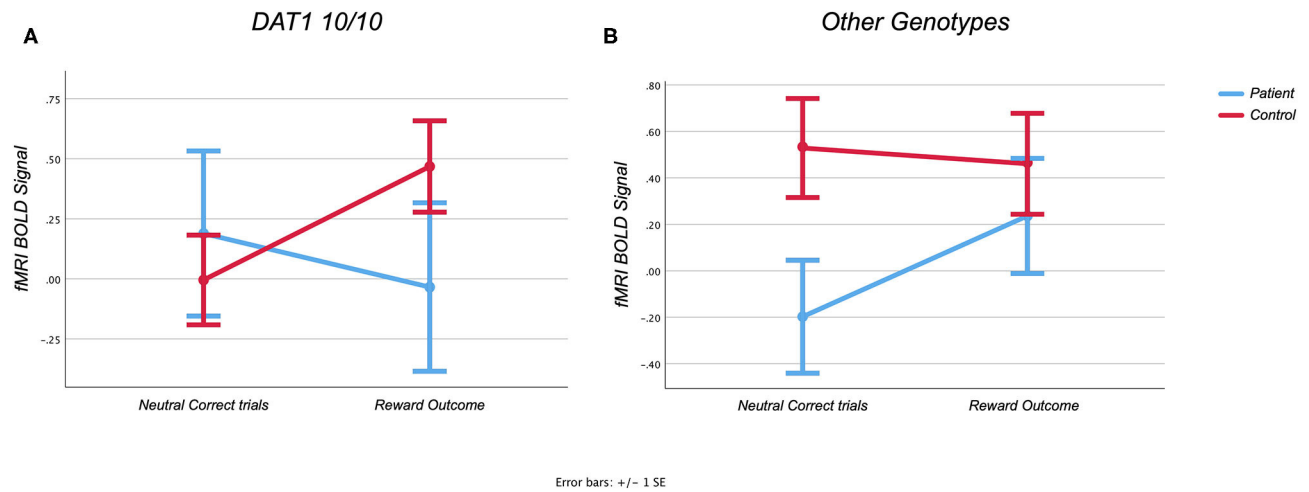

FIGURE 3 | (A,B) Region of interest analysis of the Monetary Incentive Delay task. Graphs showing task related activity during the Monetary Incentive Delay task in the orbitofrontal cortex during reward feedback.

No Difference Observed in Behavioral Data

Since the task was adjusted according to participant's performance level, patients and controls received an equal amount of reward [$t_{(43)} = -0.309$, $p = 0.759$]. Both patients and controls appropriately responded more rapidly to rewarded trials [$F_{(1, 41)} = 5.633$, $p = 0.022$], but there was no REWARD × GENOTYPE interaction effect [$F_{(1, 41)} = 0.103$, $p = 0.749$].

No Reward Anticipation Effect Observed in the Ventral Striatum

Contrary to our hypothesis, we found no main effect for reward anticipation in the ventral striatum [$F_{(1, 41)} = 1.615$, $p = 0.211$], nor a DIAGNOSIS interaction effect [$F_{(1, 41)} = 2.227$, $p = 0.143$].

Reward Outcome Effect Observed in the Orbitofrontal Cortex

Although there was no main effect for reward outcome in the orbitofrontal cortex [$F_{(1)} = 1.172$, $p = 0.285$], there was a three-way REWARD × DIAGNOSIS × GENOTYPE interaction effect [$F_{(1, 40)} = 4.666$, $p = 0.037$], while controlling for age, in line with our hypothesis (see **Figure 3**). As predicted, after *post-hoc* testing, Parkinson's patients who have the DAT1 10/10 genotype demonstrated a decrease on average in bold signal from neutral to rewarded trials, whereas those with other genotypes demonstrated a normal increase in activity [$F_{(1)} = 1.678$, $p = 0.22$, partial $\eta^2 = 0.123$). Interestingly, controls demonstrate the opposite effect, showing a relative increase in those that

have the DAT1 10/10 repeat, with an absent response for the other genotypes [$F_{(1)} = 3.017$, $p = 0.094$, partial $\eta^2 = 0.101$]. Uncorrected *post-hoc* testing however failed to yield significant results. A marginally larger effect was noted when comparing patients with and without the DAT1 10/10 genotype (partial $\eta^2 = 0.123$), than when comparing controls with and without the genotype (partial $\eta^2 = 0.101$). This suggests that the three-way interaction effect in our main analysis was driven more by the patients with and without the DAT1 10/10 genotype relationship. As *post-hoc* testing could not adequately distinguish between the various subgroups and given the small sample size, particularly that of the Parkinson's group with the DAT1 10/10 repeat ($n = 5$), these results should be considered exploratory.

The blunted response in the control group which did not have the DAT1 10/10 repeat can still be expected for this age group. Results remained unchanged, however, when the age covariate was removed. A sample using a wider age range would be needed to adequately address this question.

Exploratory Whole Brain Voxel Wise Analysis

As we had a specific hypothesis, directed at specific brain regions we did not focus on whole brain analyses. Whole brain exploratory analysis did not yield any additional information.

DISCUSSION

We investigated the potential genetic underpinnings of reward processing abnormalities seen in PD compared to healthy controls. As predicted, we found evidence for poorer reward outcome-based reactivity in the orbitofrontal cortex for patients with the DAT1 10/10 genotype compared to those with other genotypes. Contrary to our hypothesis, we found no such differences in the ventral striatum during reward anticipation. To our knowledge this is the first-time reward-related functional genotypes have been investigated in PD.

Our finding that DAT1 10/10 carriers had abnormal reward outcome related activity in the orbitofrontal cortex could reflect an increased vulnerability for PD-related dopaminergic cell loss in this region. Indeed, it has been shown that the DAT1 10/10 genotype is associated with lower synaptic dopamine availability due to possible increased levels of dopamine transporters (10). Not all studies reported this however, with some suggesting the opposite (13). Our results corroborate a potential hypo-dopaminergic state in the DAT1 10/10 group, as BOLD fMRI activity has been found to correlate with dopamine reactivity in this region (14). However, further exploration of this link with more direct methods such as the use of positron emission tomography (PET) would be needed to confirm this. Although the DAT1 10/10 genotype is one of the more common functional variants reported in the literature, it is possible that there are other unexamined variants that are stronger predictors of dopaminergic hypofunction. Our results confirm that DAT1 10/10 is a potential predictor of reward function variability in diseased states. As our present sample only includes patients on treatment, we cannot account for potential treatment effects. It could be that PD medication has an impact on the normal dopaminergic tone of the ventral tegmental area, which could also have a differential impact across genotypes. As our patients were not assessed while medication free and dopamine activity was not directly measured, we cannot comment on treatment effects. Further treatment effect studies are advised. Interestingly, although Parkinson's patients without the 10/10 DAT1 repeat showed a normal increase in reward outcome related activity, controls without the 10/10 repeat showed a relatively flattened out response. The absence of a response in the controls could be age related, as a similar flattened out response has been observed in similarly aged healthy controls in previous studies (3, 18). The relative increase in reward outcome related activity Parkinson's patients without the DAT 1 10/10 repeat could possibly reflect treatment effects. Again, future studies investigating medication and genotype interaction effects in larger number of patients and controls are needed to substantiate this finding.

Contrary to our previous findings, we did not find any reward anticipatory related activity in the ventral striatum in this particular sub-sample, nor any effect of genotype in this region for this subgroup. The absence of a reward anticipation effect could be explained by poor data quality or poor task comprehension. This was unlikely to be the case in our study as our groups also showed low levels of motion and did not differ on important measures of motion. All groups also demonstrated good task comprehension, as they increased their response times appropriately during rewarded trials. Another potential explanation is the relative older age of the current subsample, which could explain the general lack of signal for this region. Indeed, it has been found that ventral striatal but not orbito-frontal activity *per se*, decreased with normal aging (18).

Although our findings do indeed substantiate our hypothesis that reward related functioning is related, at least in part, to DAT1 genotype, our sample is small, and therefore these findings should be considered as exploratory. Although we found significant differences in the orbitofrontal regions, we cannot completely rule out similar findings for the ventral striatum due to our limited sample size. Furthermore, although our study supports functional associations with the commonly occurring *DAT1* genotype, this does not necessarily mean that it is the only or even the best predictor of reward related functioning. Future, larger studies should also explore other dopamine-related genes such as catechol-O-methyltransferase (*COMT*) (6).

Despite these limitations, our study has important implications. Reward related function loss, and PD non-motor symptoms by extension, could be exacerbated in certain vulnerable genotypes. This should be considered in future studies of genetic vulnerability and treatment in PD. Genetic risk factors could potentially play an important role in the non-motor symptoms of PD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Research Ethics Committee (HREC N13/08/115) of Stellenbosch University, Tygerberg Hospital, Cape Town, South Africa. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP: study design, fMRI task administration supervision, data processing, analysis, interpretation, and lead author on manuscript preparation. MB: genetic samples analysis, interpretation, and manuscript preparation. CB and MV: fMRI data processing, analysis, and interpretation. SS, SB, and JC: study design, interpretation, and manuscript preparation. SA: genetic sample analysis and interpretation. All authors contributed to the article and approved the submitted version.

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Patient-Derived Midbrain Organoids to Explore the Molecular Basis of Parkinson's Disease

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Induced pluripotent stem cell-derived organoids offer an unprecedented access to complex human tissues that recapitulate features of architecture, composition and function of *in vivo* organs. In the context of Parkinson's Disease (PD), human midbrain organoids (hMO) are of significant interest, as they generate dopaminergic neurons expressing markers of *Substantia Nigra* identity, which are the most vulnerable to degeneration. Combined with genome editing approaches, hMO may thus constitute a valuable tool to dissect the genetic makeup of PD by revealing the effects of risk variants on pathological mechanisms in a representative cellular environment. Furthermore, the flexibility of organoid co-culture approaches may also enable the study of neuroinflammatory and neurovascular processes, as well as interactions with other brain regions that are also affected over the course of the disease. We here review existing protocols to generate hMO, how they have been used so far to model PD, address challenges inherent to organoid cultures, and discuss applicable strategies to dissect the molecular pathophysiology of the disease. Taken together, the research suggests that this technology represents a promising alternative to 2D *in vitro* models, which could significantly improve our understanding of PD and help accelerate therapeutic developments.

Keywords: Parkinson's disease, IPS (induced pluripotent stem) cell, organoid, midbrain, dopamine, genetics

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PARKINSON'S DISEASE

Pathophysiology

Parkinson's Disease (PD) is the second most frequent neurodegenerative disorder after Alzheimer's Disease. It affects over 10 million people worldwide, with an estimated yearly cost of 52 billion dollars in the United States alone, and an increasing prevalence due to an aging population (1). Although PD has historically been characterized by its motor symptoms (bradykinesia, tremor, and rigidity), the frequent co-occurrence of cognitive and psychiatric symptoms (such as apathy, depression, and executive dysfunction) have led to the revaluation of PD as a quintessential neuropsychiatric disorder (2).

At the cellular level, the central hallmark of PD is the misfolding and aggregation of α-synuclein (α-syn), a protein involved in neurotransmitter release, membrane remodeling and vesicle recycling, into toxic ß-sheet rich fibrillar aggregates (3). While impairments in protein synthesis, folding, and degradation have been extensively linked to α-syn aggregation and toxicity, recent advances have also highlighted the importance of lipid dysregulation in its pathological

mechanisms (4). In most cases, these alterations lead to the accumulation of α-syn aggregates along with mis-trafficked lipid vesicles and organelles into inclusions termed Lewy Bodies (LB) and neurites (5, 6), which are considered to be the main histological manifestation of PD. While it remains unclear whether LB themselves play a harmful, protective or even "neutral bystander" role in PD (7), α-syn-associated pathology eventually induces the degeneration of vulnerable cells through altered mitochondrial, proteasomal, and autophagy-lysosomal pathways (6, 8).

This vulnerability has been linked to several cellular characteristics: elaborate neuronal arborization with many vesicular release sites (enriched in α-syn), elevated intracellular calcium concentrations due to autonomous pacemaker activity, and higher basal levels of mitochondrial oxidative stress (9). These characteristics are all found in the dopaminergic (DA) neurons of the *substantia nigra* (SN), which are the most affected neuronal type in PD. Their progressive degeneration leads to a massive loss of DA release within cortico-basal ganglia networks, and the emergence of both motor and psychiatric symptoms of PD (10). The symptomatology is further broadened by alterations of other neuronal types throughout the course of the disease, although to a lesser extent (11). These include other neuromodulator-producing neurons [cholinergic (12), noradrenergic (13), and possibly serotonergic (14, 15)], enteric neurons (16), as well as cortical neurons, in which comorbid Alzheimer's Disease pathology can arise in later stages (17, 18). Additionally, microglia and astrocyte-mediated neuroinflammatory processes are also known to contribute to neurodegeneration and the progression of synucleinopathy (19, 20).

PD is also a highly heterogeneous disease, as patients can present significant differences for example regarding age of onset (21), adherence to Braak staging (9), alteration in neurotransmitter systems (22–27) and symptom presentation (9, 21, 28, 29). This heterogeneity thus suggests that the etiology of PD may involve a diversity of molecular and cellular mechanisms, which remain to be fully identified.

Molecular Basis

Much of our understanding of the pathological mechanisms of PD come from the study of relatively rare, high risk/monogenic forms of the disease. To this day, 19 disease-causing genes have been identified, amongst which 10 are autosomal dominant (including mutations in *SNCA*, which encodes α-syn, and in the *Leucine Rich Repeat Kinase 2* / *LRRK2* gene), and 9 autosomal recessive (including *PRKN*, *PINK1*) (30). PD cases due to mutations in those genes however only represent 5–10% of all cases. Interestingly, the G2019S LRRK2 mutation has a variable penetrance, as it can lead to both sporadic and familial PD (31).

The most recent GWAS meta-analysis to date has identified 90 common genetic variants with medium to low effect sizes that were associated with PD (32). This study also found that the expression of candidate genes was exclusively enriched in neuronal cell types (with the strongest enrichment residing in SN DA neurons, followed by pallidal, thalamic, and cortical neurons), a striking contrast with recent reports on the genetic architecture of Alzheimer's Disease which heavily implicated peripheral and CNS glial cell types (33) (blood, spleen, lung, and microglia). Gene ontology analyses also revealed enrichment for pathways referring to cellular stress responses and suggest a potential implication of neuro-inflammatory mechanisms. Interestingly, no significant association with other neuromodulator-producing neurons (serotonergic, noradrenergic, cholinergic) was revealed in these analyses, thus highlighting the centrality of DA and DA-associated networks in PD pathophysiology. This result may nevertheless be due to the fact that this study did not account for PD subtypes (21), which may be associated with alterations in different neurotransmitter systems (22–27). In this regard, future studies integrating large cohort GWAS data with patient stratification strategies may help identify molecular mechanisms driving PD heterogeneity.

Amongst the most highly significant and best characterized risk variants are those in the *beta-glucocerebrosidase* (*GBA*) gene. Such variants seem to impair lysosomal function and can lead to an increase in PD risk between 2- and 19-fold, and are associated with a more severe clinical profile regarding symptomatology and progression rate (21, 30). Interestingly, the presence of multiple risk variants in a single patient (referred to as the "polygenic load"), has also been shown to influence age of disease onset, but not the rate of progression (34).

Recent population studies have yielded PD heritability rates ranging between 0.22 and 0.27 (32, 35), suggesting that a majority of cases may be due to the interaction of genetic and environmental factors [rural living and pesticide exposure are well-known risk factors, while tobacco, coffee, and moderate alcohol consumption may be protective, see review (36)], and to stochastic processes. Mosaicism may for instance be a non-negligible contributor to the pathogenesis of sporadic PD (37), as changes in copy numbers of the *SCNA* gene have been observed in patient SN DA neurons (38). Nevertheless, only a minor fraction of the disease's heritability (16–36% depending on its prevalence) can be explained by the most recently identified risk loci (32), indicating that much of the "missing heritability" remains yet to be uncovered. This may be partly achieved through better understanding of epistatic interactions and the functional annotation of the non-coding genome, in which a majority of the single nucleotide polymorphisms (SNPs) fall. Indeed, like many other complex polygenic human diseases, the etiology of sporadic PD is likely attributable to the interactive effects of a high numbers of variants on the regulation of large-scale genetic networks (39). A growing body of research is for example revealing how non-coding variants affecting long range enhancer/promoter interactions or non-coding RNA may be involved in PD pathophysiology (40–43). However, as non-coding sequences tend to be less conserved between species, appropriate human models of the disease are thus required to expand our understanding of the molecular basis of PD.

MODELING PD *IN VITRO*

Reproducing Midbrain Development *in vitro*

Given the importance of DA degeneration in PD, human induced pluripotent stem cell (hiPSC)-derived DA cultures constitute

highly relevant biological models to study the associated molecular mechanisms *in vitro*.

Midbrain DA (mDA) neurons are found in 3 separate nuclei: the *Substantia Nigra pars compacta* (SN, forming the A9 group), Ventral Tegmental Area (VTA, A10 group,) and Retrorubral Field (RRF, A8 group). A9 mDA neurons, which primarily project to sensorimotor and associative striatal areas (putamen and caudate nucleus), as well as some cortical areas, are particularly vulnerable to neurodegeneration in PD (10, 11).

In order to generate mDA neurons *in vitro*, several protocols have been established based on our understanding of midbrain development [see in depth reviews (44, 45)]. To summarize, stem cells are initially directed toward a neuroectodermal fate through TGFβ/activin/nodal and BMP pathways inhibition [referred to as dual SMAD inhibition (46)], using different combinations of molecules. SHH, WNT, and FGF8 signaling are then typically modulated in order to specify midbrain floor plate identity, from which mDA progenitors arise. Cells are then differentiated and matured through the use of neurotrophic factors such as brain and glial-derived neurotrophic factors (BDNF, GDNF) and Ascorbic Acid, a commonly used antioxidant. Correct specification should induce the expression of transcription factor (TF) FOXA1/2 in mDA progenitor cells, which in turn regulates the expression of LIM homeobox TFs LMX1A and LMX1B. These TFs are required for the specification and differentiation of mDA neurons, notably by up-regulating *NURR1*, *PITX3*, and *Tyrosine Hydroxylase* (*TH*), which together constitute essential markers of mDA neuron identity. The differentiation and survival of mDA neurons is then regulated by EN1/2 homeobox genes, which remain expressed in adult neurons (44). It is worth noting that these differentiations protocols do not generate SN-like mDA neurons specifically, but rather a diversity of mDA subtypes (47), out of which some neurons express markers of A9 or A10 identity.

hiPSC-Derived Models of PD in 2D

The development of these protocols triggered a wave of characterization studies aiming at identifying altered phenotypes of 2D mDA cultures derived from patient hiPSCs carrying monogenic (*PAKR2*, *PINK1, LRRK2, SNCA, GBA,* and *OPA1*) or sporadic forms of the disease. These phenotypic effects have been well-described in the literature, both at the cellular and molecular levels [see reviews (48–51)]. To summarize, several converging pathological mechanisms that contribute to the vulnerability of human mDA neurons were reproduced *in vitro*, including reductions in neuronal arborization, increases in α-syn expression, oxidative stress, and mitochondrial dysfunctions (decreased respiration and ATP production, impaired mitochondrial biogenesis), as well as altered cellular stress responses [such as the unfolded protein and integrated stress responses, which involve the endoplasmic reticulum (52–54)].

While these experiments helped validate hiPSC-derived mDA neurons as human cellular models of PD and achieve a better understanding of the cellular and molecular dysfunctions involved, only a few studies have however reported mDA degeneration (55, 56). Not surprisingly, the weeks-long differentiation of these DA neurons (up to 3 months) raises the limitations of these 2D cultures relatively to human development, in particular regarding neuronal maturity and the establishment of synaptic connections to other cell types. This has partially been taken into account using microfluidic devices that recreate direct contacts between mDA neurons and striatal medium spiny neurons (57), or using co-cultures with astrocytes (58). These approaches however do not allow the development of mDA neurons concomitantly with other cell types as it happens *in vivo*, which contributes to DA maturity and may be involved in PD mechanisms.

Developing 3D Midbrain Organoids

In this context, the rise of human stem-cell derived brain 3D organoid cultures, which recapitulate features of the brain's composition, organization, and function (59), has led to significant advances in our understanding of neurodevelopment and in disease modeling. Although midbrain and mDA markers have been found to spontaneously arise in non-directed whole brain organoids (60), the proportions of cells expressing such markers tends to be small and highly variable, thus warranting the development of more directed differentiation protocols. While some approaches have led to the development of "neurospheres," which contain an increased proportion of DA neurons (along with excitatory, inhibitory neurons as well as glial cells) (61), most efforts have been directed at specifically reproducing mesencephalic development in the dish, in order to generate mDA neurons in representative human "midbrain organoid" (hMO) structures.

Tieng et al. (62) were the first to adapt a widely-used 2D differentiation protocol (63) to 3D suspension through the use of microwells to create homogeneously sized embryonic bodies, which were then placed on an orbital shaker for 3 weeks, before being seeded and grown at air-liquid interface. Although the suspension-culture phase of their protocol was short, they proved that such an approach could efficiently generate mDA progenitor cells (∼80% of all cells expressed FOXA2 and LMX1A) as well as TH-expressing cells after only 3 weeks. Following these results, 3 new protocols were published within 1 year (64–66), describing the generation and long term maintenance of hMO (up to 5 months). These papers were the first to provide in depth characterization of the model, and proof that these organoids could be maintained in long term cultures in order to favor neuronal maturation. Although each protocol presents differences in timing, specific molecules used and their concentrations, these approaches mainly rely either on the sequential (65) or simultaneous (62, 64, 66) use of morphogens to induce midbrain floor plate identity, as described earlier (see **Figure 1** for graphical summary). In order to promote nutrient and oxygen diffusion throughout the hMO, all of these initial protocols relied on the use of orbital shakers, as well as hydrogel

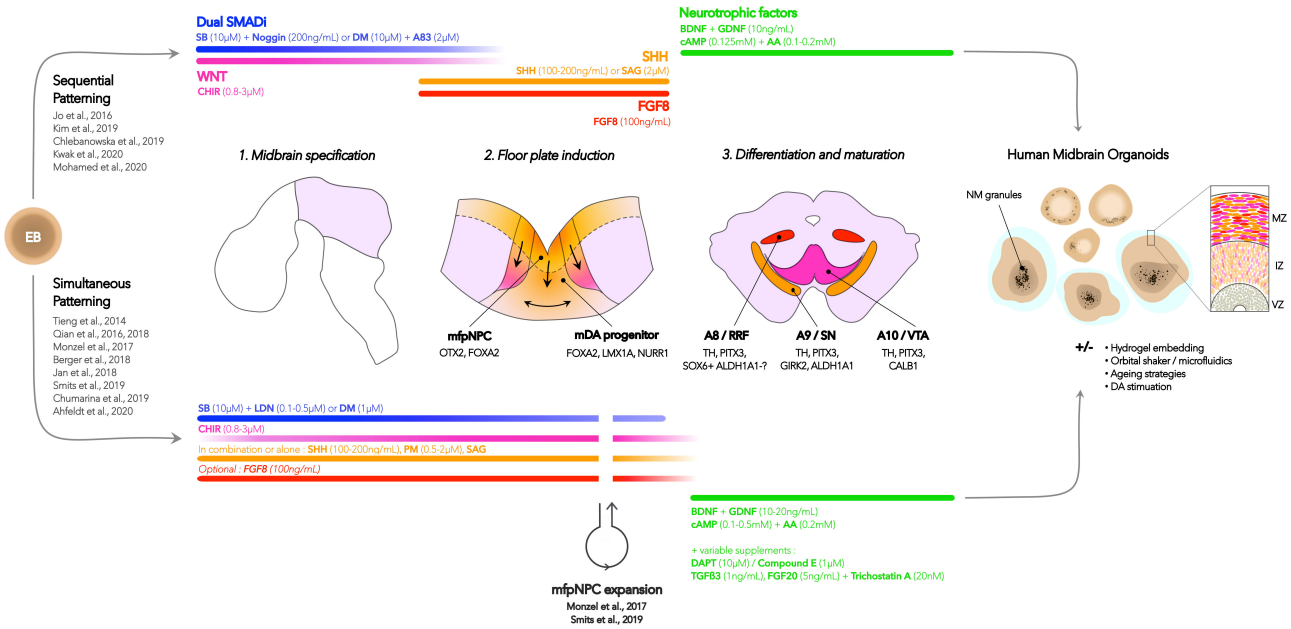

FIGURE 1 | Summarized hMO differentiation strategies. hMO differentiation protocols aim at reproducing essential dynamics of *in vivo* human midbrain development, which are represented by the drawings in the middle section: (1) midbrain specification using dual SMAD inhibition and WNT modulation; (2) midbrain floor plate (mfp) induction through modulation of SHH and FGF8, and (3) differentiation and maturation of midbrain dopaminergic neurons using neurotrophic factors (see **Table 1** for details in hMO models of PD). Each step is associated with the generation of cell types that can be identified *in vivo* and in hMO models using the mentioned markers. Starting from hiPSC-derived embryonic bodies (EB), the described protocols have either relied on the use of sequential or simultaneous patterning strategies, represented as the top and bottom branches, respectively. Optional modifications to the protocol include expansion of mfpNeural Progenitor Cells (mfpNPC), hydrogel embedding, use of orbital shakers or microfluidics devices, aging strategies, and DA treatment. The generated hMO typically contain mDA neurons expressing markers of A9 and A10 terminal differentiation, and long-term cultures may favor the apparition of neuromelanin granules, which can be enhanced through DA stimulation. Features of floor plate organization in ventricle, intermediate, and mantle zones (VS, IZ, MZ) may also be revealed using markers of mDA progenitors (65). This organization is particularly evident in hydrogel-embedded organoids, as this process favors apico-basal polarization. SB, SB431542; DM, dorsomorphin; CHIR, CHIR99021; A83, A-83-01; SHH, sonic hedgehog; SAG, smoothened agonist; FGF8: fibroblast derived growth factor 8; BDNF/GDNF, brain/glial-derived neurotrophic factor; AA, ascorbic acid; cAMP, cyclic AMP; LDN, LDN193189; PM, purmorphamine; TGFß3, Transforming growth factor beta 3; FGF20, fibroblast derived growth factor 20.

embedding in some cases to promote apico-basal orientation and cellular proliferation.

These organoids developed features of organization similar to the midbrain floor plate, namely a ventricular zone containing OTX2+ FOXA2+ cells, as well as intermediate (LMX1A+ NURR1+) and mantle layers containing progressively maturing neurons (MAP2+ TH+). Several markers of pan-mDA neuronal identity have been consistently observed in hMO, including the dopamine transporter (*SLC6A3* / DAT), DOPA decarboxylase enzyme DDC, and TF PITX3 (65, 67). While each study tried to estimate the proportions of mDA neurons in the hMO, differences in the methodologies and protocols used have led to variable results. For instance, by using FACS approaches, Jo et al. (65) found that at 2 months of differentiation, 22% of all cells were MAP2+ TH+, while Monzel et al. (66) found at the same timepoint a much higher yield of cells expressing essential markers of mDA identity: 61% were TH+FOXA2+LMX1A+. Nevertheless, both studies found that neuromelanin (NM) granules spontaneously appeared in long term cultures, their structures resembling those found in adult human SN tissue. Exogenous DA treatment could also significantly increase the accumulation of NM, suggesting that these granules may indeed be by-products of DA metabolism (65). While the authors did not try to dissect the diversity of mDA subtypes generated, which is in of itself a complex endeavor *in vivo* [see review (68)], NM-containing cells were indeed found to be enriched in transcripts expressed in A9 SN mDA neurons such as *KCNJ6 (GIRK2)* and *ALDH1A1* (47, 65, 69). Interestingly, ALDH1A1 may be particularly implicated in mDA neuron vulnerability to degeneration in PD (70). Some neurons were also found to be positive for CALB1, a marker of A10 VTA identity (65, 66). No study has however aimed at identifying A8 RRF-like neurons, likely due to the fact that they do not have a clear molecular signature (68). These organoids were also found to produce DA, and mDA neurons showed characteristic electrophysiological pacemaker activity which was responsive to the use of D2/D3 agonist quinpirole. Beyond mDA neurons and their progenitors, excitatory and inhibitory neurons (62, 65) were found in these hMO, as well as astrocytes and myelinating oligodendrocytes, consistent with the composition of the midbrain (65, 66).

hMO hold a number of advantages over their 2D counterparts. RNA sequencing of hMO for instance showed that their transcriptomic profile was closer to that of prenatal midbrain samples compared to 2D cultures (63), with higher expression of mDA markers such as *ALDH1A1* and *KCNJ6 (GIRK2)*, as well as glial markers *OLIG3* and *SLC1A3 (EAAT1/GLAST)*. Interestingly, markers of non-dopaminergic catecholaminergic neurons such as *DBH* and *SLC6A2 (NET)*, which frequently arise in 2D cultures, were also found to be significantly decreased in hMO, thus highlighting the importance of the 3D environment for proper mDA specification. Tieng and colleagues (62) also showed that mDA neurons derived from 3D cultures expressed higher levels of *TH* and presented varicose-like neurites reminiscent of A9 neuronal morphology, which had not previously been observed in 2D cultures. Furthermore, the spontaneous or dopamine-induced apparition of neuromelanin granules (65–67) is a remarkable feature as it has rarely been found in 2D cultures (55, 71).

More recent evolutions of these protocols have confirmed the initial observations, as well as aimed at increasing the quality and reproducibility of hMO (67, 72–76) and better estimating their yield of mDA neurons. For instance, using a high content image analysis approach, Smits et al. (73) showed that TH+ cells composed 62% of all cells after 1 month of differentiation, while Ahfeldt et al. (74) found using a knock-in TH:tdtomato line, that TH+ cells composed ∼38% of total cells at a similar timepoint. Such differences are likely to arise from cell line effects as well as protocol variations. Kwak et al. (67) recently aimed at establishing ideal conditions to maximize mDA neuron generation in hMO, by testing out different combinations of molecules for SMAD inhibition and modulating WNT signaling. These modifications allowed them to approximately double their yield of TH+ cells compared to commonly used molecule combinations (86% TH+ cells by day 28), and to efficiently suppress cortical marker expression. By 4 months of culture, their hMO were also producing higher concentrations of DA than previously reported. Taken together, these findings support the relevance of hMO cultures to obtain mDA neurons expressing markers of terminal differentiation (such as NM production) in a 3D environment that reproduces the neuronal and glial composition of the human midbrain.

Midbrain Organoid Models of PD

The first two in-depth reports of PD modeling in hMO focused on the effects of the *LRRK2* G2019S mutation, which has been associated with both sporadic and familial forms of the disease due to its variable penetrance (31), and which constitutes the most common genetic risk factor for PD. To do so, the researchers relied on Crispr-Cas9 gene editing to either introduce the mutation in a control hiPSC line (77), or to combine this with a correction in a mutant patient line (73). Smits et al. (73) found that while the number of mDA progenitors (FOXA2+TH- cells) was significantly increased after 1 month of differentiation in *LRRK2* vs. control hMO, an apparent impairment of differentiation led to a reduction in the number and complexity of mDA neurons (FOXA2+TH+) after longer periods of culture (day 70). Interestingly, the increase in the number of progenitors was significantly higher in *LRRK2* PD hMO compared to those from controls with the knock-in mutation. This result thus highlights the importance of the genetic background in the penetrance of the *LRRK2* G2019S variant (31). In line with these findings, Kim et al. (77) observed that while *LRRK2* G2019S hMO were no different in size compared to controls, mDA neurite length and expression of mDA identity markers were decreased (such as *TH, DAT, NURR1, PITX3, EN1*) by day 60. The *LRKK2* hMO also contained higher levels of phosphorylated α-syn in endosomal compartments, and higher expression levels of markers of mitophagy and autophagy. The authors also identified TXNIP [a thiol-oxidoreductase that induces lysosomal dysfunction and DA cell death when overexpressed (78)] as an important mediator of *LRRK2*-G2019S pathological mechanisms, and proved that knocking-down its expression reversed the accumulation of phosphorylated α-syn.

More recently, an extensive report from Ahfeldt et al. (74) used hMO to study the roles of 3 severe PD-associated mutations (in *PRKN/PARK2*, *DJ1/PARK7*, and *ATP13A2/PARK9*) through genomic editing of a healthy control hiPSC line. RNAseq analyses of TH+ cells after 1 month of differentiation found that *PRKN*–/– mDA neurons showed the highest amount of differentially expressed genes (1641) compared to controls. While proteomics analyses revealed a dysregulation of the autophagy-lysosomal pathway in all cell lines, the *PRKN*–/– mDA neurons also showed an upregulation of pathways associated with oxidative phosphorylation, mitochondrial dysfunction, and Sirtuin signaling, as well as a significant depletion of mitochondrial proteins. Supporting these results, they found a significantly higher level of mitochondrial reactive oxygen species (ROS) in TH+ cells from $PRKN^{-/-}$ hMO compared to their TH- counterparts and to control cells (both TH+ and TH–). Furthermore, while the mDA neuronal population was significant reduced in $PRKN^{-/-}$ organoids (from 40 to 17% of all cells), there were no significant differences in the other two cell lines. The authors showed that this deficit was not due to an impairment in mDA generation, but rather to the death of newly differentiated TH+ neurons, which could be linked to a 3-fold increase in SNCA protein expression in these hMO. Interestingly, the expression of VTA marker CALB1 was 4x higher in the $PRKN^{-/-}$ hMO, suggesting that A9-like neurons may have been more severely affected by the early neuronal death, thus leading to a bias in subtype generation. It is however not known if other mutations would have provoked a similar phenotype at later timepoints, although mDA neurons in $DJ1^{-/-}$ and $ATP13A2^{-/-}$ hMO also tended to show increases in mitochondrial ROS.

Reports of decreased mDA identity and impairment of mitochondrial function were supported by two additional studies which partly relied on hMO. For instance, *SNCA* A53T-mutated hMO recapitulated the increased expression of *eEF2K* mRNA found in *post-mortem* patient SN (79). eEF2K, also known as Calmodulin-dependent protein Kinase III (CamKIII), is a crucial regulator of protein synthesis and synaptic plasticity, and is involved in a-syn mediated mitochondrial toxicity (79).

Mutations in *PINK1*, which encodes a mitochondrial kinase, have also been linked to reduced TH+ counts in hMO (80). Taken together, these studies suggest that hMO constitute a valid translational model to investigate the effects of different PD-associated mutations, as they reproduce elements of cellular pathology involving oxidative stress found in *post-mortem* tissue (81) (see **Table 1** for summary).

Interestingly, a recent study focusing on a novel variation in the *POLG1* gene (Q811R), previously linked to progressive external ophthalmoplegia and parkinsonism (75), found significant increases in hMO TH+ cells after 100 days of culture compared to those from a gender-matched control. This study also reported an increased production of NM in response to DA treatment, which may have neurotoxic effects in the long run. Although no deficits in mitochondrial respiration were observed, metabolic and proteomics data indicated an increased level of glycolysis, which was specific to neurons. The striking differences from previously mentioned results (reduced mDA neuron counts and impaired mitochondrial respiration) indicate that *POLG1*-related PD may thus entail different pathological mechanisms. However, as isogenic lines were not used as controls in this study, the experiments should be replicated to confirm these findings.

hMO may also be of use to study sporadic forms of PD, including the effects of PD-associated environmental stressors. So far, only one study has aimed at deriving hMO from patients with sporadic PD (82). The authors found, in line with previous articles, a decrease in *TH* expression after 1 month of culture of hMO derived from 2 sporadic patients, compared to those from 2 healthy controls. This effect might have been linked to early decreases in *FOXA2* and *LMX1A* expression. They however also measured an increase in the expression of *PTX3*, which encodes a protein (Pentraxin 3) involved in neuroinflammatory responses that is increased in the plasma of PD patients (84). Nevertheless, as hiPSC-based studies of sporadic diseases are hard to control for, additional studies with increased statistical power are needed to further explore sporadic PD mechanisms in hMO. Sporadic PD dynamics may also be probed through exposure to mitochondrial stressors such a rotenone and MPTP, which have been shown to preferentially affect mDA neurons in hMO and related cultures (67, 77, 85).

As discussed earlier, the 3D nature of hMO favors better modeling of the *in vivo* midbrain over 2D cultures, and may by extension provide a better translational value when studying neurodegenerative disorders such as PD. A study for instance showed that in plated cultures of *LRRK2* G2019S mDA neurons, most of the PD phenotype (such as a reduction of the number and arborisation complexity of TH+ cells, impaired mitochondrial function and increased apoptosis) appeared only when Matrigel was used to recreate a 3D environment (86). Similarly, when comparing the transcriptome of hMO to 2D cultures of *LRRK2* G2019S-derived mDA neurons, Kim et al. (77) found that the genes differentially expressed in hMO were enriched for transcripts found in *post-mortem* PD tissue. In support of this finding, the expression of *TXNIP*, which they proved to be central to pathophysiological mechanism in *LRRK2* G2019S, showed 4-fold higher expression in hMO compared to 2D cultures. Altogether, these studies indicate that 3D hMO cultures may constitute a significant improvement over 2D cultures as *in vitro* platforms to model PD.

FUTURE TECHNOLOGICAL CHALLENGES

Challenges Inherent to Organoid Culture

It is somewhat surprising that several essential features of PD pathophysiology can be modeled in relatively young stem-cell derived structures, which may conceptually be better suited to study pathologies with clearly recognized neurodevelopmental components such as autism spectrum disorder (ASD), schizophrenia, lissencephaly, and many others (87). Furthermore, the reprogramming of differentiated patient cells to iPSC-states is known to have a "rejuvenating" effect by erasing many crucial aging-related epigenetic marks (88). Brain organoids have however also proven to be able to reproduce strong aging-related cellular phenotypes of Alzheimer's Disease (AD) (89–91). As tracking the earliest stages of PD or AD is an inherently difficult task, these results thus support the possibility that important neurodevelopmental aspects of such diseases may have been overlooked [see reviews (92, 93)].

However, another complementary possibility is that these severe phenotypes may partly be a by-product of organoid culture limitations. Indeed, although 3D organoid models constitute significant advances compared to their 2D counterparts, their density and size restrain the proper diffusion of oxygen and nutrients to all cells, leading to a well-known necrotic core. Brain organoids are also characterized by an upregulated reliance on glycolysis and high levels of ER stress, which may impair neuronal differentiation and promote mitochondrial stress (94), an aggravating factor in the context of neurodegeneration. Furthermore, while glial cells play an essential role of clearance in disorders such as PD and AD [see reviews (95, 96)], gliogenesis mainly happens in later stages of organoid culture (after 6 months in forebrain organoids) (97), and typically does not include microglial cells, unless differentiation protocols favor their apparition (98). In this sense, the stressful culture conditions and incomplete glial support may trigger and/or speed up pathophysiological cascades primed by genetic risk variants in PD hMO, and lead to the early apparition of severe neurodegeneration-related phenotypes.

Reducing *in vitro* Culture Artifacts

An essential endeavor to answer these questions will be to develop strategies to reduce culture-related artifacts, and to modulate cellular maturation in order to study early and later stage neurons and glia. Several studies have already started addressing these issues. For instance, it is now clear that transplantation inside rodent brains can effectively vascularise the organoids, correct artifacts linked to *in vitro* culture and significantly enhance neuronal maturation (94, 99, 100). These improvements nevertheless come at the expense of uncontrolled interactions between the host and grafted tissue, and synaptic integration into the host brain (100). It is however for now not known what effects such

TABLE 1 | Summary of studies using hMO to model PD.

References	Genetic risk variants	Cellular Stressors	Protocol						%TH+ cells (controls)	PD-related phenotype	Therapeutic approaches
			SMADi	WNT	SHH	FGF8	Maturation	Scaffolding, agitation			
Jan et al. (79)	***SNCA*** A53T	/	DM (1 μM) SB (10 μM)	CHIR (3 μM)	PM (0.5–0.75 μM)	/	BDNF, GDNF (10 ng/mL) cAMP (500 μM) AA (200 μM) TGFß3 (1 ng/mL)	Matrigel + orbital shaker	/	↑ *eEF2K* mRNA, linked to mitochondrial stress	/
Kim et al. (77)	***LRRK2*** G2019S	AO removal, MPTP (200–500 μM)	*Unclear*	CHIR (3 μM)	SHH (100 ng/mL)	FGF8 (100 ng/mL)	BDNF, GDNF (20 ng/mL) AA (200 μM) *until day 45*	Matrigel + orbital shaker	60% (Day 60)	↓ mDA neuron mRNA markers, neurite length ↑ α-syn, mitophagy & autophagy markers, MPTP sensitivity • Description of an "aging" strategy (–AO) for hMO • Identification of *TXNIP* as a mediator of LRRK2 pathology • Therapeutic strategies rescue elements of phenotype	LRRK inhibition & *TXNIP* knock-down
Smits et al. (73)	***LRRK2*** G2019S	/	LDN (250 nM) SB (10 μM)	CHIR (3–0.7 μM)	SAG (0.5 μM)	/	BDNF, GDNF (10 ng/mL) cAMP (500 μM) AA (200 μM) TGFß3 (1 ng/mL) DAPT (10 μM)	/	54% (Day 70)	↑ mDA progenitor cells ↓ Number and complexity of mDA neuons • Implication of genetic background	/
Chumarina et al. (75)	***POLG1*** Q11R	/	LDN (100 nM) SB (10 μM)	CHIR (0.8 μM)	SAG (1–2 μM) SHH (200 ng/mL)	FGF8 (100 ng/mL)	BDNF, GDNF (10 ng/mL) cAMP (500 μM) AA (200 μM) TGFß3 (1 ng/mL) *DA (50 μM) start. day 30*	/	40% (Day 100)	↑ Number of mDA and DA-induced NM accumulation ↑ Neuronal reliance on glycolysis • No alterations in mitochondrial function	/
Ahfeldt et al. (74)	***PRKN***$^{-/-}$ ***DJ1***$^{-/-}$ ***ATP***$^{-/-}$	/	LDN (100 nM) SB (10 μM)	CHIR (1 μM)	SAG (1 μM) PM (2 μM)	/	BDNF, GDNF (10 ng/mL) cAMP (100 μM) AA (200 μM) DAPT (10 μM)	SpinQ agitation	40% (Day 35)	• Different molecular & cellular phenotypes / mutation • Dysregulation of autophagy-lysosomal pathways in all lines ↑ Mitochondrial stress, SNCA expression, in *PRKN*$^{-/-}$ ↑ early death of mDA neurons in *PRKN*$^{-/-}$ (A9 specific?)	/

(Continued)

TABLE 1 | Continued

References	Genetic risk variants	Cellular Stressors	Protocol						%TH+ cells (controls)	PD-related phenotype	Therapeutic approaches
			SMADi	WNT	SHH	FGF8	Maturation	Scaffolding, agitation			
Kwak et al. (67)	/	MPTP (10–100 μM)	Best: DM (2 μM) A83 (2 μM)	CHIR (Best: 3 μM)	SAG (2 μM)	FGF8 (100 ng/mL)	BDNF, GDNF (10 ng/mL) cAMP (125 μM) AA (200 μM) *DA (50 μM) start. week 8*	Matrigel (+l/L) + orbital shaker	86% of neurons (Day 35)	↑ Vulnerability of mDA neurons to MPTP toxicity	/
Chlebanowska et al. (82)	**Sporadic PD**	/	SB (10 μM) Noggin (200 ng/mL)	CHIR (0.8 μM)	SHH (100 ng/mL)	FGF8 (100 ng/mL)	BDNF, GDNF (10 ng/mL) cAMP (125 μM) AA (100 μM)	Matrigel (+l/L) + orbital shaker	/	↓ *TH* expression ↑ *Pentraxin 3* (*PTX3*) expression	/
Monzel et al. (83)	/	6OHDA (50–500 μM)	DM (1 μM) SB (10 μM)	CHIR (3 μM)	PM (0.5–0.75 μM)	/	BDNF, GDNF (10 ng/mL) cAMP (0.5 mM) AA (200 μM) TGFß3 (1 ng/mL)	Matrigel + orbital shaker	∼50% (Day 42)	↑ Vulnerability of mDA neurons to 6OHDA toxicity	/
Jarazo et al. (80)	***PINK1*** Q456X/I368N ***PRKN*** R275W	/	DM (1 μM) SB (10 μM)	CHIR (3 μM)	PM (0.5–0.75 μM)	/	BDNF, GDNF (10 ng/mL) cAMP (500 μM) AA (200 μM) TGFß3 (1 ng/mL)	Matrigel + orbital shaker	∼45% (Day 30)	↓ Number of mDA neurons in *PINK1* hMO • HP-β-CD treatment increases mDA neuron counts in *PINK1* and *PRKN* hMO	HP-β-CD

DM, dorsomorphin; SB, SB431542; CHIR; CHIR99021; PM, purmorphamine; BDNF/GDNF, brain/glial-derived neurotrophic factor; AA, ascorbic acid; cAMP, cyclic AMP; LDN, LDN193189; SAG, smoothened agonist; l/L, short treatment with insulin (2.5 μL/mL) and laminin (200 ng/mL), mDA neuron, midbrain dopamine neuron; NM, neuromelanin.

transplantations approaches would have on neurodegeneration-related phenotypes in organoid grafts.

Animal-free approaches may however also be of use. Biophysics studies of allometric scaling have for instance highlighted the importance of culture medium, hydrogel composition and microfluidic device uses in the context of 3D cultures (101–105). First of all, the composition of commonly used media for hMO culture should be scrutinized. Indeed, such composition may be partly responsible for the cellular stress and differentiation defects observed in several brain organoid cultures (94), as the abnormally high levels of glucose used in the vast majority of hMO protocols are known to impair the normal metabolic reprogramming of neural progenitor cells to neurons (106) through increased oxidative and ER stress (107). Furthermore, recent reports suggest that using culture medium with more physiological levels of glucose may be more adapted for neuronal maturation and modeling of neurological disorders (108–110). Secondly, the development of synthetic hydrogels as alternatives to animal-derived Matrigel and Geltrex may provide enhanced control and reproducibility of the 3D environment in which organoids grow (105). Thirdly, in order to compensate for diffusion limitations in organoids, two approaches have been described. Cakir et al. (111) for instance showed that cortical organoids genetically engineered to express *hETV2*, which encodes a transcription factor involved in endothelial differentiation, spontaneously formed a vascular-like network *in vitro* which dramatically reduced markers of cell death and hypoxia without the need for transplantation. Alternatively, microfluidic devices may also help increase oxygen and nutrient diffusion throughout the organoids, as evidenced in hMO cultures (112). Finally, electromagnetic stimulation may also be of interest to enhance neuronal differentiation (113, 114), including in hMO (115).

Addressing Variability in Organoid Differentiations

Organoid cultures have also gained notoriety for being highly variable, which can be a major issue for disease modeling and testing therapeutic approaches. This variability can be traced down to several crucial factors: differences due to the heterogenous genetic backgrounds of hiPSC cell lines, variations in hiPSC culture and differentiation protocols used, as well as batch effects. Nevertheless, each of these aspects may be addressed in order to improve the reproducibility of the model.

For instance, regarding the variability imputable to genetic background heterogeneity, several approaches may be adopted. The most straightforward path when studying variants carrying a high risk and penetrance is to generate isogenic controls using Crispr-Cas9 gene editing. Alternatively, in order to study lower-risk variants with reduced penetrance, more elaborate strategies may be necessary, such as relying on hiPSC lines from related donors, or taking into account polygenic risk scores in patient and control selection criteria in order to recreate a continuous variable for risk scores (116), an approach that has seen recent applications in the field of schizophrenia research (117).

Furthermore, the way in which hiPSC cells are cultured in the lab may have an important effect on their ability to generate reproducible organoid structures. Indeed, a recent study from Watanabe et al. (118) revealed that commonly used feeder-free hiPSC culture conditions (compared to fibroblast-supported), reduced their ability to generate reproducible high-quality cortical organoids by altering their pluripotency state. The authors however showed that these defects could be alleviated through the use of TGFß superfamily agonists, which increase the quality of organoid differentiation toward different brain areas as well as reproducibility across cell lines.

Importantly, the variability in brain organoid cultures was initially identified in whole-brain organoids, which rely on very little to no exogenous patterning, and which are very sensitive to cell line and batch effects (60). Several studies have since shown that this variability could be significantly reduced through the use of cytokines to guide and restrict organoid differentiation toward a specific regional fate. While this was initially demonstrated in forebrain organoid protocols (97, 119), a recent study from Nickels and colleagues (76) showed that hMO protocol refinement could also significantly reduce cell line and batch variability. Taken together, these approaches can thus help cut back on multiple sources of variability in hMO generation and improve their translational value for PD modeling and therapeutic discovery.

Aging in a Dish

As aging is the main risk factor for PD (120), understanding its mechanisms and reproducing them *in vitro* may also help build better disease models. At the molecular level, aging is associated with changes affecting the DNA's structure, content (reduced telomere length and mitochondrial copy numbers, increased DNA damage), epigenetic modulation (methylation clocks can reliably predict chronological age), and has identifiable transcriptomic, proteomic and metabolomic signatures [see reviews (121, 122)]. At the cellular level, aging is also characterized by a progressive accumulation of oxidative stress and mitochondrial dysfunction, a global increase in the number of cells baring features of senescence, as well as chronic low-grade inflammation (122). In the context of PD, both molecular (123, 124) and cellular dynamics (9) of aging have been identified as altered.

In this regard, perhaps the most problematic limitation of hMO as model systems for neurodegenerative diseases is that they rely on the use of cellular reprogramming, which has a rejuvenating effect on these molecular and cellular processes (88). Studies of epigenetics, transcriptomics, have for instance shown that organoids reproduce fetal molecular signatures of the human brain (125, 126). While this limitation does not prevent the study of disease-associated molecular aging mechanisms using hiPSC-derived cultures—a recent report showed that retinal organoids derived from Down Syndrome patients had a faster rate of DNA aging compared to controls (127)—it is a major obstacle to study aged states *in vitro*.

In order to bypass this limitation and reproduce aging phenotypes in a dish, several types of approaches have so far been described. Vera et al. showed that

manipulation of telomere length, one of the molecular hallmarks of aging, through telomerase inhibition could induce other markers of aging (increased expression of γH2AX, a common marker of DNA damage, and increased oxidative stress) and accentuate PD phenotypes in hiPSC-derived 2D mDA cultures (128). A previous report from that group also found that a similar effect could be induced through overexpression of Progerin, the abnormal protein responsible for premature aging in Progeria syndrome (55).

Another more indirect alternative is to rely on the induction of cellular states associated with aging, for example through manipulation of oxidative stress *in vitro* with toxins (such as 6-OHDA, MPTP) or through changes in media formulation. For instance, in their 2019 paper, Kim et al. (77) removed antioxidants from the hMO culture medium after 45 days of culture (hMO-AO). While they did not provide a comparison with hMO+AO, they found that after 2 months of differentiation over a third of cells contained NM granules [compared to 7% at 146 days using a similar protocol with AO (65)], two thirds expressed markers of mature A9 neurons such as *GIRK2*, and over 40% expressed high levels of DNA damage (γH2AX+). Transcriptomics analyses also showed that control hMO-AO showed enrichment for "aged" human midbrain transcripts, and genes differentially expressed in *LRRK2*-G2019S hMO-AO were enriched for transcripts found in *post-mortem* PD midbrain tissue, thus supporting the relevance of their aging strategy. In this context, the basal level of ER stress characterizing organoid cultures (94) may also in of itself constitute an indirect aging strategy through alterations of global homeostatic mechanisms, including calcium homeostasis (129).

Finally, direct reprogramming of somatic cells such as fibroblasts into neural lineage cells (iNeurons, or iN) through transgenic expression of transcription factors (such as ASCL1 and NEUROG2), non-coding RNA, or even using small molecule cocktails, may constitute the most elegant way of inducing aging-related processes *in vitro*. Indeed, "aged" iNs preserve multi-level marks (epigenetic, genomic, transcriptomic, and proteomic) of aging and environmental interactions (130). This approach allows iN to maintain phenotypes such as defective mitochondrial function compared to hiPSC-derived neurons (131). While mDA neurons have already been generated using this approach (132), adapting this technology to hMO generation may however prove to be challenging, as published protocols for now rely on the direct conversion to post-mitotic neuronal types.

Enhanced Organoid Designs

The use of hMO can also be expanded by taking advantage of the flexibility of organoid cultures (see **Figure 2A** for graphical summary). For instance, organoids can be completed with non-neuronal lineage cells that do not typically arise during neural organoid differentiations, but which may be of interest for disease modeling. In the context of PD, co-culturing hMO with microglia-like and endothelial cells could for example enable researchers to study neuroinflammatory mechanisms involving glial activation and brain-blood-barrier disruption (133).

Several teams have indeed shown that hiPSC-derived microglia-like cells (iMG) (134–136) as well as immortalized human microglia (137) could efficiently colonize organoids when cultured together. These integrated microglial cells develop extensive ramified branching, and respond to challenges such as physical injury, stimulation with lipopolysaccharides, corticosteroids and amyloid-β-42 (amyloid-β-42) oligomers, as well as infection with Zika and Dengue viruses (135, 137–139). Two studies in particular showcase how iMG-organoid co-cultures may be of use to model neurodegenerative diseases. First of all, Lin et al. (89) showed that iMG carrying an *APOE4* genotype (a high AD risk allele of the *APOE* gene) had an altered morphology and reduced ability to clear extracellular Aβ aggregates in organoid co-cultures compared to their (low-risk) isogenic *APOE3* counterparts. Secondly, a study from Song et al. (139) proved that iMG were sensitive to the regional identity of the brain organoids they integrated (in this study, dorsal vs. ventral forebrain), and that this microenvironment impacted their response to Aβ-42 stimulation. Given that microglia play an important role in PD pathophysiology (96) and are influenced by regional specificities (140, 141), such co-cultures approaches may thus constitute a relevant strategy to study neuroinflammatory interactions.

Furthermore, as alterations in blood-brain barrier (BBB) function contribute to neuroinflammatory processes in PD, assessing the interaction between endothelial cells (ECs), pericytes and hMO may also be of interest. While this may be partly achieved through *in vivo* transplantation in rodents, the fact that the vascularization originates from the host (99, 100) may be a considerable limitation to study pathological cellular interactions. More elaborate strategies can however help overcome this issue. For instance, the transgenic induction of *hETV2* expression in organoids mentioned earlier (111) leads to the formation of a vascular structure reproducing key elements of BBB identity and function *in vitro,* which is sensitive to the disrupting effects of Aβ-42 oligomers. As an alternative, a vascular system can also be initiated *in vitro* by co-culturing organoids with hiPSC- or human umbilical vein-derived ECs, before proceeding to transplantations (142, 143).

Nevertheless, despite mDA neuron neurodegeneration being the central element of PD pathology, there is also evidence of a loss of cholinergic, adrenergic, and potentially serotonergic neurons over the course of the disease, which alters cortical and basal ganglia function and has been linked to several non-motor symptoms (11–15). Moreover, cortical regions can also be affected by Amyloid-ß and Tau pathology, which are associated with PD dementia (17, 18). In this context, using organoids differentiated toward different brain regions can help address the extended PD picture. Newly characterized brainstem organoids are particularly relevant as their composition encompasses midbrain and hindbrain structures, in which arise not only mDA, but also serotoninergic, cholinergic, and noradrenergic neurons (144). Cortical, subpallial, and thalamic organoids have also been well-characterized (145), and may be studied independently

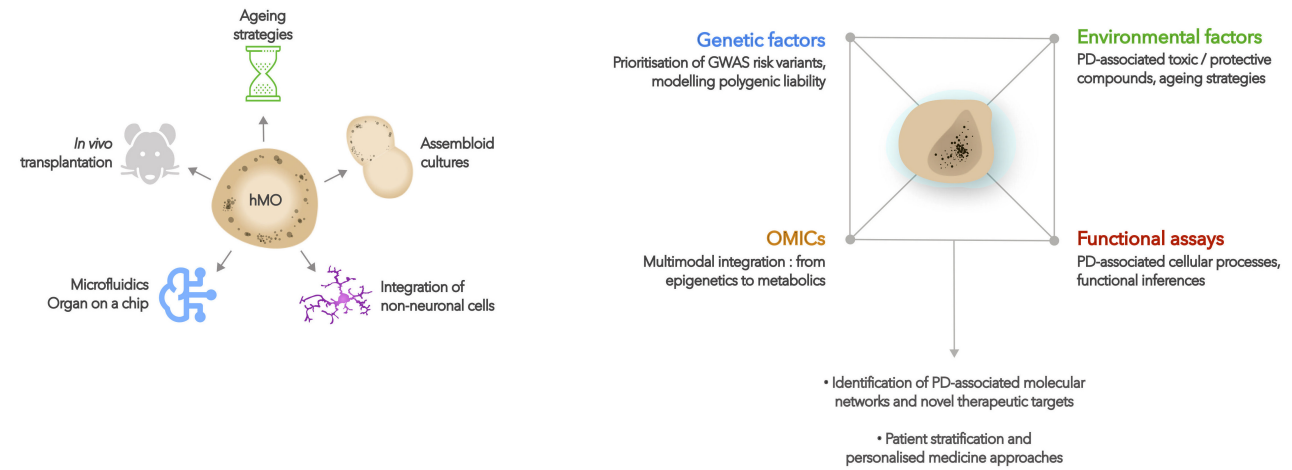

FIGURE 2 | Developments and applications of hMO cultures. **(A)** Potential developments of hMO cultures include aging strategies, fusions with other brain region organoids, co-cultures with non-neuronal lineage cells (such as microglia, endothelial cells), use of microfluidics or "organ on a chip" approaches, and *in vivo* transplantations. **(B)** hMO constitute relevant biological platforms to study the effects of PD-associated genetic and environmental factors on cellular function and molecular networks. Such approaches may lead to a better understanding of the molecular basis of PD, help identify new therapeutic targets, and develop personalized medicine approaches.

or fused with hMO/brainstem organoids to recreate elements of basal ganglia circuitry involved in PD (146–148). Such structures, named "assembloids," could thus be used to study cellular interactions and molecular phenotypes in interconnected structures and to address more complex questions *in vitro*. For instance, what are the effects of genetic and environmental risk factors on different neuromodulator-producing cell types and their connectivity to forebrain structures? Why are striatal cells, despite receiving massive inputs from the SN, seemingly less vulnerable to synucleinopathy compared to cortical neurons (9)?

Finally, beyond the central nervous system (CNS), there is increasing evidence for an important role of the enteric nervous system (ENS) in PD pathophysiology (16), which may be addressed using intestinal or engineered ENS organoids (149). In this regard, a first study comparing the transcriptomic profiles of intestinal and neural organoids derived from *LRRK2* G2019S patient hiPSCs to those from healthy controls reported a wide range of alterations in biological processes and pathways in both models, suggesting that this path should be further explored (150).

Tools to Explore the Molecular Basis of PD Using hMO

While hMO have for now mainly been used to study the effects of high-risk variants on cellular and molecular phenotypes, combining the access to human tissue provided by organoids with GWAS and -OMICs data provides an unbiased approach to further explore the genetic networks, cell types and developmental stages implicated in PD pathophysiology (see **Figure 2B** for graphical summary).

For instance, while GWAS data is often integrated with expression quantitative trait loci (eQTL) and *post-mortem* data to predict candidate risk genes with some tissue specificity, recently developed approaches may help researchers extract additional relevant information. For example, H-MAGMA (Hi-C-coupled MAGMA) can further improve candidate gene identification by incorporating chromatin interaction profiles from human brain tissue across neurodevelopmental stages (151). Cell-type specificity may also be explored more finely by integrating GWAS data with single-cell RNA sequencing (scRNAseq) datasets from the target tissue. Such an approach recently allowed Bryois et al. (152) to reveal a significant association of PD with cholinergic, monoaminergic, and enteric neurons as well-oligodendrocytes using scRNAseq data from a whole CNS. Although the main findings of this study were replicated in *post-mortem* human tissue, their identification approach relied on the analysis of protein-coding genes expressed in the CNS of adolescent mice. In this context, hMO scRNAseq datasets (153) may thus constitute more relevant tools to explore the cell types (and subtypes) involved in PD pathophysiology. Single-cell approaches may also constitute an ideal readout to assess the molecular effects of somatic mosaicism (such as *SNCA* CNVs), which can be induced in organoids through the use of transfection and mixing approaches (154).

Furthermore, considering that PD SNPs mainly fall into non-coding regions of the genome (32), combining readout modalities such as RNAseq, ChIP-seq, ATAC-seq, and proteomics can help dissect complex molecular networks by including non-coding elements and epigenetic modifications. For example, Inoue et al. (155) used a combination of multiple modalities including lentivirus-based massively parallel reporter assay to identify

key regulatory elements and dynamics involved in the neural induction of embryonic stem cells. They also found a significant enrichment of neurological disorder GWAS variants in regions with H3K27ac histone modifications. A similar approach was also recently applied to forebrain organoid models (126). The authors used a combination of RNA-seq and ATAC-seq to map changes in gene expression, chromatin accessibility, and transcription factor dynamics in purified neuronal and glial lineages over 20 months of differentiation. They then also incorporated GWAS risk gene mapping to identify specific cell types and neurodevelopment stages involved in ASD and schizophrenia. Multimodal -OMICs integration has also proven to be a useful strategy to identify the repertoires of long non-coding RNAs (lncRNA) in mDA neurons (41), in which GWAS SNP mapping identified 8 lncRNA possibly involved in PD pathophysiology. With the development of single-cell approaches [see recent reviews (156, 157)], identification of cell subtypes involved in PD pathophysiology may further increase our understanding of the disease.

CRIPSR-based technology may also be of particular use to explore the molecular networks involved in PD, notably through the use of genetic perturbation screens, and through enhanced disease modeling [see review (158)]. Indeed, Crispr-based techniques offers an unprecedented method to model the polygenic liability of complex disorders such as PD *in vitro*. In a proof of concept experiment, Schrode et al. (159) used Crispr-based allelic conversion and activation/inhibition to manipulate four risk genes associated with schizophrenia in hiPSC-derived neuronal cultures, and demonstrated a synergistic effect on synaptic function. Furthermore, combining such approaches with the use of environmental stressors associated with PD may constitute a unique opportunity to model gene * environment interactions *in vitro*. For instance, while several studies using hMO and related 3D cultures have shown mDA neuronal vulnerability to acute treatment with mitochondrial toxins such as rotenone, MPTP, and 6-OHDA (67, 77, 83, 85), studying the interaction of lower to medium risk genetic variants with chronic, low-dose environmental stressors may allow us to reproduce idiopathic trajectories of PD in a dish.

Therapeutic Opportunities

Organoids also constitute a relevant platform to identify novel therapeutic compounds and to assess their efficacy on specific phenotypes. Kim et al. (77) showed that alpha-synuclein accumulation could be reduced in *LRRK2* G2019S hMO through treatment with a LRRK2 kinase activity inhibitor (GSK2578215A), but also by knocking down the expression of *TXNIP*, which their study had identified as a central mediator of G2019S pathology. Jarazo et al. (80) also found that treatment with the HP-ß-CD compound improved mDA neuronal differentiation in *PINK1* and *PRKN*-mutated hMO, likely through increased mitophagy.

A few elements should be heeded regarding therapeutic developments using hMO. First of all, as organoid generation is prone to variability, taking measures to reduce this confounding factor (detailed in section Addressing variability in organoid differentiations) is essential to accurately assess the potential of therapeutic targets. Secondly, the organoids generated should be extensively characterized, in order to best plan the modalities of therapeutic testing and to help identify the appropriate readouts to quantify the effects. In this sense, disease-modifying treatments targeting deficits in early differentiation of mDA neurons may require different modalities and readouts than those aiming at increasing the survival of compromised, mature cells.

In the long run, hMO technology opens up perspectives for personalized medicine. The study by Ahfeldt et al. (74) identified at least two distinct molecular phenotypes in hMO derived from either *PRKN*$^{-/-}$, or *ATP13A2*/*DJ1* mutated lines, indicating that familial PD mutations induce different pathological cascades, which may call for different therapeutic strategies. Furthermore, personalized medicine approaches may also be explored in cases of non-familial PD. An initial experiment for example proved that cellular alteration in hiPSC-derived neurons from patients with Bipolar Disorder were reversed by lithium treatment only if the patients were also responsive to the medication (160). More recently, Lang et al. (161) proved that mDA neurons from patients carrying an identical variation in a common risk gene (*GBA* N370S) could be stratified based on their molecular profile using RNA sequencing. Clinical follow-up confirmed that their strategy had indeed isolated a patient who proved to be non-responsive to levodopa treatment, and who received a revised diagnosis of progressive supra nuclear palsy. They also identified a causative role of the mis-localization of a class IIa histone deacetylase (HDAC4) in the remaining cell lines, which was then also observed in 2 out of 4 idiopathic PD-derived mDA neuron lines. As modulating the activity or localization of HDAC4 alleviated the cellular PD phenotype, this study suggests that deriving personalized medicine approaches from hiPSC-derived cultures may indeed be a reality in the foreseeable future.

Finally, transplantation of hMO into PD patients' brains to compensate for their loss of mDA neurons also constitutes a promising therapeutic endeavor. Recent studies have demonstrated that stem cell-derived mDA neurons or mDA progenitors could indeed functionally integrate into striatonigral circuits (162), and provide some symptomatic relief in a non-human primate model of PD without forming tumors (163). As organoids have been shown to efficiently integrate into rodent neural circuits after transplantation (100), using dopamine-producing hMO may prove to be a useful development for therapeutic purposes. In this regard, a recent patent (115) indicates that hMO transplantation in a unilateral 6OHDA mouse model of PD could reduce turning behavior in response to an apomorphine challenge, suggesting that the hMO may have functionally integrated into the host organism.

CONCLUSION

Since their first description in 2014, hMO have proven to efficiently generate functional, NM-producing mDA neurons with A9/A10-like identity in structures that recapitulate features

of composition and organization of the human midbrain. PD modeling studies using hMO have also shown their ability to reproduce elements of the disease, such as α-syn accumulation and impairment of mitochondrial function. Interestingly, some mutations did not elicit such phenotypes, suggesting that hMO may also be suited to investigate PD heterogeneity. Whether the observed phenotypes are due to developmental or aging-related pathological mechanisms remains however unclear, as limitations inherent to hiPSC-derived organoid cultures might for now prevent the dissociation of such aspects. Nevertheless, recent studies suggest that these limitations can be overcome through optimisation of culture systems, "aging" strategies and transplantation into host organisms. Future development of hMO co-culture systems will also help study neuroinflammatory processes and interactions with other brain areas involved in PD pathophysiology. Combined with genetic engineering and multimodal molecular readouts, hMO may thus provide a crucial platform to explore the molecular basis of PD, with direct therapeutic implications.

AUTHOR CONTRIBUTIONS

BG and HC wrote the initial draft. BG, HC, and PR reviewed and edited the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Emerging Role of *RHOT1*/Miro1 in the Pathogenesis of Parkinson's Disease

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The expected increase in prevalence of Parkinson's disease (PD) as the most common neurodegenerative movement disorder over the next years underscores the need for a better understanding of the underlying molecular pathogenesis. Here, first insights provided by genetics over the last two decades, such as dysfunction of molecular and organellar quality control, are described. The mechanisms involved relate to impaired intracellular calcium homeostasis and mitochondrial dynamics, which are tightly linked to the cross talk between the endoplasmic reticulum (ER) and mitochondria. A number of proteins related to monogenic forms of PD have been mapped to these pathways, i.e., PINK1, Parkin, LRRK2, and α-synuclein. Recently, Miro1 was identified as an important player, as several studies linked Miro1 to mitochondrial quality control by PINK1/Parkin-mediated mitophagy and mitochondrial transport. Moreover, Miro1 is an important regulator of mitochondria-ER contact sites (MERCs), where it acts as a sensor for cytosolic calcium levels. The involvement of Miro1 in the pathogenesis of PD was recently confirmed by genetic evidence based on the first PD patients with heterozygous mutations in *RHOT1*/Miro1. Patient-based cellular models from *RHOT1*/Miro1 mutation carriers showed impaired calcium homeostasis, structural alterations of MERCs, and increased mitochondrial clearance. To account for the emerging role of Miro1, we present a comprehensive overview focusing on the role of this protein in PD-related neurodegeneration and highlighting new developments in our understanding of Miro1, which provide new avenues for neuroprotective therapies for PD patients.

Keywords: Miro1, Parkinson's disease, mitochondrial dynamics, mitophagy, calcium signaling

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INTRODUCTION

The mitochondrial Rho GTPase Miro1 was first described in yeast, and these studies already reported a link of Miro1 to calcium homeostasis. Yeast strains devoid of the Miro1-ortholog Gem1p displayed a calcium-dependent growth defect (1). Later, mammalian Miro1 was described as an adaptor for calcium-dependent mitochondrial transport (2–4).

The link between Miro1 dysfunction and Parkinson's disease (PD) arose from studies that identified Miro1 as a target of the PD-associated proteins PINK1 and Parkin. These proteins are mutated in autosomal recessively inherited early-onset PD, and functional studies revealed a key role of PINK1-mediated phosphorylation of Parkin for the regulation of mitophagy as a key mechanism in mitochondrial quality control (5, 6). Therefore, the functional interplay of Miro1 with these key proteins for the maintenance of mitochondrial homeostasis was the first link between mitochondrial dynamics and degradation (7, 8). Further studies *in vivo* revealed that the overexpression of Miro1 in flies led to loss of dopaminergic neurons (9), likely due to a delay of clearance of dysfunctional mitochondria via mitophagy triggered by an excess of Miro1. In contrast, knockout of Miro1 in primary mouse neurons caused a decrease in dendrite complexity as a result of impaired mitochondrial distribution (10). The link between Miro1 dysregulation and neurodegeneration was further substantiated by first studies in human patient-based models showing that impaired Miro1 degradation, and the resulting inhibition of mitophagy, was a shared phenotype in fibroblasts and neurons from different sporadic and monogenic PD patients (11, 12). Recently, our group described the first mitochondria-related cellular phenotypes in fibroblasts from PD patients carrying mutations in *RHOT1*, the gene encoding the Miro1 protein (13, 14), thereby further supporting the involvement of Miro1 in the pathogenesis of PD.

Investigations in yeast showed that Gem1p not only is involved in the regulation of mitochondrial function but also regulates the interplay between mitochondria and the endoplasmic reticulum (ER) (15, 16). This interplay came into the focus of PD research, since several PD-associated proteins were recently identified as regulators of mitochondria-ER contact sites (MERCs), i.e., PINK1, Parkin, LRRK2, or α-synuclein (17–20), all of which are also interacting with Miro1 (7, 11, 12, 21).

Moreover, Miro1 was associated with peroxisomal transport (22–24). Aberrant peroxisome-related metabolism was observed in PD patients (25), and mice with impaired peroxisome activity displayed increased aggregation of α-synuclein (26), providing another potential link between Miro1 and PD via altered peroxisome function. Together, these findings point to an emerging role of Miro1 in neurodegeneration in PD that underscores the need for summarizing the current knowledge about Miro1 and new developments that provide new perspectives for future causative therapies in PD.

STRUCTURE AND PHYSIOLOGICAL FUNCTION OF THE MIRO1 PROTEIN

In mammals, two Miro GTPases, named as Miro1 and Miro2, are encoded by the *RHOT1* and *RHOT2* genes located on chromosome 17. Miro1 and Miro2 are both ubiquitously expressed, consisting of 662 amino acid residues, and display a 60% peptide sequence homology (27–29). Miro GTPases are conserved in almost all eukaryotes containing mitochondria (30), and they were first considered as atypical members of the RAS superfamily of GTPases, particularly as two of the 23 members of the RHO (Ras homolog) protein subfamily (27).

However, in contrast to other RHO family members, Miro GTPases contain no C-terminal cysteine and they also lack the typical RHO insert, which led to their classification as a definite subfamily of small GTPases (29, 31–34).

Structurally distinct N-terminal and C-terminal GTP-binding motifs are present in both Miro proteins, with a linker region (called "MiroS") connecting two EF-hand domains to the C-terminal GTPase domain (35, 36).

In contrast to the yeast Miro1 homolog Gem1p, which needs both GTPase domains to maintain its function as an adaptor for transport (34, 37), the influence and requirement of these two GTPase domains on mitochondrial trafficking have been widely debated in metazoans, especially in neurons. Several studies suggested that the N-terminal and C-terminal GTPases of Miro1 might have different functions. For instance, it was shown in fly and rat neurons that the C-terminal GTPase domain of Miro is only involved in retrograde transport, while its N-terminal GTPase domain is essential for mitochondrial transport in both retrograde and anterograde directions (2, 38). However, another study provided evidence that alterations of mitochondrial transport in *Drosophila* neurons were exclusively caused by mutations in the N-terminal GTPase domain, but not in the C-terminal GTPase domain of dMiro (*Drosophila* homolog of mammalian Miro1) (37).

Further reinforcing this hypothesis, only mutations in the N-terminal GTPase domain led to the disruption of the mitochondrial network in mammalian cells (28). Moreover, recent work developed by Kalinski et al. describes that the deacetylation of the lysine 105 on the N-terminal GTPase domain of Miro1 could inhibit mitochondrial transport in primary mouse neurons, subsequently affecting axonal growth (39).

While earlier structural studies were performed on dMiro, recent work gave us new insights on human Miro1, demonstrating that both N-terminal and C-terminal GTPases were not only structurally but also functionally different. The N-terminal GTPase was shown to have exclusively GTPase activity, while the C-terminal GTPase also displayed NTPase activity (34, 36, 40), thus making Miro1 the only currently known human protein that contains two different GTPase domains (41). On the other hand, the C-terminal GTPase domain seems to be crucial for the calcium-related functions of Miro1, by interacting with and stabilizing the two EF-hand domains of the protein, which are involved in calcium binding (35, 42).

Miro1 also contains two ligand-mimicking α-helices (LM1 and LM2), which connect each canonical EF hand to a non-canonical "hidden" EF-hand domain (hEF) (35). The calcium-binding amino acids are exposed to the cytosol via an helix-loop-helix-motif in both EF hands, facilitating a conformational change of the protein upon binding to calcium (43, 44).

Moreover, Miro1 harbors a C-terminal transmembrane domain (TMD), which anchors the protein into the outer mitochondrial membrane (OMM), exposing the protein and the N-terminal GTPase to the cytoplasm (2, 35). Fransson et al. demonstrated that the deletion of the TMD in both

mammalian Miro proteins led to their mislocalization to the cytoplasm, proving that the TMD is required for mitochondrial targeting (28).

Our group recently described the first heterozygous mutations in the human *RHOT1* gene, found in four individuals diagnosed with PD (13, 14). The identified mutations R272Q, T351A, and R450C were located within highly conserved protein domains of Miro1: R272Q within the LM1 of the N-terminal EF-hand domain, T351A within the C-terminal EF-hand domain, and R450C within the C-terminal GTPase domain (13, 14). The T610A mutation is located within the C-terminus section of the protein, close to the TMD (14). The homology models of the 3D structure of the Miro1 protein showed that all four mutations were localized on the protein surface and exposed to the cytosol. Due to their position, these mutations could therefore impact on calcium binding and sensing, GTP hydrolysis, and mitochondrial localization features of Miro1 (13, 14).

MIRO1 AND PARKINSON'S DISEASE

Mitochondria are the main source of cellular energy, and on top of that, they have an essential role in intracellular calcium buffering and regulation of lipid homeostasis (45, 46). For these reasons, dopaminergic neurons critically depend on mitochondrial function, since they require a constant supply of energy and calcium to maintain the integrity of their long axons and to regulate their pacemaking activity for the release and recycling of neurotransmitters (47, 48).

Mitochondrial dyshomeostasis is a central factor in PD pathophysiology, and indeed several genes involved in the development of familial PD are associated with mitochondrial homeostasis (49–51). Increasing evidence indicates that proteins encoded by several PD-linked genes physically interact with Miro1, modifying its function and hence contributing to the dysregulation of neuronal integrity. For this reason, the link between Miro1 and neurodegeneration is a topic of growing interest in PD research.

Bioinformatic analyses indicated that PINK1 and Parkin are direct protein interactors of Miro1 (52). In line with this structural finding, several studies described functional links between these proteins using *in vitro* cellular models from different species. Functional connections between PINK1, Parkin, and Miro1 were first described in flies, where PD-associated deletions in *Drosophila* PINK1 were shown to cause disruption of mitochondrial transport in neuronal axons through interaction with dMiro in a Parkin-dependent manner (9, 53). On top of affecting mitochondrial movement, the loss of PINK1 and Parkin in flies promoted the disruption of other mitochondrial-related mechanisms, such as impairment of mitochondrial clearance, altered the abundance of mitochondria-ER appositions and mitochondrial calcium overload, finally leading to the death of dopaminergic neurons (9, 17). Notably, all these mentioned phenotypes were rescued by a reduction in the amount of dMiro protein in these cells (9, 17), emphasizing the importance of the multifunctional role of Miro1 for mitochondrial homeostasis in PD.

Despite the clear link between mitochondrial dyshomeostasis in PD and Miro proteins, single-nucleotide polymorphisms (SNPs) in *RHOT1/2* were not associated with PD using genome-wide association studies (GWAS) (54), and recent meta-analyses of GWAS data did not identified *RHOT1/2* as risk loci for PD (55, 56). However, GWAS are not designed to detect rare variants due to a minor allele frequency of the used SNPs of >5% in most studies, and therefore, sequencing methods to identify rare variants are needed. Of note, gene-based association clustering methods recently allowed the identification of *RHOT2*, the gene encoding for Miro2, as a PD-associated gene (57). Recently, our group identified the first heterozygous mutations in the *RHOT1* gene in four independent PD patients by exome sequencing (13, 14), further strengthening the impact of Miro1 in the development of PD and defining *RHOT1* as a potential novel risk gene for the pathogenesis of this disorder.

The knowledge about the role of human Miro1 in neurodegeneration, particularly in the pathogenesis of PD, is growing rapidly. In the next sections, we will discuss the molecular and cellular effects of mutant human Miro1 in PD.

MIRO1 AS A TARGET FOR PINK1/PARKIN-MEDIATED MITOPHAGY

An impressive number of studies over the last 30 years shed light on the so-called mitochondrial life cycle, during which these highly dynamic organelles continuously experience fission and fusion events to meet the functional needs of the cells. Maintaining this mitochondrial network requires the coordinated activity of mitochondrial biogenesis and clearance pathways, which ensure the replacement of damaged organelles with metabolically active mitochondria. Consequently, impairing this fine-tuned quality control mechanism leads to the accumulation of dysfunctional mitochondria, which in turn increases oxidative stress and deteriorates cellular activity (58, 59). Removal of damaged mitochondria is even more important in post-mitotic cells like neurons, which are not able to dilute harmful components through cell division.

In accordance, mitochondrial dysfunction plays an essential role in a large number of neurodegenerative diseases, including PD, and the accurate mitochondrial turnover is now considered a key neuroprotective mechanism against chronic disease conditions (60).

Mitochondrial degradation is a well-orchestrated process involving the two main intracellular clearance machineries, namely, the ubiquitin–proteasome system (UPS) and the autophagy pathway (61). The PD-linked PINK1 and Parkin proteins are the master and commander of this multistep mechanism: (i) the mitochondrial kinase PINK1 selectively recognizes depolarized mitochondria and rapidly accumulates on their surface, where it starts a massive phosphorylation of ubiquitinated proteins; (ii) the cytosolic ubiquitin-ligase Parkin recognizes PINK1-catalyzed phospho-ubiquitin and translocates to mitochondria, supplying further ubiquitin chains to PINK1

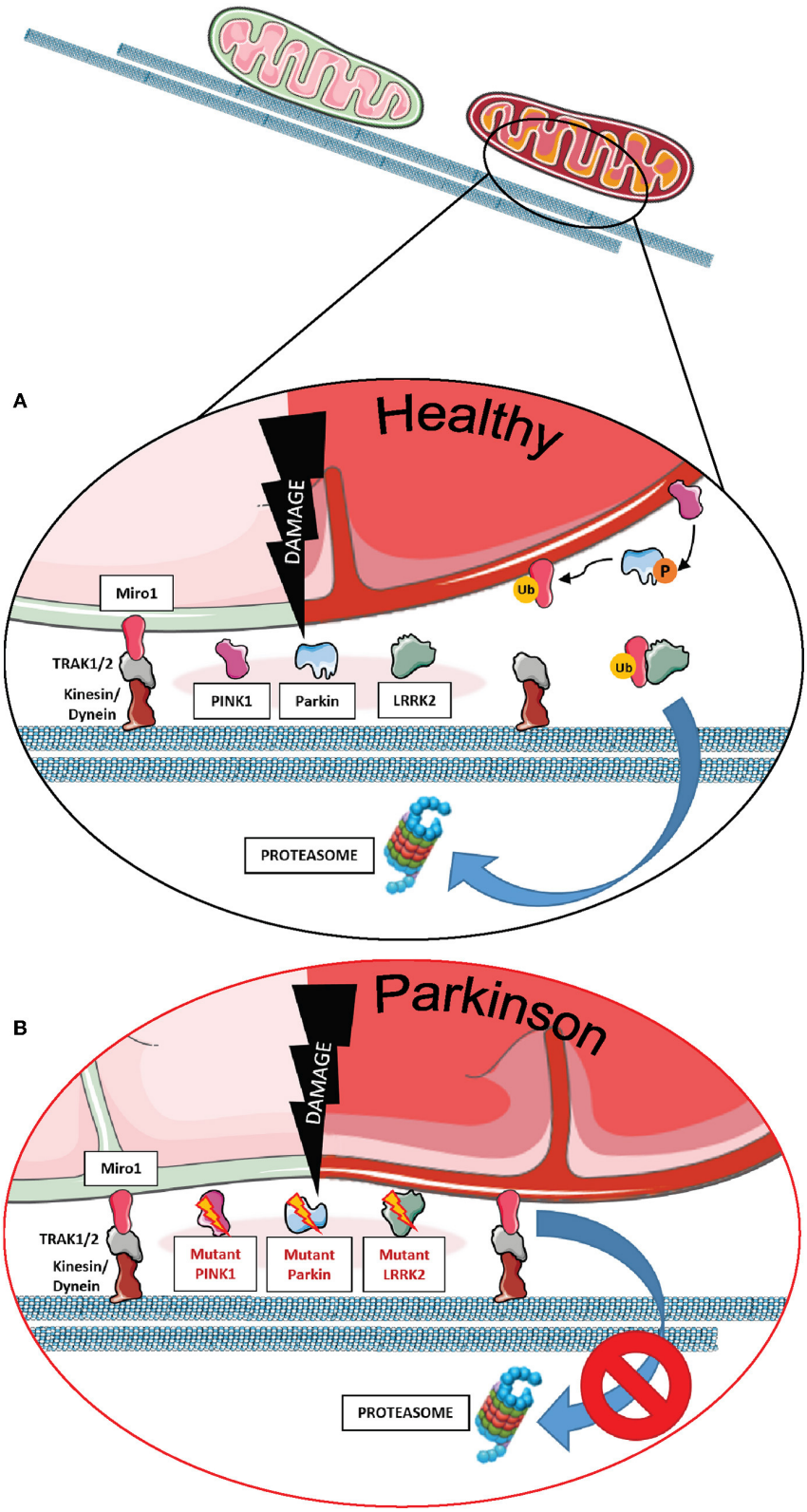

FIGURE 1 | The role of Miro1 in mitophagy. **(A)** Lysosomal degradation of dysfunctional mitochondria requires the stop of mitochondrial transport and detachment from the cytoskeleton. Mitochondrial damage leads to the accumulation of PINK1 at mitochondria and the recruitment of the E3 ubiquitin ligase Parkin. PINK1

(Continued)

FIGURE 1 | phosphorylates and activates Parkin, which in turn ubiquitinates proteins at the outer mitochondrial membrane, including Miro1. Additionally, PINK1 might also phosphorylate ubiquitin chains on mitochondrial proteins. Ubiquitinated proteins, including Miro1, are then targeted for proteasomal degradation, thereby disconnecting mitochondria from the cytoskeleton and stopping transport. Isolated mitochondria are then ready for uptake by autophagosomes. LRRK2 was shown to be involved in the removal of Miro1 from the surface of impaired mitochondria. **(B)** In cell models expressing PD-associated mutations in PINK1, Parkin, or LRRK2, the proteasomal degradation of Miro1 is impaired, consequently interfering with the arrest of mitochondrial transport and the initiation of mitophagy. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

and amplifying the signal in a positive feedback loop; (iii) Parkin ubiquitinates a number of substrates on the OMM, leading to the inhibition of mitochondrial fusion and arrest of mitochondrial movement; (iv) the coating of dysfunctional mitochondria with phospho-ubiquitin chains recruits specific components of the autophagic receptor machinery to mitochondria, which are then engulfed by autophagosomes; and (v) finally degraded into lysosomes (62).

Miro1 plays an essential role in this process, being one of the first substrates of Parkin E3-ligase activity. As a key component of the mitochondrial transport machinery that anchors the organelle to the motor proteins of the cytoskeleton, Miro1 is ubiquitinated by Parkin and consequently degraded by the UPS (**Figure 1A**), which leads to mitochondrial arrest and facilitates mitophagy (7–9, 36, 42, 63, 64). Particularly in the context of PD, PD-associated mutations in Parkin were shown to disrupt the ubiquitination of Miro1 for proteasomal degradation in patient-derived fibroblasts, leading to the inhibition of Miro1 turnover and the subsequent failure of mitochondrial arrest for mitophagy (**Figure 1B**) (7). Further supporting the functional interaction of Miro1 with other PD gene products linked to mitochondrial quality control, previous findings also showed that Miro1 physically interacts with PINK1 and is phosphorylated by the kinase on the serine 156, which could represent a signal for the following ubiquitination by Parkin (7, 65).

In addition to the PINK1-Parkin axis, Miro1 removal from the OMM of depolarized organelles could also be mediated by its association with other PD-related proteins (**Figures 1A,B**). Two studies supported this hypothesis by the discovery that LRRK2 and α-synuclein cooperate with Miro1 to stop mitochondrial movement prior to mitophagy (11, 12). In fact, the pathogenic PD mutations LRRK2 G2019S and α-synuclein A53T disrupt this process, resulting in Miro1 accumulation, delayed mitochondria arrest, and impaired mitophagy activation in patient-derived fibroblasts and induced pluripotent stem cell (iPSC)-derived neurons (11, 12).

Based on these findings, clearing Miro1 from depolarized mitochondria is emerging as a potential neuroprotective mechanism against PD, as the failure of its removal from mitochondria was demonstrated in cells from PD patients. Interestingly, in some PD cases, Miro1 degradation is impaired even in the presence of functional Parkin and LRRK2, indicating the existence of additional mechanisms accounting for Miro1 removal from dysfunctional mitochondria (66). Hence, Hsieh et al. demonstrated that genetically or pharmacologically reducing Miro1 levels improved mitochondrial arrest, activated mitophagy, and prevented dopaminergic neurodegeneration in both iPSC-derived human neurons and fly models of PD, without significantly affecting the movement of healthy mitochondria (11, 12, 66).

Further confirming an important role for Miro1 in the pathogenesis of PD, we recently described mitophagy alterations in fibroblasts from PD patients harboring heterozygous mutations in the *RHOT1* gene encoding Miro1 (13, 14). Interestingly, all mutant fibroblast lines demonstrated alterations in mitophagy flux, but the resulting phenotype was different depending on the Miro1 mutation. In fact, the R272Q and R450C mutants demonstrated increased levels of mitophagy compared to controls, reflected by increased mitochondria co-localizing with LC3 puncta and decreased Parkin protein levels under baseline conditions. CCCP treatment was not sufficient to further increase mitophagy, suggesting that mitophagy was already running at maximal capacity (13). In contrast, the T351A and T610A mutants displayed no increase in mitophagy under baseline conditions. CCCP treatment leads to increased co-localization of LC3 puncta with mitochondria in control cells, but not in Miro1-T351A or -T610A fibroblasts, suggesting an impaired mitophagy mechanism in these mutants (14).

It is worth noting that, in contrast to the increased mitophagic turnover observed in R272Q mutant fibroblasts, iPSC-derived neurons harboring the same Miro1 mutation displayed an opposite phenotype compared to the fibroblasts (67). Mitophagy was not inducible in Miro1-R272Q neurons, either by oxidative stress or by CCCP treatment. Furthermore, bafilomycin A1 treatment did not lead to an accumulation of the autophagic cargo protein p62 in these cells, suggesting a reduced autophagic turnover (68). Based on these observations, mitophagy seems to be regulated differentially in fibroblasts and neurons.

Remarkably, the mitophagy phenotype in these cells seems to be tightly related to the degree of topographic association between mitochondria and ER, as represented by the distance between both organelles (68). In 2018, McLelland et al. described that the initiation step of mitophagy in human cancer cell lines and iPSC-derived neurons occurs at mitochondria and ER appositions where the cleft that separates both organelles is wider than ∼30 nm (**Figure 2A**) (69). These "wide" appositions serve as a platform for Parkin-mediated ubiquitination of OMM proteins at depolarized mitochondria, subsequently promoting the uncoupling of mitochondria from the ER and the mitophagy process (13, 14). This hypothesis was drawn from the observation that iPSC-derived neurons from a PD patient with a deletion in Parkin did not show a decrease of these "wide" MERCs after initiation of mitophagy with CCCP (**Figure 2B**) (69). Fitting, our results showed that Miro1-mutant lines with an unchanged number of "wide" MERCs compared to controls displayed the

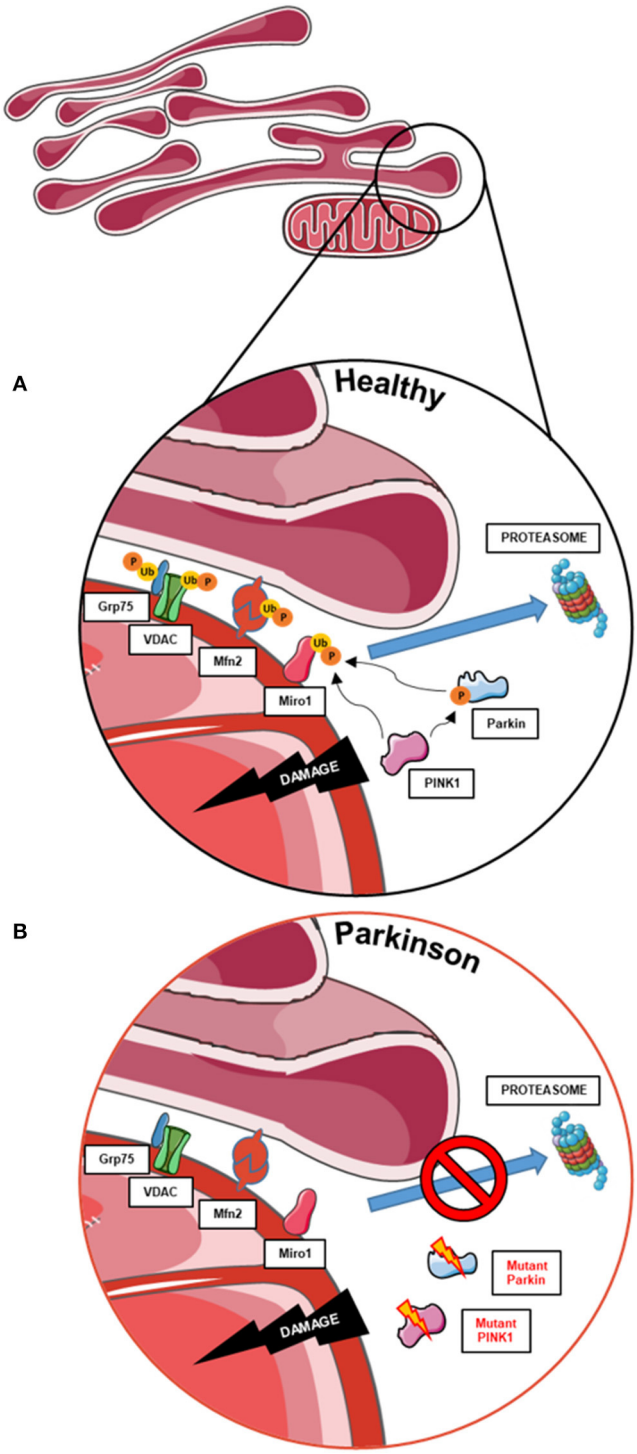

FIGURE 2 | The role of MERCs in mitophagy and the contribution of PD-associated proteins. **(A)** Mitophagy also requires the untethering of impaired mitochondria from the ER. Therefore, PINK1 and Parkin work together to ubiquitinate and phosphorylate proteins involved in subtypes of MERCs (i.e., Grp75, VDAC, Mfn2, and Miro1) for subsequent proteasomal degradation and disassembly of MERCs, allowing the degradation of impaired mitochondria. **(B)** In cells with impaired PINK1 or Parkin function, MERCs *(Continued)*

FIGURE 2 | are not disassembled upon mitochondrial dysfunction, hence hampering the initiation of mitophagy. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

ability to induce mitophagy after CCCP treatment (13, 14), while mutants with reduced amount of "wide" MERCs revealed a deficit to initiate mitophagy after treatment with CCCP (14). These findings suggest that the regulation of mitochondrial quality control by Miro1 might crucially depend on the structure of MERCs.

MIRO1 AS A REGULATOR OF MITOCHONDRIAL-ER CONTACT SITES

MERCs are discrete areas of proximity between mitochondria and the ER that coordinate essential physiological processes, such as lipid biosynthesis, cellular calcium handling, and mitochondrial homeostasis (70–72). These mechanisms are reported to be affected in neurodegeneration (73, 74); hence, MERCs are one of the most studied organelle juxtapositions and a current spotlight in PD research (75).

Several mitochondria-related proteins involved in PD pathogenesis modulate the physiological function of the MERCs by acting as regulatory factors. Overexpression of Parkin in HeLa cells was shown to increase physical and functional coupling between mitochondria and ER, stimulating mitochondrial calcium uptake and ATP production, while Parkin knockdown had the opposite effect (18). Similarly, overexpression of α-synuclein and DJ-1 proteins increased the number of MERCs in HeLa cells, subsequently increasing mitochondrial calcium uptake (18, 76). LRRK2 was recently found to also modulate MERC amount and function, since LRRK2-null MEFs express reduced MERC abundance and dysregulated mitochondrial calcium uptake (77). Moreover, α-synuclein was found in MERCs from mouse and human brain tissue, where it seems to modulate mitochondrial morphology (78). Like α-synuclein, PINK1 was recently found to also localize to MERCs, and its continuous degradation in healthy mitochondria is regulated by the interplay of mitochondria and the ER (79). It is worth noting that the PD-related proteins PINK1, Parkin, LRRK2, and α-synuclein that are involved in MERCs have also been shown to directly or indirectly interact with Miro1 (7, 11, 12, 21). Hence, impaired mitophagy and dysregulation of MERCs seems a shared feature in different cases of PD.

The relationship between Miro proteins and MERCs started to be investigated when, in 2011, two research groups identified Gem1p, the yeast ortholog of mammalian Miro1, as a crucial regulator of the ER-mitochondrial encounter structure (ERMES), a protein complex that tethers mitochondria and ER in yeast (15, 80). Association of Gem1p to ERMES controls phospholipid exchange for lipid biosynthesis between mitochondria and ER (15) and regulates mitochondrial division and morphology (81–83). The localization of dMiro at MERCs was also demonstrated

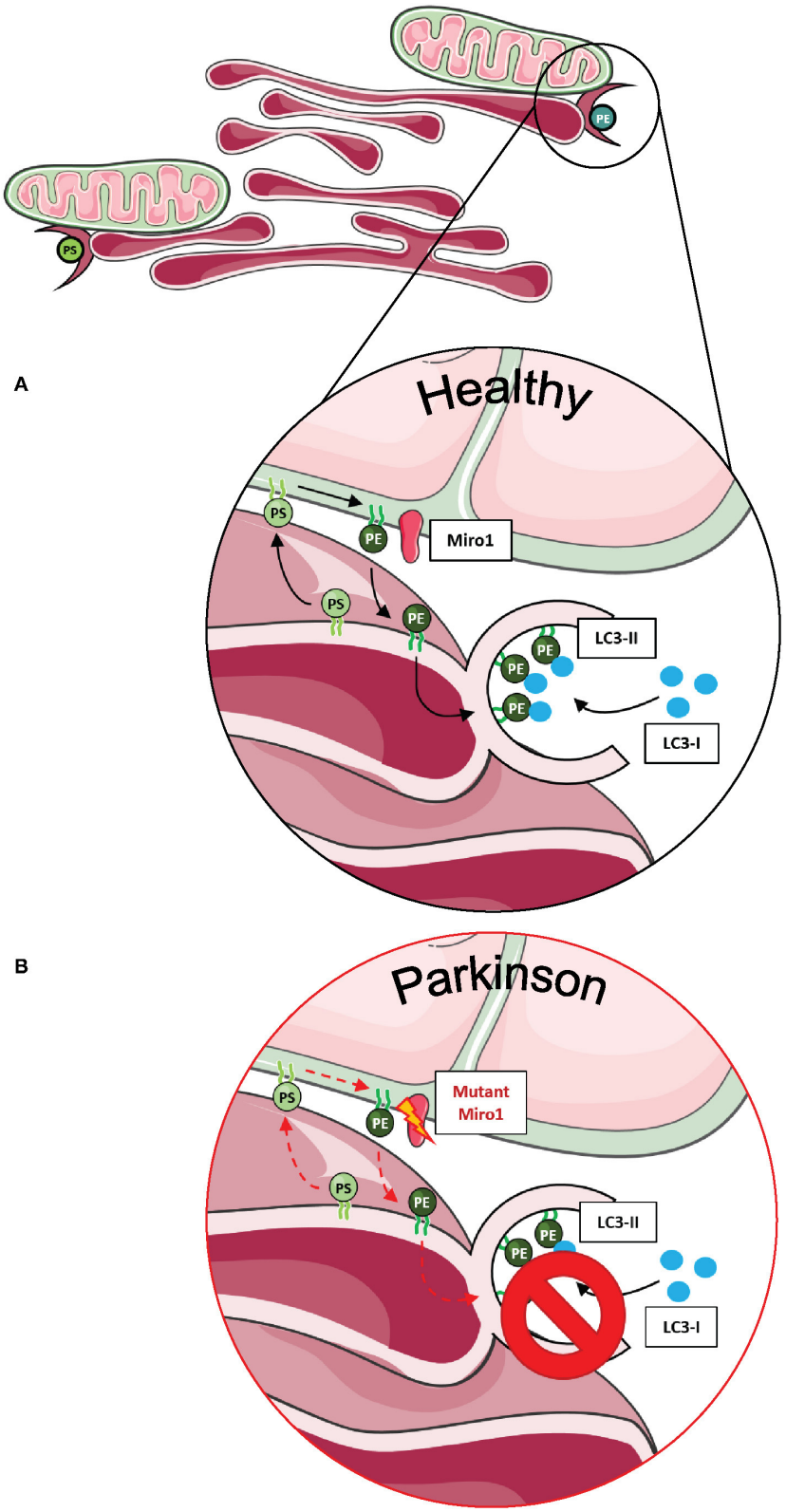

FIGURE 3 | Phospholipid synthesis and autophagosome formation at MERCs. **(A)** Phospholipids are synthesized in mitochondria and the ER, requiring the exchange of metabolites at MERCs. Phosphatidylserine (PS) is synthesized in the ER, shuttled to mitochondria via MERCs, where it is transferred into phosphatidylethanolamine

(Continued)

FIGURE 3 | (PE) and shuttled back into the ER. PE is necessary for the assembly of isolation membranes at the ER for the integration of cytosolic LC3-I into the autophagosome membrane, forming LC3-II. **(B)** A number of studies suggest that Miro1 is involved in phospholipid shuttling via MERCs. Disruption of Miro1 function might impair phospholipid synthesis, consequently interfering with the formation of autophagosomes. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

in *Drosophila* neural stem cells and dopaminergic neurons (17, 84), as well as mammalian Miro1 in COS-7 cells, HeLa cells, MEFs, human fibroblasts, and human iPSC-derived neurons (14, 15, 67, 84, 85).

In human-derived cells, the contribution of Miro1 in lipid exchange and biosynthesis was confirmed by the discovery that patient-derived fibroblasts with PD-associated mutations in Miro1 displayed an altered formation of autophagosomes, which is dependent on the conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE) at MERCs (13, 14). In mammalian cells, PS is synthesized in the ER, transferred through the MERCs to the mitochondria, and transformed into PE (72). A fraction of the mitochondrial-generated PE is then shuttled back to the ER for the generation of isolation membranes, where PE is used for the lipidation of specific adaptor proteins that recruit autophagic cargoes (**Figure 3A**) (86, 87). In our studies, none of the Miro1-mutant fibroblast lines showed an increase in the amount of newly synthesized autophagosomes following starvation conditions (13, 14). In line with these results, all Miro1-mutant fibroblast lines showed an overall reduction in MERCs, suggesting that PD-associated Miro1 mutations disturb the formation of appositions between mitochondria and ER, affecting lipid exchange and, consequently, autophagy initiation (**Figure 3B**) (13, 14).

In contrast to our observations in patient-derived fibroblasts, Miro1-R272Q neurons showed an increased number of contacts between mitochondria and ER (67). Remarkably, only control neurons exhibited accumulation of the autophagic cargo protein p62 upon bafilomycin A1 treatment, but not Miro1-R272Q neurons (67). These results point toward a functional impairment of MERCs that may affect the initiation of autophagy in patient-derived neurons, possibly triggered by the pathogenic effect of mutant Miro1.

Importantly, two Miro1 interactor proteins, PINK1 and Parkin, were also shown to be involved in the organization and lipid-related function of MERCs (**Figure 4A**). Neurons derived from flies and patients carrying mutations in PINK1 and Parkin displayed increased amounts of MERCs and a disturbed exchange of the phospholipid PS, resulting in an impaired synthesis of dense core vesicles from the ER (**Figure 4B**) (88). Altogether, these findings argue in favor of an important role of Miro1 in lipid homeostasis at MERCs and an impairment of this function in conditions linked to neurodegeneration.

Other studies in metazoans also supported a key role of Miro in the regulation of MERCs. In *Drosophila* neural-derived cultures, Polo kinase-induced phosphorylation of dMiro enhances the localization of dMiro to MERCS and the interaction with calcium transporters to regulate calcium homeostasis and the integrity of the tethering complex (17, 84). Moreover, in our studies, we were able to observe a reduced co-localization of Miro1 with MERCs in Miro1-mutant fibroblasts from PD patients compared to control fibroblasts (14) and, conversely, an increased co-localization of Miro1 with MERCs in Miro1-R272Q iPSC-derived neurons (67), underscoring the importance of Miro1 localization to MERCs and a potential role in neurodegeneration.

As mentioned in the previous section, MERCs were recently shown to act as regulators of mitophagy initiation (**Figure 2A**). Coupled mitochondria and ER in human iPSC-derived dopaminergic neurons are untethered upon Parkin-mediated ubiquitination of MERC-residing proteins, such as Mfn2 and VDAC (63, 89, 90), as a starting point for mitochondrial clearance (69). Based on the evidence that targeting of Miro1 by the PINK1/Parkin pathway is required as an initial step for mitophagy (7, 9, 91), these studies provide strong evidence that PINK1/Parkin-mediated mitophagy is organized at MERCs and that Miro1 might be directly involved in that process. Indeed, fibroblasts obtained from PD patients harboring Miro1 mutations show significant alterations in mitophagy flux accompanied by dysregulation of the abundance of specific subtypes of MERCs, supporting the previous hypothesis (13, 14).

Moreover, based on the increased amount of overall MERCs and impaired CCCP-induced mitochondrial clearance observed in iPSC-derived Miro1-R272Q neurons, we speculate that damaged mitochondria may not uncouple from the ER, consequently hampering the initiation and flux of mitophagy (67).

In conclusion, the communication between mitochondria and ER is crucial to maintaining cellular homeostasis and is a potential investigation target of growing interest in neurodegenerative diseases, such as PD. Miro1 was demonstrated to be crucially involved in the regulation of the function of the MERCs; therefore, the study of this interaction between Miro1, mitochondria, and ER will help to better comprehend the complex pathogenicity of PD.

MIRO1 AS A REGULATOR OF CELLULAR CALCIUM HOMEOSTASIS

Calcium ions act as important second messengers that control several cellular mechanisms. Therefore, cytosolic calcium levels need to be tightly regulated, and cells manage to maintain calcium homeostasis mainly via buffering calcium by specific organelles, such as the ER and mitochondria (84, 92).

One of the main functions of Miro1 is to orchestrate calcium homeostasis in mitochondria and calcium-dependent mitochondrial positioning. To fulfill this function, calcium-binding is facilitated via both of its EF-hand domains (33, 93) and

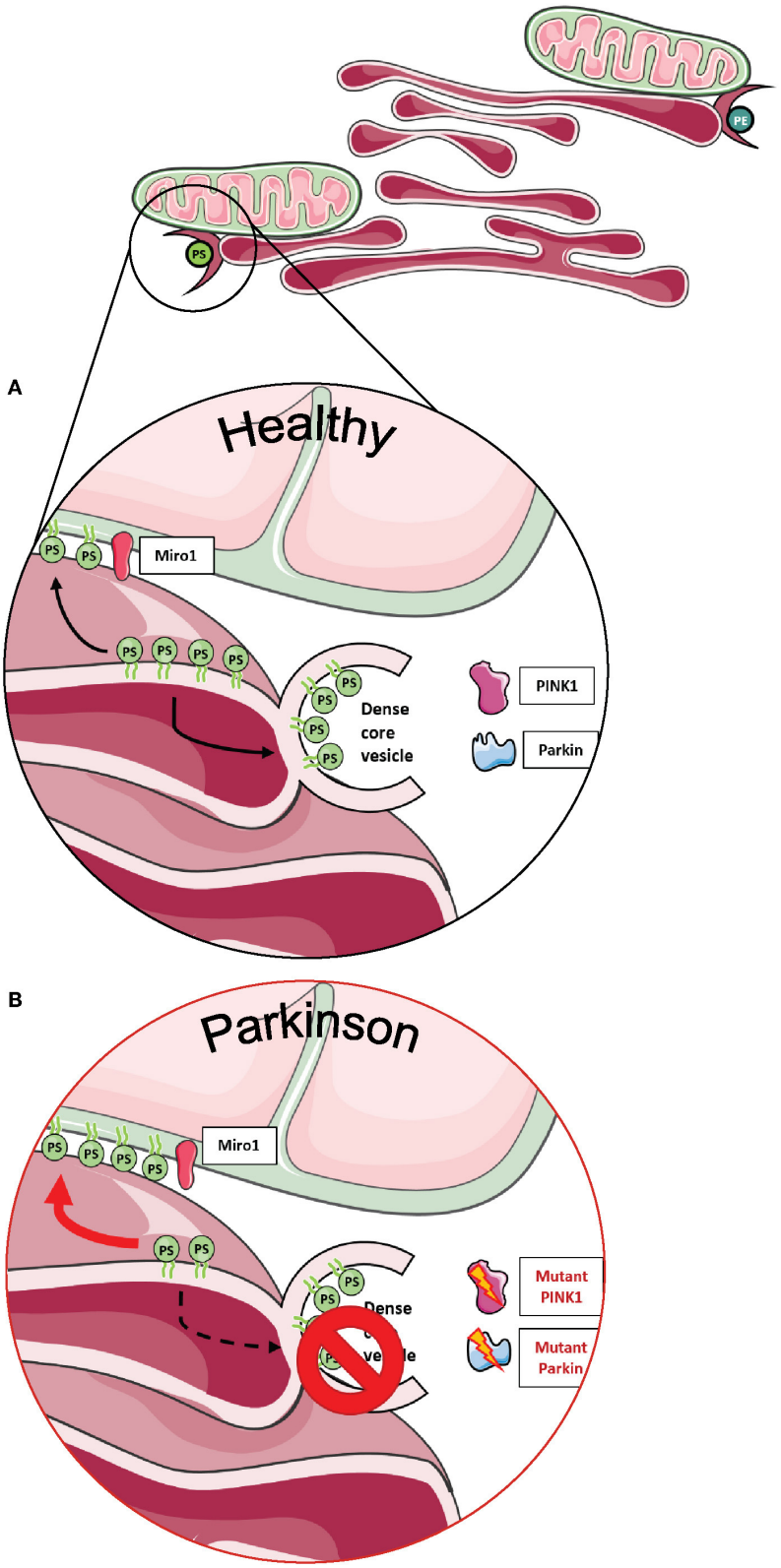

FIGURE 4 | Phospholipid synthesis and the formation of dense core vesicles at MERCs. **(A)** Phospholipids are required not only for the formation of autophagosomes, but PS is also required to provide membranes for dense core vesicles. **(B)** Fly neurons expressing mutant PINK1 or Parkin show impaired formation of dense core vesicles, resulting in alterations of neurotransmission. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

the C-terminal GTPase domain (35, 42). Miro1 was suggested to have a high calcium-binding affinity (33) and consequently binds calcium only upon elevation of cytosolic calcium levels (3, 42, 94).

Interestingly, Chang et al. found in 2011 that intra-mitochondrial calcium levels correlated with mitochondrial transport speed, suggesting that mitochondrial transport was not only controlled by cytosolic calcium transients but also by mitochondrial matrix calcium levels (4). Furthermore, primary mouse neurons overexpressing Miro1 with calcium-insensitive EF-hand domains showed a decreased influx of calcium into the mitochondrial matrix, suggesting that Miro1 also regulates intra-mitochondrial calcium levels (4).

This finding was later supported by a study in primary fly neurons. Knockdown of dMiro caused a decreased histamine-induced calcium uptake in the mitochondrial matrix, while overexpression of dMiro led to increased calcium uptake (84). Lee et al. concluded that dMiro specifically promotes the flux of calcium from the ER to mitochondria and that this mechanism is independent of mitochondrial transport and intracellular distribution (**Figure 5A**) (84).

While the effect of intra-mitochondrial calcium levels on mitochondrial transport seems surprising at first glance, it is known that increased cytosolic calcium transients lead to an unavoidable influx of calcium into the mitochondrial matrix (4). The observed regulation of mitochondrial matrix calcium by Miro1 (4, 84) raised the question how a protein bound to the OMM and facing the cytosol can possibly regulate the influx of calcium into the matrix.

Uptake of calcium into mitochondria is facilitated by the mitochondrial calcium uniporter (MCU), a protein complex of several subunits residing in the inner mitochondrial membrane. Only in 2018, Niescier et al. revealed that the N-terminus of the MCU reaches through the mitochondrial intermembrane space and the outer membrane to directly interact with Miro1 (**Figures 5A,B**). This study finally solved the question how Miro1 residing in the outer membrane is able to regulate intra-mitochondrial calcium homeostasis (95).

Regulation of mitochondrial matrix calcium levels is important for mitochondrial energy production (96–98). Hence, loss-of-function mutations in Miro1 were suggested to affect mitochondrial energy production by disrupting the mitochondrial matrix calcium uptake (86). Indeed, the ATP production was decreased in brains of *Drosophila* larvae expressing the loss-of-function mutation dMiro B682 (84), in GemA (ortholog of Miro) knockout *Dictyostelium discoideum* (30), and in fibroblasts from patients carrying PD-associated Miro1 mutations (13). Together, these findings suggest that Miro1 plays a crucial role in the maintenance of mitochondrial function via regulation of mitochondrial calcium levels.

The importance of Miro1 for the maintenance of calcium homeostasis in the context of PD was highlighted in our recently published studies with PD patient-derived fibroblasts (13, 14). In these studies, we used thapsigargin, an inhibitor of the sarco-/ER calcium ATPase (99). When the ER calcium uptake was blocked by thapsigargin treatment: (i) calcium levels in the cytosol rose rapidly due to depletion of the ER calcium store (100), and (ii) calcium buffering relied on other mechanisms, i.e., mitochondrial calcium uptake. We found that buffering of cytosolic calcium after thapsigargin treatment was delayed in patient-derived fibroblasts harboring mutations in Miro1 (13, 14), suggesting that mitochondrial calcium buffering is impaired in Miro1-mutant fibroblasts. In addition, combined treatment of thapsigargin with the MCU inhibitor Ru360 (4, 101, 102) caused a reduction in cytosolic calcium buffering in the control fibroblast lines similarly to Miro1-mutant fibroblasts. These results confirmed that calcium buffering relies mostly on mitochondria when calcium uptake via the ER is blocked (13, 14).

Our studies also supported previous observations where mutations in the EF-hand domains of Miro1 caused an elevation of the frequency of calcium spikes and an increase in the time constant of calcium transients in primary rat astrocytes (103). A similar disruption of calcium homeostasis with increased frequency and amplitudes of calcium spikes was observed in rat hippocampal cultures with deletion of Miro1 EF-hand domains (Miro1-ΔEF) (104). In contrast, overexpression of wild-type Miro1 led to decreased thapsigargin-induced calcium spikes in primary fly neuron cultures (84).

Maintenance of calcium transients is important for the function of astrocytes and neurons. High levels of calcium enter the cell at active synapses and need to be buffered via mitochondria (103, 104). Impaired cellular calcium homeostasis is a shared phenotype observed in different models of PD (105–110). In line with this, iPSC-derived neurons with heterozygous Miro1-R272Q display a significantly higher peak of cytosolic calcium and delayed buffering capacity after ionomycin treatment compared to control neurons (67). Hence, one might speculate that mutations in Miro1 drive neurodegeneration by impairing calcium homeostasis, subsequently affecting mitochondrial function and energy production in the pathogenesis of PD. Indeed, *Drosophila* expressing loss-of-function mutations in the EF-hand domains of dMiro show a decreased neuronal survival because the impaired Miro-mediated calcium-dependent mitochondrial positioning affects calcium homeostasis and thereby increasing the susceptibility to glutamate excitotoxicity (3).

Another function of Miro1 is the regulation of mitochondrial dynamics in a calcium-dependent fashion. Glutamate application to primary rat neuronal cultures caused a reduction in mitochondrial length. However, these calcium-dependent changes in mitochondrial morphology were abolished in cells expressing Miro1-ΔEF (104). In 2018, Nemani et al. revealed that this Miro1-mediated calcium-dependent mitochondrial fragmentation was independent of the mitochondrial fission protein Drp1, the mitochondrial membrane potential, or the production of reactive oxygen species (ROS) and is an important prerequisite of mitophagy (94).

In our studies, PD patient-derived Miro1-mutant fibroblasts and iPSC-derived Miro1-R272Q neurons demonstrated an increased mitochondrial fragmentation after treatment compared to control cells. This finding was not surprising given the delayed buffering of calcium transients and resulting retained high levels of calcium in the cytosol (13, 14, 67).

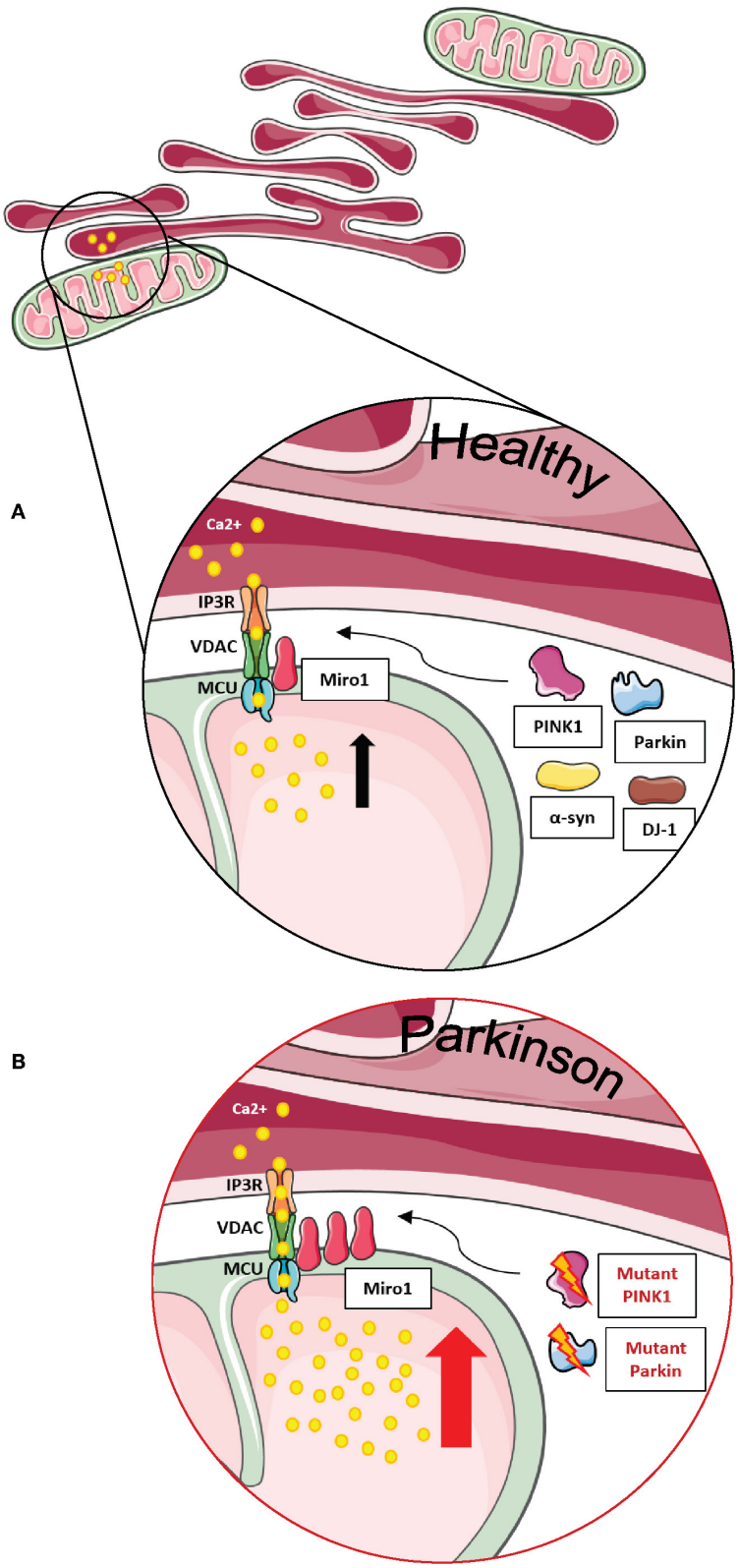

FIGURE 5 | Miro1 is involved in calcium regulation at MERCs. **(A)** Calcium homeostasis is tightly regulated. ER and mitochondria buffer cytosolic calcium transients and specialized subtypes of MERCs composed of IP3R, VDAC, MCU, and Miro1 are required for regulation of calcium uptake. Miro1 acts as sensor of cytosolic

(Continued)

FIGURE 5 | calcium levels, interacting directly with the MCU and orchestrating the mitochondrial calcium uptake at MERCs. PD-associated proteins PINK1, Parkin, α-synuclein, and DJ-1 participate in the regulation of calcium homeostasis at MERCs. **(B)** Impaired PINK1 or Parkin function leads to clustering of Miro1 and subsequent mitochondrial calcium overload that facilitates mitochondrial dysfunction and apoptosis. Additionally, impaired Miro1 function leads to disruption of cellular calcium homeostasis by alterations of mitochondrial calcium buffering. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

Our findings imply that mutations in Miro1 cause an impairment of calcium homeostasis, resulting in decreased ATP production and increased calcium-dependent mitochondrial fragmentation, thereby contributing to the pathogenesis of PD.

MIRO1 AND ORGANELLAR MOVEMENT IN PARKINSON'S DISEASE

Mitochondrial Transport

Miro1 is a well-known adaptor for the mitochondrial transport machinery, forming a complex with the motor proteins dynein, kinesin, and myosin and thereby allowing mitochondrial movement along the cytoskeleton (2, 28, 37, 111, 112).

This function of Miro1 is especially crucial in neurons, as anterograde mitochondrial transport (from soma to synapses) is necessary to provide ATP and ensure calcium buffering at highly energy-demanding areas, such as synapses (113). An appropriate mitochondrial distribution is even more essential in dopaminergic neurons, because their pacemaking activity requires high-energy supply and makes them more vulnerable to excitotoxicity (113).

Retrograde mitochondrial movement is as well essential for the physiology of neurons *in vivo*, since lysosomal degradation of damaged mitochondria takes place mostly in the neuronal soma (113, 114).

Guo et al. first demonstrated the importance of dMiro for anterograde mitochondrial transport in neurons from *Drosophila* larvae (37). However, other studies reported that the expression of the null alleles B682 and SD32 within the first GTPase domain of dMiro, which cause the loss of the protein through its premature truncation, promoted a reduction in both anterograde and retrograde transport of mitochondria (111). Interestingly, the N-terminal GTPase domain of dMiro seems to be crucial for mitochondrial transport along axons and dendrites (38). In accordance, expression of the N-terminal GTPase loss-of-function mutation dMiroT25N led to premature death and aborted development of *Drosophila puparium*, a phenotype likely due to an accumulation of dysfunctional fragmented mitochondria in the soma of their sensory and motor neurons (38). Recently, inhibition of mitochondrial transport was linked to HDAC6-mediated deacetylation of the N-terminal GTPase domain of Miro1 in rodents (39). In addition, Miro1 knockout mice displayed a reduced number of mitochondria in distal dendrites, accompanied by a lower dendritic complexity and increased neuronal death (10). Of note, these mice are still expressing Miro2, suggesting that although both isoforms (Miro1 and Miro2) are involved in mitochondrial transport, they are not able to fully substitute each other (10).

From the molecular point of view, regulation of mitochondrial transport by mammalian Miro1 and Miro2 proteins occurs through the formation of a complex with the trafficking kinesin-binding proteins 1 and 2, so-called TRAK1 and TRAK2 (2, 115, 116). In particular, the building of the Miro/TRAK1/2 complex on the mitochondrial surface leads to the recruitment of the motor proteins kinesin and dynein for anterograde and retrograde transport, respectively (**Figure 6A**) (3, 117, 118).

However, the involvement of TRAK1 and TRAK2 differentially regulates mitochondrial transport. TRAK1 predominantly facilitates anterograde and retrograde movement in axons via interaction with kinesin or dynein, while TRAK2 is mostly found in dendrites binding to dynein and thus supporting retrograde movement (41, 119).

Mitochondrial transport is regulated by cytosolic calcium levels via the calcium sensor Miro1, and in 2009, two independent studies proposed different mechanisms of how calcium binding to Miro1 regulates mitochondrial transport. MacAskill et al. showed that Kif5 directly binds to Miro1 *in vitro* (93). Upon elevation of calcium levels, the EF-hand domains of Miro1 bind calcium, inducing a conformational shift and a decoupling of Miro1 and Kif5, as shown by co-immunoprecipitation in rat brain samples. Thus, the mitochondrial transport machinery is disassembled in order to derail mitochondria from the cytoskeleton (93).

In contrast, Wang and Schwarz found that kinesin is binding to Miro indirectly via Milton (*Drosophila* homolog of TRAK1/2) (3). Elevation of cytosolic calcium levels and the subsequent calcium binding to Miro allows a direct interaction of Kif5 with Miro, thereby detaching the whole transport machinery complex from the cytoskeleton and stopping mitochondrial transport in HEK cells and rat hippocampal cells. Hence, Kif5 was associated with the Miro/Milton complex on both moving and stationary mitochondria (3). This mechanism of calcium-dependent regulation of transport enables the fine-tuned arrest of mitochondria at sites of high-energy demand and cytosolic calcium levels, i.e., synapses (**Figure 6A**) (42, 103, 104).

Dysfunction of Miro1 causes alterations in mitochondrial transport. In rat hippocampal neurons, overexpression of Miro1 caused an increased recruitment of TRAK2 to the mitochondria, increasing the number of organelles transported to neuronal processes, while disruption of the Miro1-binding domain of TRAK2 led to mitochondrial transport arrest, showing a significant decrease in mitochondrial number in neuronal processes (93).

Miro1 is also known to interact with other PD-related proteins influencing mitochondrial transport in neurons. For example, wild-type LRRK2 and α-synuclein proteins were shown to bind to Miro1 on moving mitochondria, creating a complex

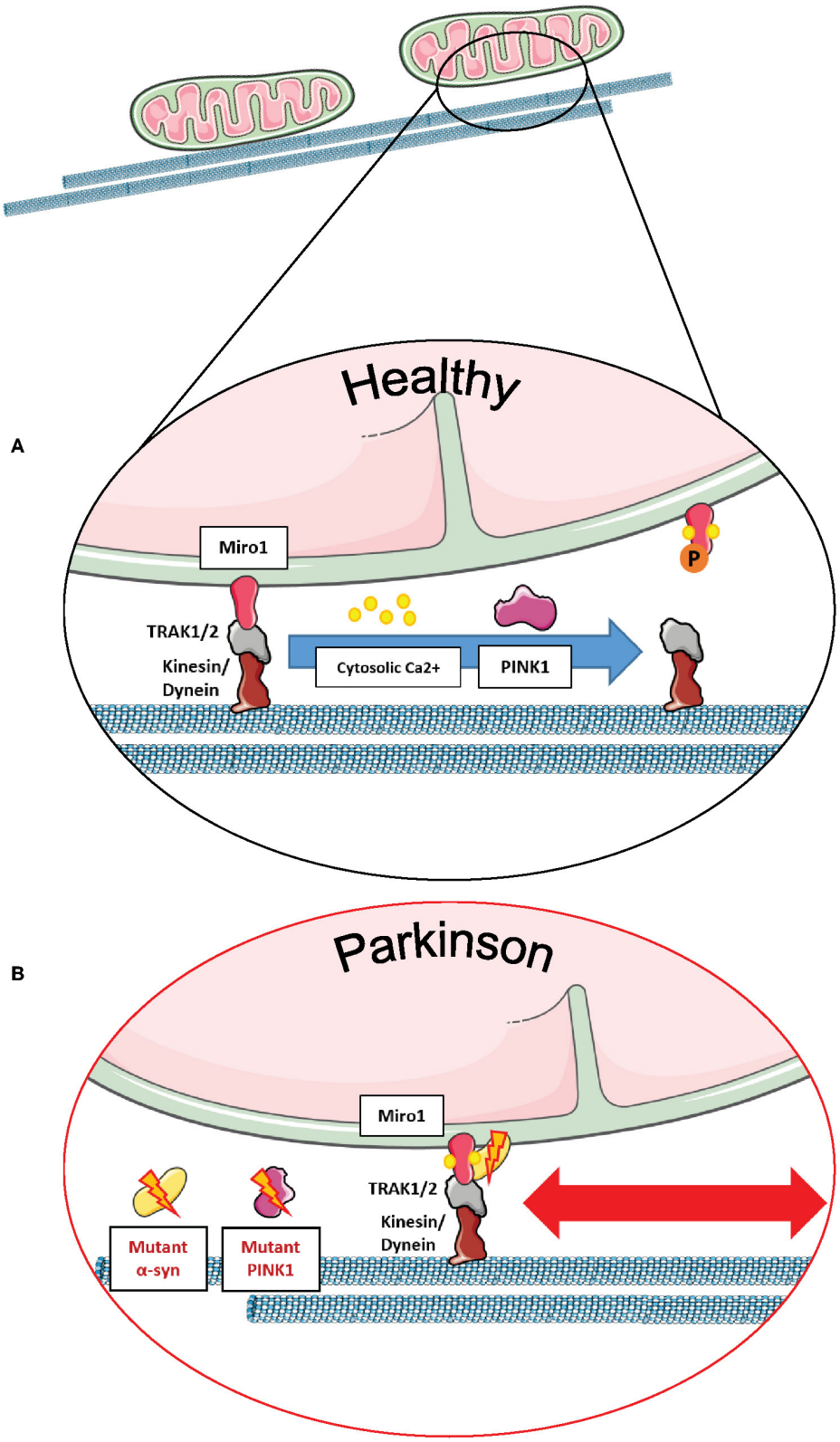

FIGURE 6 | Miro1 mediates calcium-dependent regulation of mitochondrial transport. **(A)** Miro1 is anchored to the outer mitochondrial membrane and links mitochondria to the motor proteins kinesin and dynein via interaction with TRAK1/2. Upon elevation of cytosolic calcium levels (i.e., at active synapses), calcium binds

(Continued)

FIGURE 6 | to the EF-hand domains of Miro1, leading to a conformational shift of Miro1 and a decoupling of mitochondria from the cytoskeleton. Thus, mitochondria are stopped at sites of high calcium levels, providing ATP and calcium buffering. PINK1/Parkin-mediated phosphorylation and ubiquitination of Miro1 and its subsequent proteasomal degradation also leads to arrest of mitochondrial transport, allowing lysosomal degradation of dysfunctional mitochondria. **(B)** Cells expressing PD-associated mutations of α-synuclein or PINK1 failed to remove Miro1 from the surface of impaired mitochondria, causing dysregulation of mitochondrial transport and delayed mitophagy. This figure was created using elements from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (www.smart.servier.com).

that regulates the removal of Miro1 from the OMM and, therefore, promoting the detachment of mitochondria from the transport machinery (11, 12). Interestingly, the G2019S PD-associated mutation in LRRK2 disturbed this complex, inhibiting Miro1 removal from the transport machinery, thus delaying mitochondrial arrest in PD patient-derived fibroblasts and neurons (11). Moreover, the overexpression of wild-type α-synuclein and/or the PD-associated A53T mutation led to the stabilization of the α-synuclein-Miro1 complex in PD flies and human iPSC-derived neurons, preventing mitochondria to detach from the transport machinery and subsequently leading to a delayed mitochondrial arrest (**Figure 6B**) (12).

Furthermore, the PD-related protein PINK1 was also identified as an interaction partner of Miro1 in human neuroblastoma cells and primary fly neurons for the trafficking of mitochondria, and the loss of PINK1 led to aberrations in mitochondrial morphology and dynamics (7, 65). In addition, PD-associated PINK1 deletions promoted the movement of mitochondria via the stabilization of dMiro (**Figure 6B**), consequently leading to synaptic overgrowth and death of *Drosophila* dopaminergic neurons (21). Liu et al. showed in *Drosophila* muscle and dopaminergic neurons that downregulating dMiro could rescue mitochondrial transport and distribution defects observed in mutant PINK1 flies, whereas overexpressing dMiro alone led to mitochondrial enlargement and dopaminergic neuronal death (9).

Intercellular Mitochondrial Transfer

Converging evidence supports the notion that mitochondria can be transferred between mammalian cells, in order to replace damaged organelles and prevent death of the recipient cell. There are different approaches to analyze the transfer of mitochondria between cells in co-cultures. One way is to label mitochondria of donor cells and recipient cells in red or green, respectively, allowing the detection of cells with mixed mitochondria after co-culture (120). Another approach is to label only the mitochondria from the donor cells in order to detect their transfer into recipient cells labeled with GFP, phalloidin, or cell tracker dyes (121–124).

Since the first observation of this phenomenon in human stem cells (125), intercellular transfer of mitochondria was also noticed between healthy and cancer cells (126–128) and, more relevant to neurodegeneration, between astrocytes and neurons. In particular, by using co-culture experiments, Hayakawa et al. demonstrated that transfer of mitochondria from astrocytes to neurons improved mitochondrial function of the latter, resulting in increased neuronal recovery and survival after stroke (129). The molecular mechanisms ensuring intercellular mitochondrial transfer between neuronal cell types have not been fully elucidated yet; either the selective formation of tunneling nanotubes (TNT), the establishment of gap junctions, or the release of extracellular microvesicles containing mitochondria have been observed in different non-neuronal models (126, 128, 130, 131).

Despite the fact that the last stage of the transfer is still being debated, it is widely accepted that intercellular mitochondria donation requires a fully functional mitochondrial transport machinery. In light of its fundamental role in regulating mitochondrial movement along microtubules, a strong body of evidence indicates that Miro1 also plays a key role in mitochondrial transfer between different cell types, including non-neuronal cells (120–123). For instance, inhibition of mitochondrial complex I activity by rotenone treatment significantly decreased Miro1 protein levels in mesenchymal stem cells (MSCs), leading to impaired transfer of mRFP-labeled mitochondria to recipient primary mouse epithelial cells containing mGFP-labeled mitochondria. Consequently, Miro1-depleted MSCs displayed reduced donor activity compared to control cells, a phenotype specifically linked to impaired mitochondrial movement along microtubules (120).

Importantly, Gao et al. recently demonstrated that Miro1, as well as Miro2, participates in the transfer of mitochondria between brain cells, as displayed by a reduced mitochondrial transfer efficiency from neurons expressing mito-DsRed to GFP-labeled astrocytes upon shRNA-mediated Miro1 or Miro2 downregulation. Conversely, mitochondrial transfer increased when Miro1 or Miro2 were ectopically expressed (124).

Since mitochondrial transfer is activated by loss of respiratory function in recipient cells, the presence of functional Miro1 in donor cells is crucial to enhance mitochondrial transfer capacity and rescue mitochondrial dysfunction in injured cells. This mechanism is extremely important for many neurodegenerative diseases including PD, as it may represent a key neuroprotective approach in stem cell-based regenerative medicine. At the same time, the transfer of damaged mitochondria between different cell types may also trigger the spread of PD pathology to other brain regions and therefore needs to be taken into account for the design of targeted therapies (132).

Peroxisomal Transport

Until recently, Miro1 was described as an entirely mitochondrial protein (27, 28, 133). However, in 2017 Costello et al. revealed that Miro1 was also localized to peroxisomes in COS-7 cells (22). The peroxisomal receptor/chaperone PEX19 was found to be necessary for the integration of Miro1 into the peroxisomal membrane (23). Recent findings suggest that the N-terminal GTPase domain regulates the direct interaction of the transmembrane domain of Miro with Pex19 (134).

In 2018, Okumoto et al. showed that the localization of Miro1 to mitochondria or peroxisomes depends on the alternative splicing of exons 19 and 20. The resulting different insertions between the C-terminal GTPase domain and the transmembrane domain determine the organelle-targeting specificity of Miro1. Variant-1 of Miro1 does not contain either the exon 19 or the exon 20 and localizes exclusively to mitochondria. Variant-3 contains exon 20 and is likewise found only on mitochondria. In contrast, variant-2 contains exon 19 and localizes partially to peroxisomes, whereas variant-4 containing both exons 19 and 20 is localized mostly to peroxisomes and to a minor extent to mitochondria (24).

In human cancer cells, Miro1 variants−1 and−2 were predominantly expressed, while variants−3 and−4 showed low expression levels of 10% compared to variants−1 and−2 (24). However, in contrast to this study, Covill-Cooke et al. recently showed that Miro1 variants lacking exon 19 as well as Miro2 are able to localize to peroxisomes in MEFs (134).

Initially, the study of Castro et al. suggested the main function of Miro1 in the regulation of peroxisomal transport and transport-dependent peroxisomal proliferation in fibroblasts (23). Nevertheless, the role of Miro in peroxisomal movement was questioned later. The knockout of Miro1 or Miro2 or a double knockout of both proteins did not reveal any effect on long-range microtubule-dependent peroxisome transport in MEFs (134). Interestingly, knockout of Miro2 revealed a significant reduction in median net displacement of peroxisomes in MEFs. This short-range peroxisomal movement was independent of the integrity of the actin and microtubule cytoskeleton but followed the oscillating movements of the ER, suggesting that Miro might regulate short-range peroxisome transport via the interaction with the ER (134).

While the results of the study by Covill-Cooke suggest that the main function of Miro at peroxisomes is independent of transport, their study demonstrated a major impact of Miro on peroxisome size and number. Double knockout of Miro1 and Miro2 in MEFs caused a significant reduction in peroxisome size, accompanied by increase in peroxisome number (134). This phenotype is likely caused by an increased interaction of the fission proteins Drp1 and Fis1 at peroxisomes, indicating that Miro proteins regulate Fis1-/Drp1-dependent fission not only of mitochondria (135) but also of peroxisomes (134).

Of note, the single knockout of Miro1 or Miro2 had no effect on peroxisome size or number, while overexpression of Miro1, but not Miro2, caused an increase in peroxisome size. This result suggests that peroxisome morphology is mainly regulated by Miro1, and Miro2 has the ability to compensate for Miro1 impairment in peroxisomes (134). This is interesting because other studies demonstrated that Miro2 was not able to compensate for the lack of Miro1 on mitochondrial level in murine brains (10, 136). Future investigations will be necessary to uncover the differential functions of Miro1 and Miro2 in mitochondria and peroxisomes and their impact in neurodegeneration. Peroxisomes are critically involved in lipid metabolism and defense against ROS (137, 138). The physical link to mitochondria and the ER is important for peroxisomal proliferation and function (139, 140). Given the crucial roles of Miro1 at mitochondria and MERCs, further investigations are needed to elucidate Miro1 functions outside of mitochondria.

To date, peroxisomal dysfunction in the pathogenesis of neurodegenerative diseases like PD is not well understood. A previous study showed a reduction in plasmalogen levels in blood plasma of PD patients (25). Plasmalogens are phospholipids synthesized in peroxisomes, which are involved in the defense against ROS. Interestingly, mice deficient in the peroxisomal proteins Pex2, Pex5, or Pex13 showed an elevation of α-synuclein oligomers and α-synuclein phosphorylation in brain tissue. The observed α-synuclein aggregation correlated with changes of peroxisomal lipid synthesis instead of being associated with mitochondrial dysfunction or oxidative stress (26).

The calcium handling function of peroxisomes is largely unknown. Previous studies showed that increased cytosolic calcium concentrations lead to an elevation of peroxisome calcium levels, suggesting that peroxisomes might play a role in calcium homeostasis (141). The newly identified role of Miro1 as adaptor for peroxisomal transport, together with the known function of Miro1 in calcium homeostasis, raises the question whether Miro1 is also involved in peroxisomal calcium handling. Furthermore, it remains to be investigated how calcium transients regulate Miro1-mediated peroxisomal transport and distribution and how this would influence peroxisomal function in the healthy state and in the context of PD.

OUTLOOK

The emerging role of Miro GTPases in brain health and disease provides unique opportunities for a better understanding of neuronal homeostasis and indicates these proteins as potential therapeutic targets and entry-points for precision medicine. Especially from the perspective of neurodegeneration, the roles of Miro1 as an adaptor for mitochondrial transport and as a PINK1/Parkin-mediated mitophagy substrate are of high relevance in the context of brain disorders, in particular for PD. In this review, we showed that Miro1 is not only a crucial component of the mitochondrial transport machinery and mitochondrial quality control, but it is also an important regulator of mitochondrial and cytosolic calcium homeostasis, mitochondria and ER interface, and peroxisomal dynamics. Based on a variety of *in vitro*, *ex vivo*, and *in vivo* studies performed in yeast, animal, and human models, Miro GTPases stand no longer as exclusive mitochondrial proteins, but their recently discovered key functions further extend their physiological role to other organelles and cellular compartments.

Based on genetic studies, a direct link of Miro1 to neurodegeneration in PD was established. Together with the fact that Miro1 physically and functionally interacts with a number of PD-related proteins, Miro1 has recently been proposed both as a molecular signature in PD and as a therapeutic target, which could be used as a biomarker for the diagnosis and treatment of PD (66). Hsieh et al. were able to rescue impaired mitophagy and neuronal cell death by pharmacologically removing excess Miro1. Treatment with the compound called "Miro1 reducer" in combination with CCCP lead to Miro1 degradation and

induction of mitophagy in fibroblasts derived from PD patients. Additionally, iPSC-derived neurons from PD patients displayed significant death after induction of oxidative stress via antimycin, while control neurons did not show cell death under these conditions. Remarkably, treatment with the "Miro1 reducer" rescued iPSC-derived neurons from PD patients under antimycin stress, thereby demonstrating that the removal of excess Miro1 is neuroprotective (66).

Another possible approach for pharmacological intervention was demonstrated by Lee et al. in 2016. This study revealed that the recruitment of Miro1 to MERCs depends on the phosphorylation of the N-terminal GTPase domain by Polo kinase, thereby critically regulating mitochondrial calcium uptake and mitochondrial energy production (84). Specific inhibition of Polo kinase with BI2536 reduced the localization of Miro1 to MERCs and also caused a destabilization of MERCs (84). This finding is especially interesting in the light of increased numbers of MERCs and enhanced localization of Miro1 to MERCs observed in iPSC-derived Miro1-R272Q neurons (67). Thus, pharmacologically targeting regulators of Miro1 function such as Polo kinase offers another promising approach for personalized medicine but also bears the risk of unwanted side effects.

Future studies should focus on the impact of Miro1 on neuronal homeostasis, and the establishment of screening campaigns on cellular phenotypes in patient-based cellular models should be performed to rescue impaired Miro1 function. Identified compounds may be applicable to patients beyond monogenic PD, as impaired Miro1 function was also identified in sporadic PD (11, 12, 66). Further applications beyond PD may be also considered, as functional associations between Miro1 and key proteins causative of other neurodegenerative diseases, such as Alzheimer's disease (142, 143), amyotrophic lateral sclerosis (144, 145), and Charcot–Marie–Tooth disease (146), were discovered during the past years.

AUTHOR CONTRIBUTIONS

DG and RK designed and organized the structure of the review. DG, CB-E, AC, and GA developed the writing of the original review draft. DG, CB-E, GA, and RK performed the revision and editing of the original draft. DG, CB-E, AC, and GA developed all the literature research for the writing of this review. Finally, CB-E performed the figures contained in the review.

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Identification of Disease-Associated Variants by Targeted Gene Panel Resequencing in Parkinson's Disease

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Background: Recent advanced technologies, such as high-throughput sequencing, have enabled the identification of a broad spectrum of variants. Using targeted-gene-panel resequencing for Parkinson's disease (PD)-associated genes, we have occasionally found several single-nucleotide variants (SNVs), which are thought to be disease-associated, in PD patients. To confirm the significance of these potentially disease-associated variants, we performed genome association analyses, using next-generation target resequencing, to evaluate the associations between the identified SNVs and PD.

Methods: We obtained genomic DNA from 766 patients, who were clinically diagnosed with PD, and 336 healthy controls, all of Japanese origin. All data were analyzed using Ion AmpliSeq panel sequences, with 29 PD- or dementia-associated genes in a single panel. We excluded any variants that did not comply with the Hardy–Weinberg equilibrium in the control group. Variant frequencies in the PD and control groups were compared using PLINK. The identified variants were confirmed to a frequency difference of $P < 0.05$, after applying the Benjamini–Hochberg procedure using Fisher's exact test. The pathogenicity and prevalence of each variant were estimated based on a public gene database.

Results: We identified three rare variants that were significantly associated with PD: rs201012663/rs150500694 in *SYNJ1* and rs372754391 in *DJ-1*, which are intronic variants, and rs7412 in *ApoE*, which is an exonic variant. The variants in *SYNJ1* and *ApoE* were frequently identified in the control group, and rs201012663/rs150500694 in *SYNJ1* may play a protective role against PD. The *DJ-1* variant was frequently identified in the PD group, with a high odds ratio of 2.2.

Conclusion: The detected variants may represent genetic modifiers or disease-related variants in PD. Targeted-gene-panel resequencing may represent a useful method for detecting disease-causing variants and genetic association studies in PD.

Keywords: next generation sequencing, panel resequencing, genetic association study, Parkinsion's disease, missing heritability

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INTRODUCTION

Parkinson's disease (PD) is the second-most frequent neurodegenerative disorder, associated with motor and non-motor symptoms (1). Clinical symptoms are characterized by tremor, rigidity, bradykinesia, and gait disturbances. To date, advanced genetic methods have revealed several genes associated with both familial and sporadic PD (2). Initially, genes were identified based on large pedigrees associated with Mendelian forms of PD, using positional cloning and linkage analyses, which resulted in the identification of *SNCA* (3), *LRRK2* (4, 5), *PRKN* (6), and *PINK1* (7). Later, next-generation sequencing (NGS) was used to identify additional causative genes, such as *ATP13A2* (8), *CHCHD2* (9), *VPS13C* (10), and *PSAP* (11). Furthermore, genome-wide association studies (GWASs) have identified single-nucleotide variants (SNVs) and other rare variants associated with sporadic PD (12–14). The explained heritability ranged from 16 to 36%, even in the latest large GWAS, as reported in 2019 (15).

Many genome-association studies, including GWAS, have been conducted for PD; however, some unrevealed genetic background remains, referred to as "missing heritability" (13, 14, 16). Missing heritability is the difference between heritability estimated from twin studies and GWAS, as GWAS has only been able to detect some of the heritability estimated from twin studies (17). Many explanations for missing heritability have been proposed, including unrevealed variants with smaller effects, rarer variants that are poorly detected by the currently available genotyping arrays, copy number variants that cannot be detected by available arrays, and the low power to detect gene–gene interactions (16). Variants of *GBA* are known to be strong risk factors for sporadic PD but have not been detected by GWAS, likely due to a low minor allele frequency.

We have developed a targeted-gene-panel resequencing protocol to screen 29 PD-associated genes, simultaneously. Panel resequencing has both advantages and disadvantages because it can identify multiple types of variants, including pathogenic variants, risk-associated variants, and rare variants of uncertain significance. Therefore, determining which variants are disease-associated can be difficult. A previous report describing Mendelian genes showed that rare functional variants occurred more frequently in sporadic PD cases than in control cases, indicating that Mendelian genes may be associated not only with familial PD but also with sporadic PD, which may be assessable using panel resequencing (18). In our analyses, through targeted-gene-panel resequencing, rare variants were identified in ∼40% of PD patients with a family history or early-onset PD (data not shown), and pathogenic variants were found in an even smaller percentage of patients. We also identified several putative disease-associated variants in PD patients. We hypothesized that these variants may play a role in PD onset and could account for some degree of missing heritability. Thus, we aimed to implement target-panel resequencing, to identify associations between SNVs and familial or early-onset PD. Our method contributes to expanding the understanding of missing heritability among familial and early-onset PD patients.

MATERIALS AND METHODS

Participants

The present study was approved by the ethics committee of Juntendo University, Tokyo, Japan, and all participants provided written informed consent to participate in the genetic research. We collected DNA samples from the Juntendo PD DNA bank, which included 766 patients with PD, who were clinically diagnosed using standard criteria (1), and 336 healthy control subjects. Among these, 407 PD patients had a family history of PD (average age at onset: 54.6 ± 15.77 years, range 6–88), and the remaining 359 PD patients were without family history (average age at onset: 42.0 ± 11.22 years, range 9–83). We also collected data regarding the Hoehn and Yahr stages for each PD patient. The healthy controls were defined as individuals without any individual or family history of neurodegenerative disorders. An overview of the clinical characteristics of the included PD patients and healthy controls is shown in **Table 1**.

Processing Data Output From the Ion Torrent System

The sequencing analysis of the Ion AmpliSeq panel (Thermo Fisher Scientific, Waltham, MA, USA) was performed using the Ion Chef System (Thermo Fisher Scientific) and the Ion S5 Sequencer (Thermo Fisher Scientific), according to the manufacturer's instructions. Our Ion AmpliSeq panel (Thermo Fisher Scientific, IAD103177_182) included 29 PD- and dementia-related genes (**Table 2**), and its coverage was 98.34% (829 amplicons, missed: 1,646 bp) (manuscript in preparation). The output data were obtained as a variant call format (VCF) file from the Ion torrent system. VCF files were processed using vcftools (19).

Statistical Analysis to Compare the Frequencies of Non-rare Variants

We confirmed all samples with a mean depth >100 and excluded those amplicons with read depths smaller than 10. The analyzed variants were confirmed to exist among the target sequences and to have read depth of coverages >45. We also calculated the coverage percentage. During the variant-screening stage, we excluded all variants that did not comply with Hardy–Weinberg equilibrium (HWE; $P < 0.05$) within the control group (**Figure 1**). We analyzed only the control group during the variant-screening stage because performing HWE analysis while including PD patients would introduce bias. During the analysis stage, the variant frequencies observed for the PD and healthy non-PD groups were compared using PLINK 1.9 (20). To verify this comparison, variants with a frequency difference of $P < 0.05$, based on the performance of the Benjamini–Hochberg procedure and Fisher's exact test, were analyzed using the genotyping data available in 4.7KJPN, from the Japanese Multi Omics Reference Panel (jMorp) (21), and a genome aggregation database (gnomAD) (22). The scheme used for the analysis is presented in **Figure 1**. To confirm the presence of significant variants identified during the association study, we conducted Sanger sequencing on three cases with the variant and three cases without the variant, during the panel resequencing experiment.

TABLE 1 | Demographic data of the analyzed subjects.

	PD patients	Controls
Total number of subjects	766	336
Gender (female:male)	366:400	114:222
Average age at onset	48.6 ± 15.35	NA
Average age at examination	57.0 ± 14.14	62.2 ± 16.36
Hoehn and Yahr stage (On phase)	2.32 ± 1.06	NA
Hoehn and Yahr stage (Off phase)	3.09 ± 1.83	NA
Subjects with known pathogenic mutations	61	0
Subjects with family history	407	NA
Average age at onset	54.6 ± 15.77	NA
Average age at examination	62.6 ± 13.57	NA
Subjects without family history	359	NA
Average age at onset	42.0 ± 11.22	NA
Average age at examination	50.7 ± 11.88	NA

PD, Parkinson's disease; NA, not applicable.
The data are presented as the mean ± standard deviation.

TABLE 2 | PD- and dementia-related genes analyzed by resequencing.

Genes related to PD	Genes related to dementia
SNCA (PARK1,4)	*MAPT*
parkin (PARK2)	*PSEN1*
UCH-L1 (PARK5)	*GRN*
PINK1 (PARK6)	*APP*
DJ-1 (PARK7)	*APOE*
LRRK2 (PARK8)	
ATP13A2 (PARK9)	
GIGYF2 (PARK11)	
HTRA2 (PARK13)	
PLA2G6 (PARK14)	
FBXO7 (PARK15)	
VPS35 (PARK17)	
EIF4G1 (PARK18)	
DNAJC6 (PARK19)	
SYNJ1 (PARK20)	
DNAJC13 (PARK21)	
CHCHD2 (PARK22)	
VPS13C (PARK23)	
GCH1	
NR4A2	
RAB7L1	
BST1	
C19orf12	
RAB39B	

RESULTS

The percentage of coverage was calculated, showing that 99.7% of the total dataset was read at a depth of least 1×, 98.9% at 20×, 97.9% at 100×, and 84% at 500×. During the variant screening stage, we identified 796 variants in our healthy controls, of which 749 were retained after screening for HWE compliance ($P < 0.05$) and were included in the association analysis performed using PLINK. We conducted Sanger sequencing on nine significant variants with *p*-values below 0.05 after performing the Benjamini–Hochberg procedure for Fisher's exact test, and five of them (chr1:65830299 T>G, chr1:65830300 T>G, chr3:184033555, chr2:233620927-233620929, and chr1:205743943) were not validated and excluded from the analysis. All of the false-positive variants were positioned around the tandem repeat of mononucleotides that was considered to cause false positives. **Table 3** shows the top 15 variants that had the lowest *p*-values based on Fisher's exact test.

Four variants were significantly associated with PD: rs201012663 and rs150500694 in *SYNJ1*, rs372754391 in *DJ-1*, and rs7412 in *ApoE* (**Table 4**). The two *SYNJ1* variants, rs201012663 and rs150500694, were considered to represent a single variant because they are located four bases apart and demonstrated the same frequency in our subjects and public gene databases, which suggests that these variants are strongly linked (**Tables 3**, **4**). The *SYNJ1* variants are both located in an intron, with an odds ratio of 0.37. The *DJ-1* variant (rs372754391) was also intronic and was more frequently identified in the PD cohort than in controls, with an odds ratio of 2.2. However, its frequency in the public database was quite large compared with the frequency in our data. The *ApoE* variant was exonic and was more frequently observed in the control group than in the PD group, with an odds ratio of 0.39. The *ApoE* variant was one of the single-nucleotide polymorphisms (SNPs) that determine the *ApoE* genotype. The E2 *ApoE* genotype was more frequently observed in the control group, whereas the E4 genotype was more frequently observed in the PD group

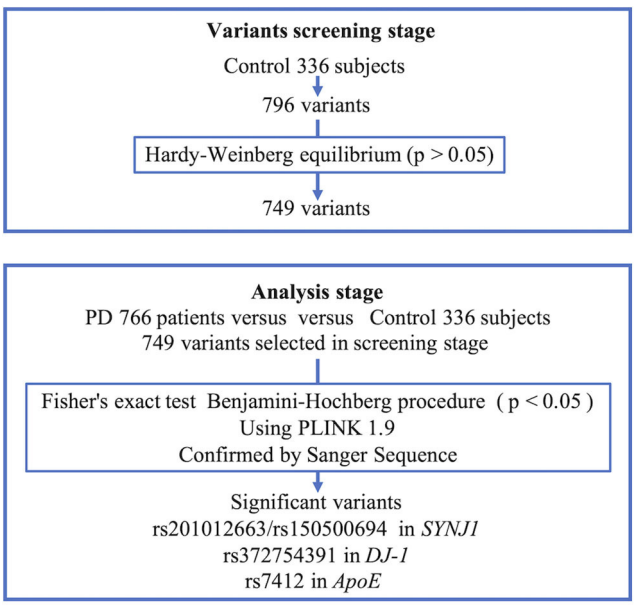

FIGURE 1 | Scheme of analysis. PD, Parkinson's disease; *SYNJ1*, Synaptojanin 1; *APOE*, Apolipoprotein E.

TABLE 3 | Top 15 variants detected in genome association studies.

Ranking	Position	rs number	Gene	AF in PD	AF in control	UNADJ	GC	BONF	SIDAK_SS	SIDAK_SD	FDR_BH	Confirmed by Sanger sequencing
1	chr21:34050937	rs201012663	*SYNJ1*	0.2004	0.4048	1.06E-23	1.66E-08	7.76E-21	INF	INF	7.76E-21	Yes
1	chr21:34050941	rs150500694	*SYNJ1*	0.2004	0.4048	1.06E-23	1.66E-08	7.76E-21	INF	INF	7.76E-21	Yes
3	chr1:8029508-8029510	rs372754391	*DJ-1*	0.09465	0.04464	6.41E-05	0.02459	0.04708	0.04599	0.04563	0.006726	Yes
4	chr19:45412079	rs7412	*APOE*	0.01762	0.04315	0.0004541	0.04863	0.3337	0.2838	0.2815	0.04172	Yes
5	chr1:17328732	NA	*ATP13A2*	0	0.00744	0.0007247	0.05734	0.5327	0.4131	0.4097	0.05919	N/A
6	chr22:32887150	rs9726	*FBXO7*	0.2454	0.186	0.002182	0.08487	1	0.7993	0.7953	0.1316	N/A
7	chr21:34037203	rs142813430	*SYNJ1*	0.003264	0.01488	0.002259	0.08592	1	0.8103	0.8059	0.1316	N/A
8	chr3:132221076	rs11293788	*DNAJC13*	0.03264	0.01042	0.002481	0.08886	1	0.8389	0.8345	0.1316	N/A
9	chr3:132194001	rs11920646	*DNAJC13*	0.06462	0.03274	0.002485	0.08892	1	0.8394	0.8345	0.1316	N/A
10	chr1:17323061	NA	*ATP13A2*	0	0.005952	0.002507	0.08919	1	0.8419	0.8367	0.1316	N/A
11	chr1:65830301	rs372128441	*DNAJC6*	0.01828	0.002976	0.004319	0.1085	1	0.9585	0.9559	0.21	N/A
12	chr21:27369838	rs2051503	*APP*	0.1012	0.1429	0.00457	0.1108	1	0.9655	0.9631	0.21	N/A
13	chr12:40758652	rs371863	*LRRK2*	0.4204	0.4836	0.005873	0.1214	1	0.9868	0.9855	0.2539	N/A
14	chr21:27348447	rs3737414	*APP*	0.3551	0.4152	0.007258	0.1311	1	0.9953	0.9946	0.2835	N/A
15	chr15:62226277	rs1009641857	*VPS13C*	0.06919	0.04018	0.008459	0.1387	1	0.9981	0.9977	0.2835	N/A

UNADJ, Unadjusted, asymptotic significance value; GC, Genomic control-adjusted significance value; BONF, Bonferroni-adjusted significance value; SIDAK_SS, Sidak single-step-adjusted significance value; SIDAK_SD, Sidak step-down-adjusted significance value; FDR_BH, Benjamini and Hochberg (1995) step-up FDR control; N/A, not applicable.

(**Table 5**). No significant differences in age, age at onset, or Hoehn and Yahr scores were observed between patients with and without detected variants (**Table 6**).

We do not have data for four variants (rs16856139, rs11931532, rs11931074, and rs1994090) that were previously identified in a GWAS performed in Japanese PD patients because these variants were absent from our target panel (13). *LRRK2* G2385R (rs34778348), which is a risk factor for PD in East Asian individuals, was the 21st most significant variant identified among our cohort (23). Except for rs34778348, none of the currently known risk variants for PD were detected.

DISCUSSION

We performed a genetic case–control analysis, using NGS data from our Ion AmpliSeq panel. We identified three variants in three different genes: the combination of rs201012663 and rs150500694 in *SYNJ1*, rs372754391 in *DJ-1*, and rs7412 in *ApoE*. None of these three variants were reported as PD-related variants when we searched a GWAS catalog on June 8, 2020 (24). Our identified variants might account for missing heritability in PD. Targeted resequencing could perform deeper reads of selected genes associated with phenotypes than the microarrays that are normally used in GWAS. Thus, targeted resequencing-based association studies may be able to identify risk variants that have not been previously identified by GWAS (17).

The three identified variants have never previously been reported as variants associated with PD. In our study, variants in *SYNJ1* (rs201012663 and rs150500694) showed a higher frequency in the control group than in the PD group. *SYNJ1* is known to be a causative gene for early-onset Parkinsonism, with atypical characteristics, such as seizures, dystonia, and dementia, with an autosomal-recessive inheritance pattern (25, 26). This gene encodes the protein Synaptojanin 1, a polyphosphoinositide phosphatase that is concentrated at synapses (27, 28). Synaptojanin 1 is associated with synaptic vesicle endocytosis. The variants identified in *SYNJ1* (rs201012663/rs150500694) in this study have not previously been reported to be pathogenic variants. Synaptojanin 1 is also known to play a role in the pathogenesis of Alzheimer's disease (AD), associated with a PI (4, 5)P_2 imbalance. The haploinsufficiency of *SYNJ1* protects cells from the neurotoxic actions of Aβ42 (29). The variants rs201012663/rs150500694 might play a similarly protective role against alpha synuclein-mediated neurotoxicity.

The identified variant in *DJ-1* might be interesting, due to the high odds ratio of 2.2. However, this variant may be specific to ethnicity because the frequency of this variant among our healthy controls was lower than that observed in public databases. This variant was not recorded in jMorp, one of the largest genomic databases in Japan, suggesting its rarity in the Japanese population. *DJ-1* was initially identified as an oncogene and was later found to cause familial PD (30). *DJ-1* has also been associated with other disorders, including stroke, familial amyloidotic polyneuropathy, and type 2 diabetes

TABLE 4 | Details of the detected variants with significant value.

Gene symbol	*SYNJ1*	*DJ-1*	*APOE*
dbSNP153	rs201012663 rs150500694	rs372754391	rs7412
Position	chr21:34050937-34050941	chr1:8029508-8029510	chr19:45412079
Reference/alternative	AATATA/AATT	GGG/GAA	C/T
Exon or intron	Intron	Intron	Exon
Allele frequency in PD	0.2004 (307/1,532)	0.09465 (145/1,532)	0.01762 (27/1,532)
Genotype in PD (alt/alt, alt/ref, ref/ref)	70, 167, 529	4, 137, 625	0, 27, 739
Allele frequency in control	0.4048 (272/672)	0.04464 (30/672)	0.04315 (29/672)
Genotype in control (alt/alt, alt/ref, ref/ref)	61, 150, 125	1, 28, 307	0, 29, 307
Odds ratio	0.367	2.237	0.398
gnomAD 2.1 EAS	NA	NA	0.07511 (947/12,608)
gnomAD 3.0 EAS	0.4181 (1,297/3,102) 0.4178 (1,297/3,104)	0.6263 (1904/3040) 0.6352 (1,936/3,048)	0.08029 (251/3,126)
jMorp	0.4257 0.4257	NA	0.044
p-value (PD vs. gnomAD 3.0)	<0.001	<0.001	<0.001
p- value (control vs. gnomAD 3.0)	0.2767	<0.001	0.0003

SYNJ1, Synaptojanin 1; APOE, Apolipoprotein E; PD, Parkinson's disease; gnomAD, genome aggregation database; EAS, east Asia; NA, not applicable; jMorp, Japanese Multi Omics Reference Panel; dbSNP, the Single Nucleotide Polymorphism Database; alt, alternative allele; wild, reference allele.
The variant in SYNJ1 is recorded separately as rs201012663 and rs150500694 in dbSNP153, in gnomAD, and in jMorp.
The variant in DJ-1 is recorded as rs372754391 in dbSNP153 and registered separately as two single variants in gnomAD.

TABLE 5 | Allele frequencies in patients, according to *APOE* genotype.

	PD ($n = 1,532$)	Controls ($n = 672$)	
Genotype of *APOE*	Allele frequency (%)	Allele frequency (%)	*p*-value (PD vs. control)
E1	0	0	NA
E2	1.76	4.32	0.0006
E3	86.88	87.5	0.3728
E4	11.36	8.18	0.0137

E, epsilon; PD, Parkinson's disease; NA, not applicable; APOE, Apolipoprotein E.

(30–33). DJ-1 has several functions, including transcriptional regulation, antioxidative stress reactions, chaperone, protease, and mitochondrial regulation (30). DJ-1 is expressed in almost all cells, including neurons and glial cells. DJ-1 protein contains three cysteine residues, C46, C56, and C106. C106 is likely to be influenced by oxidative stress and oxidized into SOH, SO_2H, and SO_3H (34–36). DJ-1 containing a C106 residue that has been oxidized to SO_3H is thought to represent an inactive form (37). In the brains of PD patients, excessively oxidized forms of DJ-1 have been observed (38). The identified mutation might facilitate oxidation, inactivating DJ-1.

APOE genotypes have previously been associated with an increased risk of AD (39, 40). rs7412 is one of two SNVs that have been defined in common allelic *APOE* variants. *APOE4* is known to represent a strong risk factor for AD. The variant (rs7412) identified in our study is included in *APOE1 or APOE2*, which are known to decrease the risk of AD. rs7412 was significantly rare in the PD group in our study. In our study, *APOE2* was significantly rare in the PD group, whereas *APOE4* was significantly frequent in the PD group. Larger research studies have concluded that *APOE* epsilon had no association with PD onset (41). Differences between our study and past studies may be due to the smaller sample size included in our study and differences in the ethnicities of the participants.

In our study, SNVs detected in previous GWAS were not identified in our cohort because most of the reported risk-associated SNVs have been identified in non-coding regions, which were not included in our targeted panel (42). Targeted resequencing can cover more SNVs within the targeted exons than DNA microarrays, which are commonly used in GWAS. Our method might enable the detection of SNVs in exons or near exons that are not included in the SNP chips used for GWAS. Our target panel was designed to include all exons and the 25 bp up- and downstream of the exon–intron boundaries. Therefore, our method allowed the discovery of PD-related variants that were not detected by GWAS. The inclusion of patients with a family history or early-onset PD in our cohort might facilitate the detection of susceptibility-associated variants, with deep genetic backgrounds. For example, mutations in *GBA* are more frequently identified in familial PD patients than in sporadic PD patients (43). However, our panel resequencing approach also has several disadvantages. This approach cannot be used to identify novel genes associated with PD and does not cover the majority of introns and transcriptional regulatory regions. The variants detected in this study may also be associated with sporadic PD, similar to GWASs that identified causative genes associated with sporadic PD that were previously reported to be causative genes for familial PD (*SNCA, MAPT*, and *LRRK2*) (44).

Our study includes the following limitations: (i) the sample size is too small to satisfy genome-wide significance, (ii) the lack

TABLE 6 | Clinical characteristics of patients, according to the presence of the identified variants.

	Presence of the variant	Age	Age at onset	Hoehn and Yahr scale	Disease duration (years)
SYNJ1 rs201012663 rs150500694	+	58.0 ± 17.73	48.5 ± 15.67	2.3 ± 1.11	9.35 ± 9.18
	−	56.6 ± 15.28	48.7 ± 15.22	2.33 ± 1.05	8.01 ± 8.14
	p-value	0.209200773	0.96096438	0.81537244	0.05363004
DJ-1 rs372754391	+	57.0 ± 15.06	49.9 ± 15.48	2.41 ± 1.11	7.07 ± 6.96
	−	57.0 ± 24.72	48.4 ± 23.42	2.30 ± 1.06	8.73 ± 8.78
	p-value	0.972443834	0.304154621	0.315455549	0.016077725
APOE rs7412	+	58.8 ± 14.44	49.3 ± 15.92	2.10 ± 0.88	9.48 ± 9.75
	−	57.0 ± 14.14	48.7 ± 15.25	2.33 ± 1.07	8.39 ± 8.45
	p-value	0.60125477	0.84599687	0.22211054	0.57046758

SYNJ1, Synaptojanin 1; APOE, Apolipoprotein E.
Data are presented as the mean ± standard deviation.
The p-values were calculated by Student's t-test, comparing PD patients with the variant with those without the variant.

of a second cohort to confirm our results, (iii) the possibility of sampling bias in the control group because the allele frequencies of variants in the public database were different from those identified in our healthy control group, (iv) the absence of any functional analysis to support our results, and (v) the lack of copy number variant evaluations.

We developed a new approach for surveying susceptibility-associated variants by using targeted resequencing, which may represent an effective method for revealing hidden disease-associated variants. Further studies that include additional patients remain necessary to confirm the suitability of this approach for the identification of disease-associated variants.

DATA AVAILABILITY STATEMENT

The DNA sequence data of 1,102 participants used in this study are based on the informed consent of genetic testing from all participants according to the formal procedure approved by the Juntendo University School of Medicine Ethics Committee. However, some participants have refused to publish their DNA sequence data in public databases. Therefore, if the reader wishes to use the raw data used in this paper, please request directly to the corresponding author Manabu Funayama, funayama@juntendo.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Juntendo University School of Medicine Institutional Review Board (No. 2019227). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KD designed and performed the experiments, analyzed the data, wrote the first manuscript, and revised the manuscript. MF designed the study, wrote the first manuscript, and revised the manuscript. YL, HY, AH, AI, KO, and KN performed the experiments, analyzed the data, and revised the manuscript. NH directed the research project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Clinical and Epidemiological Aspects of Parkinson's Disease in the South of Western Siberia

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Background: The article is devoted to one of the most common neurodegenerative diseases in the world—Parkinson's disease (PD), the prevalence of which in Russia reaches 140–150 people per 100,000 people. The clinical and anamnestic profile of a patient with PD is presented, the prevalence of motor and non-motor symptoms is reflected, and a comparative characteristic of the neurological deficit in the Siberian population of patients with other cohorts of patients with Parkinson's disease in different countries and ethnic groups is presented.

Methods: We studied 140 patients with Parkinson's disease. A comprehensive assessment of neurological status was performed using the "Unified Parkinson's Disease Rating Scale (UPDRS)." In addition, we used the Beck Depression and MoCA scale test. Assessment of the presence and severity of olfactory dysfunction was performed using the Sniffin Stick odor identification test. The stage of PD was evaluated according to the classification of M. M. Hoehn and M. D. Yahr.

Results: The cohort of the study was dominated by overweight patients with a higher level of education, with concomitant arterial hypertension, coronary heart disease, and dyslipidemia. The severity of motor and most non-motor symptoms directly correlates with the duration of PD and the stage of the disease. The predominant form of the disease was a mixed form, which was also noted in research cohorts in Canada and the UK. The Siberian cohort tends to be more prevalent in hyposmia, daytime sleepiness, orthostatic hypotension, and depressive and REM disorders.

Conclusion: Our data show the importance of a comprehensive assessment of both motor and non-motor neurological deficits as well as the analysis of comorbid disorders and risk factors for the occurrence and progression of Parkinson's disease. They also show the prevalence of certain motor and non-motor symptoms in the Siberian cohort of patients with Parkinson's disease.

Keywords: Parkinson's disease, Siberia (Russia), hyposmia, cognitive impairment, depression

BACKGROUND

Parkinson's disease is a chronic progressive brain disease mainly associated with the degeneration of dopaminergic neurons of the substantia nigra with intraneuronal accumulation of the α-synuclein protein and the formation of intracellular inclusions (Lewy bodies). These are manifested by a combination of hypokinesia with rigidity, resting tremor, and postural instability, as well as a wide range of non-motor manifestations (mental, autonomic, sensory, etc.) (1–3).

Parkinson's disease is classified as a predominantly motor disorder, although clinical non-motor polymorphism in recent years has been the subject of extensive research (4–6). This is explained by a significant influence of non-motor symptoms on the quality of life, the progression of disability sometimes exceeding the negative effects of motor neurological deficit (7–9).

To date, there are no specific instrumental or laboratory markers of this disease that could be reliably used in everyday clinical practice. Despite the rapid scientific and technological advances in medicine, the diagnosis of PD in routine practice remains clinical and relies on the identification of cardinal motor signs of parkinsonism (hypokinesia, rigidity, rest tremor, and postural disorders) and the absence of atypical symptoms. In relation to this, a rigorous study of the clinical features and characteristics of the course of the disease is extremely important and is the key to success in a complex diagnostic process.

The study of the features of the course of various progressive diseases, including Parkinson's disease, is relevant in connection with the climatogeographic features of the Omsk Region, located in the south of the West Siberian Plain. The geographical location features open this territory for the interference of air masses: northern Arctic, warm southern Central Asian, dry western Central Asian, and cold eastern. The different nature of the air flow leads to sharp changes in temperature, making the whole weather of the region unstable. The climate of Omsk is continental temperate. According to https://world-weather.ru, obtained during long-term observations of air temperature, it is possible to present all the features of the climate in the Omsk region:

The lowest average temperature is −16.9°C (in January).
The highest average temperature is +18.9°C (in July).
Absolute minimum is −50°C.
Absolute maximum is +40°C.

From these data, it can be seen how large the temperature fluctuations during the year: 40° between average values and 90° between the minimum and maximum. This is one of the features of the continental climate. Omsk is characterized by the predominance of clear sunny days even in the autumn-winter period—from 223 to 300 during the year. The cloudiest month is December; in May, the number of such days is minimal. These climatogeographic features may probably influence the course of chronic progressive diseases, such as Parkinson's disease.

In accordance with the urgency of the problem, a retrospective clinical and epidemiological assessment of the clinical course of Parkinson's disease in the south of western Siberia was performed at Omsk State Medical University.

In accordance with the urgency of the problem presented at the Omsk State Medical University, a retrospective clinical and epidemiological assessment of the clinical course of Parkinson's disease in the south of western Siberia was carried out. The aim of our study is to assess the impact of climatic, geographical, and ethnic factors on the course of Parkinson's disease in the Siberian cohort of patients.

METHODS

Patients

All patients (Russians residing in the Siberian part of Russia) were diagnosed with PD at the Omsk State Medical University. All patients with PD were selected and studied according to the international Unified Parkinson's Disease Rating Scale (UPDRS) and Hoehn and Yahr scores (10, 11). The diagnosis of PD was based on the UK PD Brain Bank Criteria (12). In addition, the Beck Depression Scale and Montreal Cognitive Assessment (MoCA) test was used (13). The severity of olfactory dysfunction was performed using the Sniffin Stick odor identification test.

In this work, we evaluated the clinical and epidemiological features of the course of the disease in 140 patients with Parkinson's disease. A retrospective analysis of medical documentation and an assessment of clinical parameters at the onset of the disease and at the time of the examination were carried out.

These were the criteria for inclusion of patients in the study:

- A reliable diagnosis of Parkinson's disease in accordance with the criteria of the European Federation of Neurological Societies (EFNS) in conjunction with the Movement Disorders Society 2013.
- Signed informed consent to participate in the study.
- The absence of other neurodegenerative diseases in the patient.

Criteria for exclusion of patients from the study:

- Secondary parkinsonism and parkinsonism—plus identified at the initial visit.
- The presence of severe concomitant somatic pathology in the stage of decompensation.
- The patient's refusal to participate in the study.
- Patient involvement in other clinical studies.
- The patient has other diseases that have a genetic component in the pathogenesis, due to the high risk of distorting the information received.

Statistical Analysis

Descriptive statistics for qualitative accounting features are presented in the form of absolute values, percentages, their standard errors (m), and standard deviations (σ). Data for variational series with non-parametric distribution are described as medians and quartiles (Me [25th; 75th percentile]). For comparison of non-parametric data, the Mann–Whitney *U* criteria were used. The critical level of significance of the tests is determined at $p \leq 0.05$. Statistical processing of the results was carried out using Statistica 10 licensed software packages (StatSoft, USA).

TABLE 1 | Risk/protective factors for patients with Parkinson's disease.

Risk/protective factors	Patients with PD (abs)	Patients with PD (%)
Industrial contact with pesticides	8	5.7
Household contact with pesticides	44	31.4
Smoking	31	22.1
Quit smoking after the onset	6	4.3
Never smoke	103	73.6
Drink at least 1 cup of coffee per day	78	55.7
Do not drink coffee	62	44.3
Mild traumatic brain injury	14	10

TABLE 2 | Comorbidity of patients with Parkinson's disease.

Diseases	Patients with PD (abs)	Patients with PD (%)
Ischemic stroke	4	2.9
Malignant neoplasms	3	2.1
Coronary heart disease	44	31.4
Arterial hypertension	55	39.3
Dyslipidemia	28	20
Diabetes	12	8.6
Hyperthyroidism	3	2.1

The study was approved by the Ethics Committee of the Omsk State Medical University.

RESULTS

According to inclusion/exclusion criteria, 140 patients with Parkinson's disease were included in the study, including 55 men and 85 women (the majority of patients had PD with the overage duration of 3–10 years with the symptoms effectively managed by combination of adequate treatment options (dopamine agonists, levodopa, amantadine). The ratio of men and women in the group was 1:1.5, with a predominance of females. The age of patients ranged from 37 to 82 years (median [25th; 75th percentile], 67 years [61; 73]): women, 85 (66.3 ± 9.5; age, 68 years [61; 72]), and men, 55 years (66.5 ± 9.7; age, 68 years [61; 74]). The average age of all patients at the time of the examination was 66.4 ± 9.5 years. Treated patients with PD received different medications (dopamine receptor agonists: pramipexole in a dosage of 1.5 mg/day or piribedil in a dosage of 150 mg/day, L-dopa in a dosage of 150–200 mg/day, and amantadine in a dosage of 300 mg/day), either as monotherapy or in various combinations.

The studied group of patients consisted of 14 (10%) Mongoloids and 126 (90%) patients of the European race. By the level of education, the group was divided into the following categories: 13 (9%) people had secondary education, 25 (18%) patients received secondary special education, and 102 (73%) patients graduated from higher educational institutions.

The average weight in the main group was 76.0 ± 14.3 kg (73.5 [65; 86]). The average height in the study group was 165.4 ± 8.0 cm (164.0 [160; 170]). Thus, the average body mass index is 27.8 ± 4.7 (27.0 [24.7; 30.7]), which indicates the predominance of patients with overweight.

The study conducted an analysis of risk factors for the development of PD and protective factors. The data are presented in **Table 1**.

The analysis of anamnestic information about the presence of concomitant diseases showed that patients with PD are quite comorbid and usually have several nosological forms in the structure of the diagnosis. The data are presented in **Table 2**.

The presence of depressive symptoms was reported by 4 (2.9%) patients with PD, but depression of varying severity during the assessment of the Beck depression scale conducted in the framework of this study was detected in 46 (71%) of the 65 patients examined. The average level of depression in the group was 15.6 ± 9.1 points, which corresponds to mild depression.

MOTOR SYMPTOMS IN THE SIBERIAN COHORT

In the study group of patients, the stage was determined according to the classification of Hen-Yar (1967): in 31 patients, one stage of the disease was established, in 63 patients, two stages, in 45 patients, three stages, and in one patient, four stages of the disease.

The average disease duration in the observed group of patients was 6.9 ± 4.9 years. The debut of PD with motor symptoms was noted in 129 (92.1%) patients, the debut with non-motor symptoms was detected in 11 (7.9%) patients with PD. Of the motor symptoms of the debut, the obligate symptom was hypokinesia, which was anamnestically established in all 140 (100%) patients. Other motor symptoms of PD debut in frequency were arranged in decreasing order as follows: resting tremor−76 (54.3%) patients, muscle rigidity−65 (46.4%) patients, and postural instability in PD debut was not noted.

In the analysis of the current clinical picture in patients with PD, hypokinesia was detected in 140 (100%) patients, resting tremor was diagnosed in 95 (67.9%) patients, rigidity in 97 (69.3%), and postural instability in 43 (30.7%) patients with PD.

Thus, the increase in the frequency of occurrence of the main motor symptoms of Parkinson's disease in the Siberian cohort over a 7-years period was 14% for resting tremor, 23% for muscle rigidity, and 31% for postural instability.

In our cohort of patients, 98 (70%) patients with a mixed form of the disease, 22 (16%) patients with a rigid-trembling form, and 20 (14%) patients with an akinetic-rigid form of Parkinson's disease were observed. We compared our data on motor deficiency with other cohorts of patients that were collected and systematized this year (14). The data are presented in **Table 3**.

Table 3 presents numerous studies of motor deficiency in Parkinson's disease in various ethnic groups (14–26).

TABLE 3 | Motor subtype in *de novo* PD cases by country.

Study	Year	Country	Total study participants	Mean Age (year)	Sex M/F	TD (No.)	TD (%)	Rigid akinetic or PIGD (No.)	Rigid akinetic or PIGD (%)	Indeterminate or mixed (No.)	Indeterminate or mixed (%)	Method of subtyping
Reinoso	2014	Singapore	576	63.8	328/248	19	3.3	383	66.5	174	30.2	Lewis method and Rossi modifications
Rajput	2017	Canada	156	65.0	98/58	10	6.4	45	28.8	101	64.7	Novel method
Ramani	2016	UK	42	67.0	29/13	7	16.7	17	40.5	18	42.9	Novel method
Poletti	2011	Italy	42	65.0	28/14	10	23.8	24	57.1	8	19.0	Lewis method
Alves	2006	Norway	171	71.3	112/87	43	25.1	92	53.8	36	21.1	Jankovic
Auyeung	2012	Hong Kong	171	62.2	93/78	46	26.9	62	36.3	63	36.8	Novel method
Yuan	2013	China	51	61.9	24/24	20	39.2	19	37.3	12	23.5	Jankovic method with Korchovinov modifications
Mocciia	2016	Italy	63	60.6	38/25	27	42.9	18	28.6	18	28.6	Jankovic
Konno	2018	USA	1,003	64.0	637/366	439	43.8	386	38.5	178	17.7	Most prominent symptom at diagnosis
Seong-Min Choi	2018	South Korea	192	66.2	94/98	87	45.3	82	4.1	23	12.0	Jankovic
Muller	2011	Norway	207	67.9	122/85	95	45.9	88	42.5	24	11.6	Jankovic
Hiorth	2013	Norway	207	67.9	122/85	95	45.9	89	43.0	23	11.1	Novel UPDRS ratio
Nicoletti	2016	Italy	485	65.6	292/193	311	64.1	104	21.4	70	14.4	Most prominent symptom at diagnosis
Rozhdestvensky	2020	Russia	140	66,4	55/85	22	16	20	14	98	70	Most prominent symptom at diagnosis

Lewis method with Rossi modifications, ratio of the mean tremor scores (TD) (items 20 and 21) and the mean akinetic-rigid score (AR) (items 18, 19, 22, and 27–31): if the ratio TD/AR is >2.0, it was defined as TD and if AR/TD is more than 2.0, it was defined as AR. Mixed type was any indeterminate result. Jankovic: ratio of mean TD scores divided by mean of postural instability and gait items (falling, freezing, subjective gait difficultly, gait, and postural instability): if ratio is >1.5 TD, PD; if ratio is <1.0 PIGD, PD. Other subtyping methods are as described in the reference materials.
TD, tremor dominant; PIGD, postural instability and gait disorder.

NON-MOTOR SYMPTOMS IN THE SIBERIAN COHORT

The most frequent non-motor symptoms of PD debut in the study group were constipation in 47 (33.6%) patients, cognitive impairment in 24 (17.1%) of 140 patients, behavioral disturbances in the REM phase of sleep in 24 (17.1%) of patients, insomnia diagnosed in various variations in 38 (27.1%) patients, daytime sleepiness in 43 (30.7%) patients, subjective olfactory sensation dysfunctions at the time of the motor debut of PD in 19 (13.6%) patients, unscheduled weight loss in 8 (5.7%) patients, anhydrosis of the skin in 7 (5%) patients, sweating already in the debut of PD in 21 (15%) patients, seborrhea (indicated as an additional non-motor symptom of the debut of PD) in 4 (2.9%) patients, orthostatic hypotension in 35 (25%) patients, dysphagia in 5 (3.6%) patients, and a dysfunction of the pelvic organs by the type of night urination and fecal incontinence in 17 (12.1%) patients.

In the analysis of current non-motor symptoms, cognitive impairment was detected in 61 (43.6%) patients, psychotic disorders in the form of hallucinations in 7 (5%) patients, behavioral disturbances in the REM phase of sleep in 65 (46.4%) patients, olfactory dysfunction in 28 (20%), daytime sleepiness in 84 (60%), insomnia in various variations in 59 (42.1%) patients, unplanned weight loss in 12 (8.6%) patients, orthostatic hypotension in 80 (57.1%), dry skin in 6 (4.3%), hyperhidrosis in 29 (20.7%), dysphagia in 21 (15%), constipation in 85 (60.7%), and night urination in 41 (29.3%) patients.

Thus, the increase in the prevalence of non-motor symptoms of Parkinson's disease in the Siberian cohort for a 7-years period was constipation in 27%, cognitive impairment in 27%, REM disorders in 29%, hyposmia in 6%, orthostatic hypotension in 32%, insomnia in 15%, daytime sleepiness in 29%, and nocturia in 17%. BP progression over a 7-years period is shown in **Figure 1**.

Olfactory test (Sniffin Stick) or Sniffin Sticks odor identification test (Bürghard firm, Hamburg, Germany) was performed in 83 patients, and the average score was 7.6 $\pm$ 3.3, which corresponds to anosmia. Identification test: the ability to detect odors from the proposed four names. The patient is given a pencil to inhale the smell and are offered four options, one of which he must choose. The result is the sum of the positive answers. A result of <8 points corresponds to anosmia, 9–11 points to hyposmia, and 12–16 points to normosmia. In this group of patients, olfactory dysfunction reaching the degree of anosmia was diagnosed in 47 (56.2%) patients, hyposmia in 26 (31.3%) patients, and normosmia in 11 (12.5%) patients. The data are presented in **Figure 2**.

A statistical analysis of a group of patients with Parkinson's disease at the onset of the disease and at the time of the study, taking into account the average duration of the disease course of 6.9 $\pm$ 4.9 years using the non-parametric Mann-Whitney *U*-test, showed significant differences in the severity of the main motor and non-motor symptoms. At the time of the study, patients had more pronounced resting tremor ($U = 1{,}021.5$; $p < 0.0000001$), general bradykinesia ($U = 1{,}854$; $p < 0.001$), and rigidity ($U =$

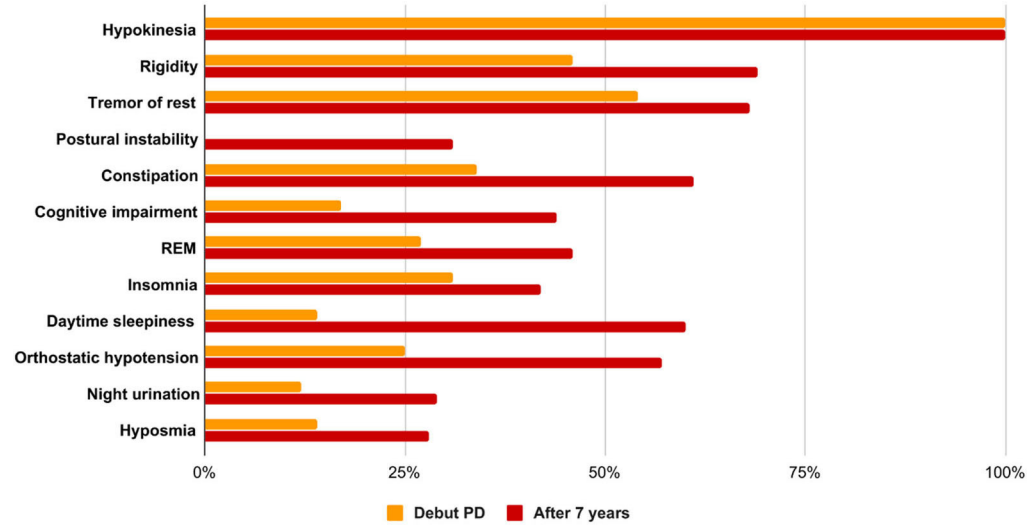

FIGURE 1 | Features of the clinical picture in the debut of PD and at the time of the examination, taking into account the average duration of the disease.

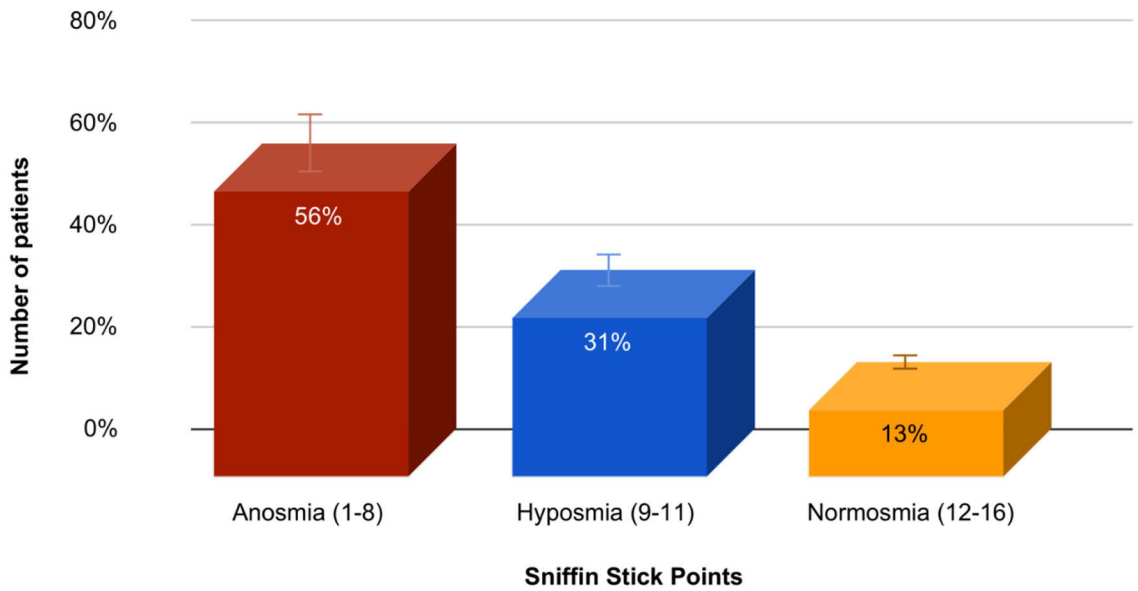

FIGURE 2 | The prevalence and severity of olfactory dysfunction in the study group of patients with PD.

1,630.0; $p < 0.0007$). When comparing the severity of non-motor symptoms in the debut of BP and after 7 years, more pronounced constipation ($U = 1{,}387.5$; $p < 0.000001$), cognitive impairment ($U = 1{,}683.5$; $p < 0.0002$), behavioral disorders were noted in the REM phase of sleep ($U = 1{,}775.5$; $p < 0.0004$), insomnia ($U = 1{,}082.5$; $p < 0.0000001$), daytime sleepiness ($U = 1{,}534.0$; $p < 0.000008$), orthostatic hypotension ($U = 1{,}826.0$; $p < 0.000008$), and hyposmia ($U = 836.0$; $p < 0.00004$). There is also a tendency to increase the severity of night urination ($U = 1{,}814.5$; $p < 0.02$).

We compared the results of evaluating some non-motor symptoms in our cohort of patients with Parkinson's disease with symptoms in other cohorts of patients around the world; data for which were systematized this year (27). The data are presented in **Table 4**.

Table 4 shows the prevalence of major non-motor symptoms identified during studies in different corners of the globe (8, 28–37).

The average score during neuropsychological testing of the cognitive sphere using the Montreal scale for assessing cognitive functions was 23.4 ± 3.9, which corresponds to moderate cognitive impairment. A score of 20 and lower was scored by 13 (15.1%) patients, and in the range of 21–25 points, 43 (51.8%)

TABLE 4 | Some non-motor symptoms in patients with PD by country.

Study	Year	Country	Total study participants	Mean years since diagnosis	Anosmia (%)	Dysphagia (%)	Constipation (%)	Nocturia (%)	Weight change (%)	Memory problems (%)	Hallucinations (%)	Depression (%)	OH (%)	EDS (%)	Insomnia (%)	RBD (%)	Hyperhidrosis (%)
Duncan	2014	UK	158	0.5	44	20	42	25	23	54	22	37	32	25	18	35	10
Romenets	2012	USA	70	3.8	21	16	30	68	21	42	12	38	38	14	41	38	19
Hui-juan Li	2015	China	82	5.1	45	33	67	87	29	95	15	67	38	73	78	52	65
Cosentino	2013	Peru	300	5.8	36	22	56	77	53	61	20	81	48	33	48	36	43
Khedr	2013	Egypt	112	6.2	10	24	52	60	33	30	13	47	54	39	46	15	21
Cheon	2008	S Korea	74	6.4	28	31	66	68	35	61	18	65	64	26	56	35	60
Rodríguez-Violante	2011	Mexico	232	6.6	34	33	58	62	28	47	19	67	46	28	47	33	39
Martinez-martin	2007	International	545	7	29	28	53	62	18	45	23	50	28	31	46	36	30
Tanveer	2018	Pakistan	97	7	26	28	60	77	38	59	30	52	53	41	53	36	37
Bostantjopoulou	2013	Greece	166	7.1	26	14	46	52	7	31	2	42	28	9	26	27	21
Chaudhuri	2010	UK, Germany, Spain	242	8	43	27	48	65	23	51	17	49	39	35	47	39	31
Rozhdestvensky	2020	Russia	140	6.9	56	15	61	41	9	44	5	75	57	60	42	46	21

Any data that was not available is replaced with a dash.
OH, orthostatic hypotension; EDS, excessive daytime sleepiness; RBD, REM sleep behavior disorder.

patients were observed; the normal values of this test [i.e., 26–30 were noted in 28 (33.1%) patients with PD].

CONCLUSION

Of course, factors such as ethnicity (38), geographical location of the region of residence (39), diet (40), overall life expectancy (41), genetic characteristics of the population (42, 43), and presence of concomitant diseases (44) affect the development and nature of the course of both neurodegenerative and other chronic progressive neurological diseases. A comparative assessment of the clinical parameters of Parkinson's disease in populations is to some extent difficult due to the variability of approaches for obtaining clinical data and their interpretation. In addition, the UK PD Brain Bank Criteria used in most studies provide a diagnostic accuracy of only 82.7% for the diagnosis of Parkinson's disease (45). This indicator, of course, affects the statistical reliability of clinical and epidemiological studies of Parkinson's disease and can lead to some distortion, especially in the early stages, when the clinical picture is usually incomplete, the response to dopaminergic drugs is uncertain, or signs of atypical parkinsonism have not yet appeared. Despite significant advances in understanding the pathogenetic aspects of this disease, we still lack clear imaging or biochemical markers to accurately diagnose Parkinson's disease.

In our work, we estimated the prevalence of motor and non-motor neurological deficits, as well as some risk factors and concomitant diseases in the Siberian cohort of patients with Parkinson's disease. The predominant debut of BP is the motor debut. The progression of the disease is characterized by an increase in the frequency of both motor and non-motor symptoms. When comparing the prevalence of motor symptoms with other cohorts of patients (**Table 3**), we found the greatest similarity with Canadian (24) and, to a lesser extent, UK (15) cohorts of patients, in which mixed forms of Parkinson's disease also prevailed. This similarity may be due to the close climatic and geographical features of western Siberia and Canada with a sharply continental climate, and the predominance of patients of the European race in our cohort. The analysis also noted the predominance of tremor in the structure of the clinical picture of Parkinson's disease in the Italian (14, 23), Norwegian (18, 26), and South Korean (16) cohorts. Against this background, a study of Reinoso et al., conducted in 2014, which shows the predominance of an akinetic-rigid form or postural instability with gait disorders of up to 66.5% in a cohort of patients with Parkinson's disease (21), looks quite interesting. The ratio of disease forms in the US (22) and China (19) cohorts was approximately equal.

In this article, we compared the prevalence of non-motor symptoms of Parkinson's disease in Siberian and other cohorts around the world. Based on these data, the Siberian cohort demonstrates a greater prevalence of olfactory disturbances, daytime sleepiness, orthostatic hypotension, depressive disorders, and behavioral disorders in the REM phase of sleep compared with cohorts similar in terms of the duration of Parkinson's disease (29–33, 35, 36). However, the prevalence of

dysphagia, nocturia, weight changes, and hallucinations turned out to be lower than in the cohorts of patients similar in terms of sample size and duration of the disease. It is possible to consider these parameters only tentatively, since they have differences in the methodology for assessing non-motor symptoms, cohorts have ethnic differences, which probably affect the final result.

In the study group of patients, mild depressive symptoms, mild cognitive impairment, and severe olfactory impairment (anosmia) were noted.

The severity of motor and most non-motor symptoms directly correlates with the duration of PD and the stage of the disease, which is confirmed by most clinical trials of this disease (46–48).

The comorbidity of patients with Parkinson's disease is an urgent public health problem. A study by Mollenhauer et al. reflected the negative dynamics of the rapid progression of Parkinson's disease in patients with cardiovascular risk factors, impaired regulation of blood glucose levels, impaired uric acid metabolism, and inflammation (49). According to Huang Y.F. in patients with PD, the risk of stroke is higher than in the population (50). In the Siberian cohort of patients with Parkinson's disease, women with higher education, overweight, concomitant arterial hypertension, coronary heart disease, and dyslipidemia predominated. The predominance of females in the analyzed cohort of patients with Parkinson's disease is probably associated with regional features of a higher medical demand for females. The data obtained during the study are consistent with the results of other studies in various cohorts and in different parts of the world (51–53).

Smoking as a protective risk factor in Parkinson's disease is confirmed by a large number of scientific studies (54–57). Our data indirectly confirm and are consistent with previously published studies. The analysis found that 75% of respondents never smoke. The study found that household contact with various pesticides is relevant for 30% of respondents.

The Siberian cohort of patients with Parkinson's disease has its own peculiarities in the clinical picture of the disease, in questions of comorbidity, which necessitates further studies of various clinical, epidemiological, and genetic aspects of the disease using unified protocols to better understand the nature of the disease. The results presented in the article indicate the main directions of further deeper study of this pathology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Requests to access the datasets should be directed to Roman Andreevich Delov, delov_roman@mail.ru.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Omsk State Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR: study conception and design. IG, EK, EM, and RD: acquisition of data. RD: analysis and interpretation of data, drafting of manuscript, and critical revision.

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BRIEF RESEARCH REPORT
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Novel Variants in *LRRK2* and *GBA* Identified in Latino Parkinson Disease Cohort Enriched for Caribbean Origin

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Background: The Latino population is greatly understudied in biomedical research, including genetics. Very little information is available on presence of known variants originally identified in non-Hispanic white patients or novel variants in the Latino population. The Latino population is admixed, with contributions of European, African, and Amerindian ancestries. Therefore, the ancestry surrounding a gene (local ancestry, LA) can be any of the three contributing ancestries and thus can determine the presence or risk effect of variants detected.

Methods: We sequenced the major exons and exons of reported Latino-specific variants in *GBA* and *LRRK2* and performed genome-wide genotyping for LA assessments in 79 Latino Parkinson disease (PD) patients, of which ∼80% identified as Caribbean Latino.

Results: We observed five carriers of LRRK2 p.G2019S, one GBA p.T408M, and three GBA p.N409S on European as well as three GBA p.L13R on African LA backgrounds. Previous Latino variant GBA p.K237E was not observed in this dataset. A novel highly conserved and predicted damaging variant LRRK2 p.D734N was identified in two unrelated individuals with African LA. Additionally, we identified rare, functional variants LRRK2 p.P1480L and GBA p.S310G in one individual each heterozygous for European/Amerindian LA.

Discussion: Additional functional analysis will be needed to determine the pathogenicity of the novel variants in PD. However, the identification of novel disease variants in the Latino cohort potentially contributing to PD supports to importance of inclusion of Latinos in genetics research to provide insight in PD genetics in Latinos specifically as well as other populations with the same ancestral contributions.

Keywords: Parkinson disease, Hispanic/Latino, genetics, diversity and inclusion, health disparities

INTRODUCTION

Parkinson disease (PD) is the second most common neurodegenerative disease next to Alzheimer disease (AD), affecting individuals of all races and ethnicities. Most studies of PD, however, have been conducted in individuals of European (non-Hispanic whites, NHW) and Asian descent. Interestingly, incidence rates of PD are slightly higher in Latinos than for NHW (1, 2), indicating a clear disregard of the field to include Latinos in PD research. The bias toward NHW leads to health disparities for PD diagnosis and treatment. Many of the disparity reports however make no distinctions for NHW vs. Latinos, compared to for example NHW vs. African Americans (3). Therefore, the health disparities experienced by Latinos are likely understudied and underestimated despite the fact they are the fastest-growing and now largest minority in the US (18.3%) (4).

To date, >50 genes/loci have been identified for PD in European or Asian-descent studies (5). It is not known at what frequency NHW PD variants occur in other racial/ethnic groups or if entirely different variation or separate genes play a role in these other groups. Variants unique to a specific racial background have been reported for PD, such as *PINK1* variants that are predominantly identified in Asian patients (6). Ethnic-specific mutations have been found in several genes influencing complex disease, most notably in late-onset AD, and the effects of these genetic differences vary between populations (7–11).

Interestingly, genetic research in admixed populations such as the Latino population can provide insight in genetic contribution on many backgrounds because of their complex and variable genetic admixture. Latino populations collectively trace their ancestry to three continental groups; European, Amerindians, and West African (12–14), though contributions to contemporary Latino populations vary geographically (15–18). Interestingly, specifically for the Caribbean, there is high variability in ancestry contribution among and even within different Latino groups of this region (19). These contributions of various origins also lead to the observation that even though an individual's global ancestry ("average" ancestry) might mostly resemble European, African (American) or Amerindian, their genome is a mosaic of contributions. Therefore, local ancestry (LA), or the ancestral background of a particular ("local") chromosomal region or haplotype (i.e., *LRRK2* locus), can be highly variable between different genomic regions and between individuals of the same population group. More recently, different variant size effects have been demonstrated for the same variant on different LA, i.e., lower risk of APOEε4 for AD on African vs. European or Japanese background (20), clearly indicating the importance of understanding LA for disease variants.

A small number of studies have reported results of genetic analyses in small (secondary) Latino datasets (21–26). These analyses often summarize across all Latino PD patients, regardless of ancestry, due to the small sample size. Given the high variability of admixture in these populations (described above), caution is warranted for the interpretation and extrapolation of these results. The only larger cohort, Latin American Research Consortium on the Genetics of PD (LARGE-PD, PI Dr. Mata) consisting of 1,150 Latino patients originating from southern South America, reports an enrichment of a novel variant in PD gene LRRK2 (p.Q1111H, rs78365431) in Peruvian and Chilean PD patients and controls (27) as well as a GBA mutation (p.K237E, rs773409311) in Colombian patients only (28), suggesting these variants originated from the Amerindian genetic background in these patients. Though these studies are an important first step, more elaborate analyses in the full range of Caribbean, Central, and South America are needed. The data presented here is the first report on variants in a cohort highly enriched for Caribbean Latino patients, complementing the reported dataset of LARGE-PD.

MATERIALS AND METHODS

Human Subject Research Compliance

The presented study was approved by the Institutional Review Board at the University of Miami and informed consent for the survey was obtained from all participants.

Sample Dataset

All PD participants were enrolled locally in Miami, FL, through collaboration with the University of Miami Department of Neurology Movement Disorders Division (Drs. Singer and Luca) or through ascertainment efforts in Puerto Rico through collaboration with Dr. Vinuela of the Movement Disorders Group at Manatí Medical Center in Manatí, PR.

Genotyping Chip

We performed genome-wide genotyping using Illumina's Global Screen Assay (GSA) with Multiple Disease content version 2 (GSAMDv2), at the Center for Genome Technology at John P. Hussman Institute for Human Genomics. Quality control analyses were performed using the PLINK software, v.2 (29). Samples with a call rate <90% and with excess or insufficient heterozygosity (± 3 standard deviations) were excluded. Sex concordance was checked using X chromosome data. To eliminate duplicate and related samples, relatedness among the samples was estimated by using identity by descent (IBD). SNPs available in samples with the call rate <97%, or those not in Hardy–Weinberg equilibrium ($p < 1x\,e\text{-}5$), were eliminated from further analysis.

Illumina's CNVpartition program (Illumina, San Diego, CA) was used with default settings to evaluate presence of copy number variations in the genotyping data.

The genotyping data was used for determination of ancestries as well as presence of few variants (potentially) contributing to PD included on the chip (LRRK2 p.G2019S, p.Q1111H, and PARK2 p.R275W).

Global and Local Ancestry Determination

Standard principal component analysis (PCA) using the Eigenstrat program (30) was performed to establish global ancestry for the participants. Reference datasets from the Human Genome Diversity Project (HGDP) data, i.e., European (/NHW), West African, Amerindian, were used in the analysis (31).

To determine LA at the genomic region surrounding the known PD genes, we phased the genotyping data using SHAPEITtoolver.2 (32) and the same reference datasets as for the PCA. We then used the RFMix ancestry software (33) to estimate LA for the whole genome (for reference) and around *LRRK2*, *GBA*, and *PARK2* in particular. These LA blocks are defined by variants common in specific ancestral populations spread across a large region, up to several Mb, depending on LD structure. The same reference populations (NHW, West African, Amerindian) used for phasing are used in the LA estimation. RFMix then compares each genomic region to the reference populations to infer the ancestral origin of each haplotype. Admixture plots identifying overall percentage of ancestral contributions are created using the ADMIXTURE program (34).

Sanger Sequencing

We performed Sanger sequencing for exons in major PD genes for late-onset PD harboring known pathogenic variants (LRRK2 p.R1441 hotspot codon, p.G2019S, GBA common variants, SNCA), as well as harboring newly identified variants putatively contributing to Latino PD reported by Velez-Pardo et al. (28). Additionally, we extended LRRK2's analyses to all exons coding for functional domains Roc and Kinase, as well as exons harboring putative pathogenic variants identified in NHW patients in-house and by collaborators (personal communication). In total, these exons include LRRK2 exon 17-19, exon 29-31, exon 34, exon 36, exons 38-44, GBA exons 2-11, and SNCA exons 2-3 (primer sequences are available upon request).

TaqMan Genotyping

To confirm the observed homozygous status of variant PARK2 p.R275W on the genotyping chip, we performed TaqMan genotyping (C__27532069_20, Thermo Fisher Scientific) on all participants using the recommended protocol. Data were analyzed on QuantStudio (Life Technologies).

Variant Annotation

Novel variants are annotated for conservation (PhastCons/GERP, values over 2 and 0.5 are considered conserved by consensus) and functional effect in the protein using PolyPhen2 (35) as well as Combined Annotation Dependent Depletion algorithm (CADD) score. A score over 20 indicates top 1% of highest CADD scores (most evidence for functional potential of the position) genome-wide. Additionally, we queried the genome aggregation database [gnomAD, (36)] holding exonic/genomic data of 140,000 individuals, including 17,000 "Latino" individuals.

RESULTS

A total of 79 Latino patients are included in this report, 79.7% identified as Caribbean (originating from Cuba, PR, Dominican Republic, or mixed/undefined). Other countries of origin reported by participants include Colombia, Peru, Ecuador, El Salvador, Guatemala, Brazil, Mexico, or unknown. Sample characteristics are described in **Table 1**. Nineteen out of 79 patients reported a first or second degree relative with PD (positive family history, FamHx+; 24%). Analyses of global ancestry (**Figure 1**) and ancestral contributions (admixtures, **Figure 2**) determined that the vast majority of this cohort has a high percentage of European ancestral contribution, though highly variable contribution from both African and Amerindian ancestry is observed (0 to ∼80%, **Figure 2**). Contribution of other ancestries (e.g., East Asian) was minimal (<2%, data not shown).

TABLE 1 | Sample characteristics.

	N (%)	Avg AAO (range)	M/F ratio	FamHx P/N+U
ALL	79 (100)	54.4 (29–69)	42/37	19/60
Caribbean	63 (79.7)	63 (40–69)	36/27	16/47
Puerto rico	37 (46.8)	55.6 (40–67)	19/18	14/23
Cuba	22 (27.8)	53.6 (42–69)	15/7	1/21
Dominican republic	2 (2.5)	49 (1 unknown)	1/1	0/2
Undefined	2 (2.5)	57.5 (53–62)	1/1	1/1
Other	16 (20.3)	53.2 (29–68)	6/10	3/13
Colombia	2 (2.5)	55.5 (55–56)	0/2	0/2
Peru	2 (2.5)	54 (49–59)	0/2	1/1
El Salvador, Guatemala, Brazil, Ecuador, Mexico (1 each)	5 (6.3)	55.5 (44–68)	2/3	1/4
Unknown	7 (8.9)	50.2 (29–64)	4/3	1/6

avg AAO, average age at onset; M/F, male/female; FamHx, family history (defined as first- or second-degree relative); P, positive; N, negative; U, unknown.

Detection of Known Variants in Selected Exons of Major PD Genes

We set out to determine the frequency of rare (MAF<1%) known variants originally identified in NHW patients in the Latino cohort. Using genotyping and Sanger sequencing data, we identified five heterozygous carriers of LRRK2 p.G2019S (5/79 = 6.3%, 1/19 FamHx+ = 5.3%) of various origins, two heterozygous carriers of GBA p.A495P (2/79 = 2.5%, 0/19 FanHx+) from Puerto Rico, three carriers of GBA p.N409S (3/79 = 3.8%, 0/19 FamHx+) of various origins, one heterozygous carrier of GBA p.T408M (1/79 = 1.2%, 0/19 FamHx+) from Cuba, three carriers of GBA p.L13R from Puerto Rico (3/79 = 3.8%, 1/19 FamHx+ = 5.3%), and a homozygous carrier of PARK2 p.R275W (confirmed by TaqMan genotyping, 1/79, 0/19 FamHx+ or 2/158 alleles = 1.2%) from Puerto Rico (**Table 2**). We did not observe any variants in *SNCA*, on the LRRK2 p.R1441 (C/G/H/S) hotspot or GBA p.L483P. No larger copy number variations in major PD genes detectable by the genotyping chip were observed.

We also evaluated presence of reported putative Latino specific and/or Latino PD contributing variants, i.e., LRRK2 p.Q1111H and GBA p.K237E. We did not observe either of these variants in the current dataset.

When examining the LA for *LRRK2*, *GBA*, and *PARK2* for the variant carriers, we determined that all variant

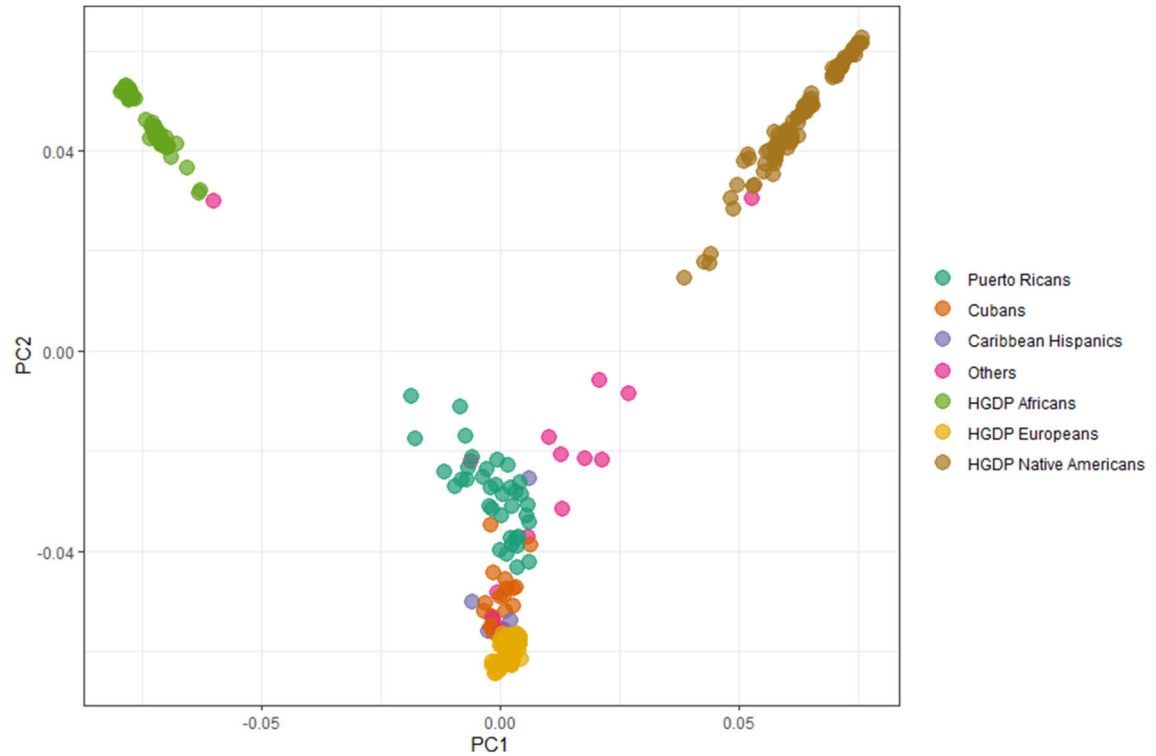

FIGURE 1 | Principal component analyses. Estimation of relationship to ancestral groups from Human Genome Diversity Project (HGDP). Aqua = Puerto Ricans. Orange = Cubans. Purple = Caribbean Latinos (including Dominican Republic, mixed or undefined Caribbean origin). HGDP datasets included Europeans (yellow), Africans (green), and Amerindians (brown).

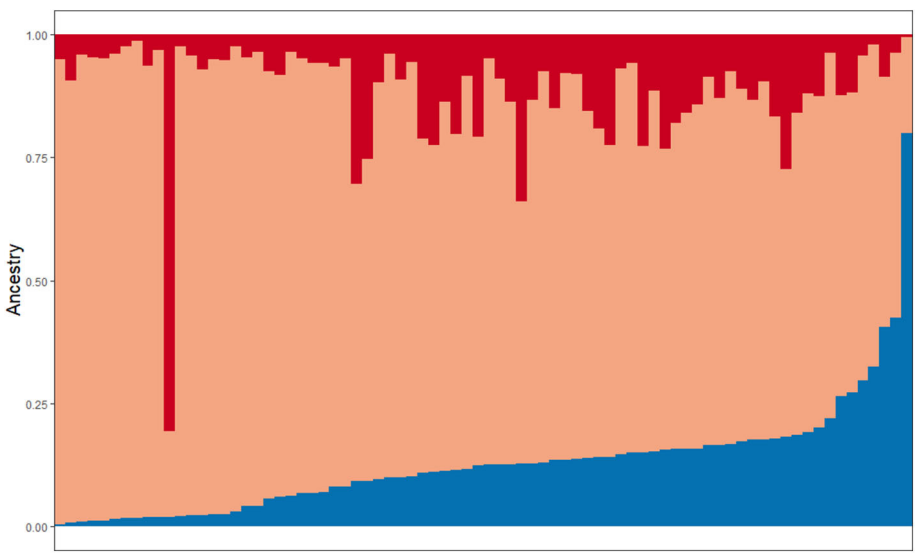

FIGURE 2 | Representation of ancestral admixture in Latino cohort. Subjects are sorted on the X-axis based on percentage of Amerindian ancestry contribution (0–80%, displayed in blue). Colors in each vertical line represent that individual's ancestral admixture. Red = African, Orange = European, Blue = Amerindian.

carriers are homozygous for European LA at the genomic location where they carry a variant, except for one carrier of GBA p.A495P (Amerindian/European) and all three carriers of GBA p.L13R (2 × African/European and 1x African/Amerindian). Interestingly, p.L13R is common in the African population (7.7% in gnomAD), vs. <0.5% in other population groups, and considered benign for GBA function in ClinVar.

TABLE 2 | Known rare variants (MAF<1%) identified in major exons of PD genes.

Gene	Variant	#carriers	Countries of origin	Avg AAO	Likely local ancestry	gnomAD overall (%)
LRRK2	G2019S	5	2x PR, Brazil, Guatemala, unknown	56 (47–61)	European	0.05
GBA	T408M	1	Cuba	58	European	0.6
	N409S	3	Cuba, PR, unknown	55.7 (45–64)	European	0.2
	A495P	2	PR	Unknown	Amerindian/European	0.01
	L13R	3	PR	51.67 (41–59)	African	0.007

MAF, minor allele frequency; Avg AAO, average age at onset; PR, Puerto Rico; gnomAD, genome aggregation database.

Identification of Additional Variants in Selected Exons of Major PD Genes

We identified five heterozygous carriers of rare new variants (**Table 3**) with varying levels of *in-silico* support for pathogenicity (**Table 4**); LRRK2 p.D734N (2 individuals), p.P1480L, p.R1941H, and GBA p.S310G.

The two individuals carrying the LRRK2 p.D734N variants are from PR; only one reports a positive family history. No DNA of the other affected in the family was available for segregation analyses. This variant has only been reported once in gnomAD in an additional Latino individual. LA analyses in these individuals (Amerindian/African and African/European) suggest that this variant might be located on an African background. The variant is predicted to be highly deleterious and is conserved. This variant has not been reported in PD context before, so no information is available in ClinVar. Both individuals presented with mild idiopathic PD with predominant tremor and postural changes and reported loss of smell and constipation. One further presented with short-term memory problems and the other with possible REM sleep behavior disorder.

The patient carrying LRRK2 p.P1480L variant identified Ecuador as country of origin and reported no positive family history to their knowledge. The variant was not present in 140,000 individuals from gnomAD (including 17,000 Latinos), though p.P1480S on the same codon is reported in only one European individual (0.000004% overall in gnomAD). The position is highly conserved, and the variant is predicted to be damaging. LA analyses showed that this patient is heterozygous for Amerindian and European ancestry at the *LRRK2* locus. The patient presented with idiopathic PD with tremor, bradykinesia, and rigidity and underwent successful deep brain stimulation surgery.

Variant LRRK2 p.R1941H was identified in a Cuban patient with no family history for PD, is classified as a Variant of Unknown Significance to PD in ClinVar, and has been observed in European, Latino, and African genomes in gnomAD (0.0001%). *In silico* predictions are inconsistent in supporting a damaging role of this variant. The individual carrying the variant is homozygous for European ancestry at the *LRRK2* locus. The patient presented with idiopathic PD with mild tremor, rigidity of the neck and leg, postural instability, and moderate facial hypokinesia.

The patient carrying GBA p.S310G is from PR and reports no family history for PD. The variant has been reviewed to be (likely) pathogenic for GBA function in ClinVar and has not been observed in European or Latino individuals from gnomAD but is rare in East Asian individuals. LA analyses identified both Amerindian and European ancestry at the *GBA* locus for the carrier. The patient presented with mild idiopathic PD with predominant tremor and postural changes.

DISCUSSION

Here we sequenced exons with reported pathogenic or strong risk variants for PD in three known PD genes (*LRRK2*, *GBA*, and *SNCA*) to evaluate presence of these variants originally identified in NHW patients in a Latino cohort enriched for Caribbean patients. Additionally, we extended LRRK2's analyses to all exons coding for functional domains Roc and Kinase, as well as exons harboring putative pathogenic variants identified in NHW patients in-house and by collaborators (personal communication). We used genome-wide genotyping data to determine ancestral background of identified variants and presence of few extra variants included on the chip (e.g., PARK2 R275W). We identified five carriers of LRRK2 p.G2019S as well as more common GBA p.T408M and p.N409S in one and three patients, respectively, all on putative European background. As these variants have been frequently reported in European patients, this suggests these variants were introduced to the Latino population through their European ancestor. Additionally, we identified benign variants GBA p.L13R common in African populations, in three individuals, and p.A495P in one individual. LA analyses supported p.L13R was indeed introduced through an African ancestor. p.A495P was identified in two patients who are heterozygous for European and Amerindian LA at the variant location. No ancestry-specific variants were located close enough (∼10 kb) to the variant to allow us to phase the variants with its ancestral background (defined by variants across up to several Mb surrounding the gene) in cloning experiments. Independent of the reported observation here of GBA p.A495P, this variant has been identified across populations with rare instances reported in Africans, Latinos, East Asians, and Europeans in gnomAD. This reoccurrence on different backgrounds might indicate a tolerance of GBA for changes on this position, suggesting this variant is likely benign, which is also reflected in ClinVar's assessment of its relevance to GBA function.

Interestingly, we identified four more variants with varying levels of evidence for impact in PD. The presence of LRRK2 p.R1941H in individuals of all populations in gnomAD suggests

TABLE 3 | New variants (MAF<1%) identified in selected exons of major PD genes.

Gene	Variant	*N*	Country of origin	AAO	FamHx P/N	Likely local ancestry	gnomAD overall (%)
LRRK2	D734N	2	PR	67 / 60	1/1	African	0.000008
	P1480L	1	Ecuador	44	0/1	Amerindian/European	Not observed
	R1941H	1	Cuba	42	0/1	European	0.000127
GBA	S310G	1	PR	58	0/1	Amerindian/European	0.000021

MAF, minor allele frequency; AAO, age at onset; FamHx P/N, family history positive/negative; U, unknown; PR, Puerto Rico; gnomAD, genome aggregation database.

TABLE 4 | *In silico* evidence for novel variants.

Gene	Variant	GERP	Phast Cons	PolyPhen2	CADD	gnomAD NFE (%)	gnomAD AFR (%)	gnomAD Lat (%)	gnomAD EAS (%)
LRRK2	D734N	5.94	0.579	Probably damaging	25.5	0	0	0.00003	0
	P1480L	5.53	0.935	Probably damaging	29	–	–	–	–
	R1941H	4.85	1.000	Possibly damaging	23.6	0.00019	0.00004	0.00028	0.00005
GBA	S310G	3.51	0.985	benign	26.5	0	0.00004	0	0.00020

gnomAD, genome aggregation database; NFE, non-Finish Europeans; AFR, African; Lat, Latinos; EAS, East Asian.

tolerability for this variant, thus reducing the likelihood that this variant is a major player in PD. Data on LRRK2 p.D734N and p.P1480L and GBA p.S310G however support potential pathogenic roles of these variants. LRRK2 p.D734N is predicted to be highly functional and is very rare in the general population being identified only once in another Latino individual. Though one patient presented with positive family history, unfortunately no DNA was available for the others affected for segregation analyses. However, the observation of this variant in two independent PD patients on African LA, rarity in the general population (including African individuals), and strong *in silico* evidence supports the hypothesis that this variant might be a novel pathogenic variant for PD in individuals with African background. The identification in just Latino individuals, and not European or African groups, could suggest that this variant was introduced more recently in Latin history.

Both variants LRRK2 p.P1480L and GBA p.S310G have been identified each in one patient who is heterozygous for European and Amerindian LA at the variant location and does not report family history preventing segregation analyses. No ancestry-specific variants were located close enough (∼10 kb) to either variant to allow for phasing of the variants on its ancestral background. Both variants are highly conserved and are predicted to have a (strong) effect on protein function. LRRK2 p.P1480L has not been reported previously in any general population, though a variant on the same codon (p.P1480S) was observed in one European individual. No information on this variant is available; however, it is located in the highly conformational Roc domain of LRRK2 and affects a proline residue, which are often involved in providing curvature in protein structures, suggesting a potential consequence for the domain structure due to this variant. Additional data of other carriers or families or functional analyses would be needed to assess its impact for PD. In contrast, GBA p.S310G has been observed in Gaucher's disease patients before and has been reviewed to be pathogenic by ClinVar. It has been seen very rarely in East Asian individuals in gnomAD. LA analyses in the variant carrier did not identify East Asian ancestry in this region (<1% in patient overall), indicating that this variant might have arisen independently in different populations. All patients carrying these new rare variants presented with classic idiopathic PD without atypical features; often with predominant tremor; and reporting no hallmarks differentiating them from other idiopathic PD. Screening in more (Caribbean) Latino PD cohorts or extensive single molecule sequencing will be needed in the future to confirm pathogenicity of these new potential PD variants and determine the ancestral origin of these variants in the Latino population. This first report on identification of novel variants in selected exons with higher likelihood of impactful variants in major PD genes in a Caribbean enriched cohort indicates that we can identify novel variants in the Latino population with variable evidence for involvement in PD pathogenesis. This is supported by the identification of the Colombian-specific variant GBA p.K237E (28) when querying GBA in a larger continental Latin dataset. Extending these analyses to more exons, more genes and larger cohorts will greatly increase the number of novel variants we identify in Latino PD patients and will further the field's understanding of PD in the Latino population.

Furthermore, inclusion of admixed population in genetic research is especially valuable because of their varied ancestry. As evidenced here by potential pathogenic variant LRRK2 p.D734N and previously by Velez-Pardo for GBA p.K237E, variants identified in Latino populations specifically can provide insight in variants on African and Amerindian background, both of which also play a major role in other, equally underserved, populations (African American/Amerindian).

Generally, the lack of information for other racial and ethnic populations (albeit in genetics specifically or biomedicine overall) leads to health disparities as study of a limited population pool creates biases in findings and only benefits the limited population in the end (37). Expanding genetic studies of complex diseases, such as PD, to Latino populations is crucial to meeting the needs of this increasing US demographic. The identification of novel variants in Latino cohorts not previously identified

further support the importance of inclusion of participants across race/ethnicity.

DATA AVAILABILITY STATEMENT

All genotyping data will be available through dbGAP, accession number: phs000908.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at the University of Miami. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KN, WS, MP-V, and JV contributed conception and design of the study. PB, KC, CS, CL, and AV were responsible for participant enrolment and data collection. FR performed the chip quality control and ancestry analyses. KN performed the sequencing analyses and wrote the first draft of the manuscript. WS, JV, CS, CL, and AV critically reviewed the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Association of the Plasma Long Non-coding RNA *MEG3* With Parkinson's Disease

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Objective: To investigate the expression level of the maternally expressed gene-3 (*MEG3*) of the free long non-coding RNA (lncRNAs) in the plasma of Parkinson's disease (PD) patients and its relationship with the disease.

Methods: Thirty PD patients (PD group) who treated at Xuanwu Hospital of Capital University of Medical Sciences between January 2017 and December 2019 were selected as the research objects and 30 healthy subjects were enrolled in the study during the same period as the control group. Cognitive function was assessed according to the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) were used to evaluate cognitive function, Non-Motor Symptoms Scale (NMSS) was used to evaluate severity of non-motor symptoms. The relative expression of lncRNAs *MEG3* in plasma was measured by PCR, and the levels of neuron-specific enolase (NSE), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in plasma were measured by ELISA, and the relationship with these all indexes was analyzed.

Results: The NMSS score of PD group was significantly higher than that of the control group, while the MMSE and MoCA scores were significantly lower than that of the control group ($P < 0.05$); The relative expression of lncRNAs *MEG3*, NGF and BDNF levels of PD group were significantly lower than that of the control group, and NSE level was significantly higher than that of the control group ($P < 0.05$); The H&Y stage and NMSS score in PD group were negatively correlated with the relative expression of lncRNAs *MEG3*, the levels of NGF and BDNF ($P < 0.05$), and positively correlated with NSE ($P < 0.05$); The MMSE and MoCA scores in PD group were positively correlated with the relative expression of lncRNAs *MEG3*, NGF, BDNF levels ($P < 0.05$), and negatively correlated with NSE ($P < 0.05$); The relative expression of lncRNAs *MEG3* in PD group was positively correlated with NGF, BDNF levels ($P < 0.05$), and negatively correlated with NSE ($P < 0.05$).

Conclusion: The expression of lncRNAs *MEG3* in the plasma of PD patients was downregulated compared to that of healthy control subjects, and its expression level was closely related to the aggravation of non-motor symptoms, cognitive decline, and PD

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stage. These associations may reflect the synergism of the increase of NSE and decrease of NGF and BDNF levels, highlighting plasma lncRNA *MEG3* as a new candidate biomarker of PD.

Keywords: Parkinson's disease, long non-coding RNAs, *MEG3*, cognitive function, non-motor symptoms

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease and the second most common neurodegenerative disease worldwide, following Alzheimer's disease, affecting 2–3% of the global population ≥65 years of age, and the incidence rate has been increasing steadily every year (1, 2). The number of PD patients in China was estimated at about 5 million in 2019, accounting for half of the world's total number of cases, affecting quality of life for the patients and imposing an economic burden on society (3). Clinical manifestations include resting tremor, bradykinesia, muscle rigidity,balance disorders, and postural instability. With non-motor symptoms, other symptoms appear such as REM sleep behavior disorders (or RBD), olfactory dysfunction, constipation, pain, fatigue, sleep disorders, autonomic dysfunction, and cognitive dysfunction that seriously affect the quality of life of patients; in particular, the incidence of cognitive dysfunction reaches 80% or more among patients with PD (4–6).

The main pathogenic mechanisms include phasic stimulation of dopamine receptors, non-physiological levodopa-to-dopamine conversion in serotonergic neurons, hyperactivity of corticostriatal glutamatergic transmission, and overstimulation of nicotinic acetylcholine receptors on dopamine-releasing axons (7). However, the pathogenesis of PD is complex, involving genetic, environmental, and other factors, remaining a topic of continuous research and exploration. Current research suggests that the pathogenesis of PD includes oxidative stress leading to mitochondrial dysfunction, endoplasmic reticulum stress leading to abnormal protein folding, neuroinflammation, and alterations in the microecology-gut-brain axis and genes. The central link of these mechanisms involves a multi-molecular pathway network, and synergistic effects induce the degeneration of dopaminergic neurons (8). The majority of PD patients have typical motor symptoms, and about half of the dopaminergic neurons are lost irreversibly. Therefore, mining early biomarkers is of great significance for the diagnosis, treatment, and prognosis of PD.

With the rapid development of gene detection technology, the role of epigenetic modification has emerged as an important link between genetic and environmental interactions in central nervous system diseases, becoming a hot spot in clinical research in recent years. Epigenetic modifications include DNA methylation, histone modifications, and non-coding RNA (ncRNA)-mediated expression regulation. NcRNAs do not encode proteins and are now increasingly recognized to play an important role in gene transcription and in disease. Indeed, in the last decade, unprecedented numbers of ncRNAs with novel functions have been discovered. Among the various types of ncRNAs, long non-coding RNAs (lncRNAs) play important regulatory roles in physiological processes such as neuronal differentiation, and brain development and function, along with pathological processes such as cerebral ischemia-reperfusion injury, glioma, neurodegeneration, and sex-related diseases (3, 9).

LncRNAs are protein-free transcripts, but occupy a large part of the transcription output. They are involved in the regulation of epigenetic, transcription, and post-transcriptional processing in cell homeostasis, and have attracted increased research attention in biomedicine, with particular focus of the roles of lncRNAs in normal neurodevelopment and neurogenerative diseases (including Alzheimer's disease, Huntington's disease, and PD) (10, 11). However, this field is still in its infancy, and the function of most identified lncRNAs remains unclear.

The lncRNA maternally expressed gene-3 (*MEG3*) is a maternal-encoded allele that is imprinted in a gene cluster in the distal part of murine chromosome 12, corresponding to human chromosome 14. Previous studies have shown that *MEG3* acts as a tumor suppressor; its expression is lost in a variety of cancer tissues, and overexpression of *MEG3* could inhibit tumor formation (12–14). Recent studies have shown that *MEG3* is overexpressed in patients with ischemic stroke and induced the apoptosis of neurons, but is downregulated in patients with glioma and Huntington's disease (15, 16). These findings suggest that *MEG3* may be an important epigenetic regulatory factor of the brain and neurons. To explore the potential role of the lncRNA *MEG3* in the pathogenesis of PD, in this study, we compared *MEG3* plasma expression levels in patients with PD and healthy subjects, and evaluated the relationship with clinical characteristics and disease severity.

MATERIALS AND METHODS

Subjects

Plasma samples were obtained from 30 patients diagnosed with PD who were treated at Xuanwu Hospital of Capital University of Medical Sciences, Beijing, China (kind gift from Professor Shun Yu) between January 2017 and December 2019. All patients met the PD diagnostic criteria according to the diagnostic reference standard of the neurology branch based on United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria (UKPDSBB) (17). All patients had been newly diagnosed with PD and had not received any relevant treatment before enrollment in the study. The exclusion criteria were patients with severe heart, liver, kidney, and other organ dysfunction; patients with mental illness, malignant tumor, or other central nervous system diseases; and a history of substance or alcohol abuse. In addition, 30 healthy subjects were enrolled in the study during the same period as the control group. There was no statistically significant difference in age and gender between the two groups ($P > 0.05$), and the general data were comparable (**Table 1**).

The severity of the disease in PD patients is evaluated by the modified H&Y scale. Stage 0 means no symptoms or signs. Stage

TABLE 1 | Comparison of general information between the two sets of data.

Groups	Samples	Age	Gender		Basic diseases	
			Male	Female	Arterial hypertension	Type 2 diabetes
PD group	30	67.19 ± 8.12	17	13	19	13
Control group	30	68.63 ± 7.17	15	15	18	15
T/X^2		0.728	0.268		0.071	0.268
P		0.469	0.605		0.791	0.605

Duration of disease (years)	H&Y classification					Motor symptoms		
	1	1.5	2	3	4	Tremor	Non-tremor	Postural instability
4.13 ± 1.50	4	4	8	9	5	8	12	10
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-

1 means unilateral limb involvement. Stage 1.5 means unilateral limb involvement with symptoms of limb muscle involvement. Stage 2 means bilateral limbs are involved but there is no balance disorder. Stage 2.5 is mildly involved both limbs with mild balance disorder. Stage 3 is moderately involved both limbs with obvious postural disorder, but can take care of themselves and turn around slowly. Stage 4 is severely affected both limbs, barely able to walk or stand independently. Stage 5 is bedridden or living in a wheelchair.

Fasting peripheral blood was collected from all subjects on the morning after admission, and plasma was separated for analysis of lncRNA *MEG3* levels. All subjects provided informed consent for participation in the study, which was approved by the hospital ethics committee.

Polymerase Chain Reaction (PCR)

Total RNA in the plasma was extracted by the Trizol method, which was used as a template for reverse transcription to obtain cDNA. cDNA was then used as a template for real-time fluorescence PCR with the lncRNA *MEG3* upstream primer 5′-GCATTAAGCCCTGACCTTTG-3′ and downstream primer 5′- TCCAGTTTGCTAGCAGGTGA-3′, synthesized by Sangon Biotech (Shanghai) Co., Ltd. *GAPDH* served as the internal reference. The relative expression levels were calculated according to the cycle threshold (Ct) value using the formula $2^{-\Delta\Delta Ct}$.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma levels of nerve-related factors, including neuron-specific enolase (NSE), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), were measured by ELISA. ELISA kits were purchased from Shanghai Thermo Scientific and Biological Co., Ltd. The specific operation steps were carried out in accordance with the instructions. Within 30 min, the absorbance value at 450 nm was measured with a microplate reader (Thermo Scientific, FC type), and a standard curve was drawn based on the standard substance. Measure the corresponding sample concentration, repeat the measurement for each sample three times and take the average value as the final concentration.

Disease Severity Evaluation

Cognitive function was assessed according to the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA). The Non-Motor Symptoms Scale (NMSS) was used to evaluate the severity of non-motor symptoms. The NMSS score mainly reflects the severity of the patient's non-motor symptoms, such as sleep disorders, autonomic dysfunction, cognitive and psychiatric symptoms, etc. The higher the score, the more severe the above symptoms; both MMSE and MoCA are widely used in clinical practice. The cognitive function screening scale reflects the mental state and the degree of cognitive impairment. The higher the score, the better the cognitive function, and the MMSE<27 or MoCA<17 points indicate the presence of cognitive impairment; all three scores can be used evaluation of neurodegenerative state in PD patients.

Statistical Analysis

Measurement data are presented as the mean ± standard deviation, and were compared between the groups using Student *t*-tests. Count data are presented as percentage and were compared using the chi-square test. The plasma biochemical indices were analyzed by Spearman and Pearson correlation coefficients and multivariate Logistic regression was used to analyze the relationship between H&Y scale and other indicators. The relationship between plasma lncRNA *MEG3* levels and various quantitative indices was assessed by a linear regression model. SPSS 16.0 software was used for all statistical analyses; $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Scores and Plasma Biochemical Markers Between Groups

The PD patient group included 17 males and 13 females, ranging in age from 57 to 78 years with a mean age of 67.19 ± 8.12

TABLE 2 | Comparison between various scores and plasma biochemical indexes between two groups.

Variable	Groups		*t*	*p*
	PD group	Control group		
NMSS score	58.38 ± 33.89	24.69 ± 17.27	4.851	0.000
MMSE score	24.63 ± 4.41	28.69 ± 0.70	4.980	0.000
MOCA score	18.44 ± 4.62	26.44 ± 1.46	9.044	0.000
MEG3 expression level	0.57 ± 0.19	0.94 ± 0.36	4.979	0.000
NSE (μg/mL)	17.55 ± 7.13	11.08 ± 4.53	4.195	0.000
NGF (pg/mL)	25.92 ± 3.26	30.78 ± 3.54	5.531	0.000
BDNF (pg/mL)	27.38 ± 3.52	32.19 ± 3.78	5.101	0.000

years. Among them, there were 19 patients with hypertension and 13 patients with diabetes. The control group comprised 15 males and 15 females, aged 55–79 years with a mean age of 68.63 ± 7.17 years, including 18 patients with arterial hypertension and 15 patients with Type 2 diabetes. There were no significant differences in age, gender, and basic diseases between the two groups (all $P > 0.05$), and the general data were comparable.

The NMSS scores of the PD group were significantly higher than those of the control group, whereas the MMSE and MoCA scores were significantly lower in the patient group. The relative expression levels of plasma lncRNA *MEG3*, NGF, and BDNF in the PD group were significantly lower than those in the control group, whereas the NSE level was significantly higher than that of the control group (**Table 2**).

Relationship Between Disease and Plasma Markers in PD Patients

There was a significant positive correlation between the H&Y stage and NMSS score in PD patients, and a significant negative correlation between MMSE and MoCA scores. The H&Y stage and NMSS scores of PD patients were negatively correlated with the relative expression level of lncRNA *MEG3*, and with the levels of NGF and BDNF, and were positively correlated with plasma NSE levels. By contrast, MMSE and MoCA scores in PD patients were positively correlated with the levels of lncRNA *MEG3*, NGF, and BDNF, and were negatively correlated with NSE levels. In addition, the relative expression level of plasma lncRNA *MEG3* was positively correlated with NGF and BDNF levels, and negatively correlated with NSE levels (**Table 3**).

Multivariate Logistic Regression Analysis of H & Y Scale and Other Indexes

All the above-mentioned factors related to H&Y scale were used as independent variables to assign values. For H&Y scale, "≤2" was regarded as mild and ">2" was regarded as moderate to severe (18). Multivariate Logistic regression analysis was performed. The results showed that age, disease course, NMSS and NSE was significantly positively correlated with disease stage, while MMSE, MoCA scores and the relative expression of lncRNA *MEG3* levels were significant negatively correlated with disease stage, and the differences were statistically significant ($P < 0.05$) (**Table 4**).

Linear Relationship Between Plasma lncRNA *MEG3* and Other Indicators

As shown in **Figures 1A–F**, linear correlation analyses showed that the plasma lncRNA *MEG3* level in PD patients was negatively correlated with NMSS score ($r = -0.284$, $P = 0.002$), and positively correlated with MMSE ($r = 0.255$, $P = 0.004$) and MoCA ($r = 0.186$, $P = 0.017$) scores. Plasma lncRNA *MEG3* levels were negatively correlated with NSE levels in PD patients ($r = -0.181$, $P = 0.019$), and positively correlated with NGF ($r = 0.131$, $P = 0.049$) and BDNF ($r = 0.351$, $P = 0.001$) levels.

DISCUSSION

Recent studies have shown that the lncRNA *MEG3* is associated with glioma, Huntington's disease, stroke, and other neurological disorders, suggesting a potentially new clinical biomarker (15, 16), it can block the cell cycle by activating the p53 pathway, leading to cell replication senescence or apoptosis. In this study, we compared the levels of lncRNA *MEG3* and biochemical brain markers in the plasma of PD patients with those of healthy control subjects. The results showed that the relative expression levels of plasma lncRNA *MEG3* are reduced in PD patients compared to those of the healthy population, shows that lncRNA *MEG3* is one of the most important potential molecular in the diagnosis of PD or selection of therapeutic potential targets.

The mature *MEG3* lncRNA is composed of 10 exons and is abundantly expressed in the brain, adrenal glands, placenta, breast, and liver tissues. Previous studies have demonstrated that the expression level of *MEG3* is reduced in cancer cells, and upregulation of *MEG3* expression could inhibit tumor growth; therefore, *MEG3* was initially considered to function mainly as a tumor suppressor (12, 13). Lin et al. (12) found that upregulation of *MEG3* expression in the HeLa cervical cancer cell line can inhibit the PI3K/AKT/Bcl-2/Bax/P21 signaling pathway, thus inhibiting the proliferation, invasion, and migration of HeLa cells and promoting their apoptosis. Han et al. (13) found that the lncRNA *MEG3* methylation level increased successively in the serum of healthy subjects, patients with low-stage cervical cancer, high-stage cervical cancer, and cervical cancer with lymph node metastasis, indicating that *MEG3* methylation might be a marker of disease progression in cervical cancer. Subsequently, lncRNA *MEG3* expression was found to be downregulated in glioma tissue cells. Zhang et al. (10) reported that lncRNA *MEG3* inhibited glioma cell growth *in vitro* by regulating the mir-96-5p/MTSS1 signaling pathway, and was involved in cell proliferation and apoptosis regulation. Earlier studies indicated that lncRNA *MEG3* also showed a downregulated trend in the brain tissues of patients with Huntington's disease, and *Meg3*-knockout affected the expression of genes in the cerebral cortex of mice, leading to increased cortical microvascular density and enhanced expression of genes related to angiogenesis (19). Collectively, these studies suggest that *MEG3* may also be an important epigenetic regulatory factor in brain development by regulating the gene expression profile to correspond to neuron activity. However, clinical studies on its regulatory pathway are scarce, and no consensus has been reached to date.

TABLE 3 | The relationship between scores and plasma biochemical indexes in PD patients.

		NMSS score	MMSE score	MOCA score	*MEG3* expression level	NSE	NGF	BDNF
H&Y Grade	*r*	0.594**	−0.824**	−0.592**	−0.559**	0.834**	−0.651**	−0.396*
	P	0.001	0.000	0.001	0.001	0.000	0.000	0.030
NMSS score	*r*	1.000	−0.810**	−0.708**	−0.532**	0.362*	−0.643**	−0.662**
	P		0.000	0.000	0.002	0.049	0.000	0.000
MMSE score	*r*	−0.810**	1.000	0.698**	0.505**	−0.648**	0.630**	0.597**
	P	0.000		0.000	0.004	0.000	0.000	0.001
MOCA score	*r*	−0.708**	0.698**	1.000	0.431*	−0.450*	0.449*	0.508**
	P	0.000	0.000		0.017	0.013	0.013	0.004
MEG3 expression level	*r*	−0.532**	0.505**	0.431*	1.000	−0.426*	0.362*	0.593**
	P	0.002	0.004	0.017		0.019	0.049	0.001
NSE	*r*	0.362*	−0.648**	−0.450*	−0.426*	1.000	−0.557**	−0.469**
	P	0.049	0.000	0.013	0.019		0.001	0.009
NGF	*r*	−0.643**	0.630**	0.449*	0.362*	−0.557**	1.000	0.719**
	P	0.000	0.000	0.013	0.049	0.001		0.000

***Significantly correlated at 0.01 level (bilateral).*
**Significantly correlated at the 0.05 level (bilateral).*

TABLE 4 | Multivariate Logistic regression analysis of H&Y classification and other indexes.

Relevant factor	β	SE	Wald χ^2 value	OR (95%CI)	*P*-value
Age	1.057	0.413	6.549	2.878 (1.132 – 4.558)	0.010
Duration of disease	1.289	0.382	11.385	3.629 (1.473 – 6.052)	0.001
NMSS score	0.783	0.348	5.060	2.188 (1.440 – 3.038)	0.024
MMSE score	−1.331	0.369	13.011	3.785 (1.064 – 6.057)	0.000
MOCA score	−0.751	0.341	4.850	2.119 (1.113 – 3.036)	0.028
NSE	1.289	0.382	11.385	3.629 (1.473 – 6.052)	0.001
MEG3 expression level	−0.668	0.329	4.122	1.950 (1.205 – 2.671)	0.043

We found that the NMSS score of PD patients was significantly higher, whereas the MMSE and MoCA scores were significantly lower than those of the control group.

The main pathological characteristic of PD is dopaminergic neuron degeneration, which will lead to striatum dopaminergic denervation and loss of function of the dopaminergic nerve, resulting in secondary effects to the temporal lobe, cortex, thalamus, with reduced neurotransmitter synthesis and secretion in the hypothalamus, ultimately resulting in impaired cognitive function. Accordingly, in the H&Y scale of patients with PD, NMSS scores are positively correlated, whereas MMSE and MoCA scores are negatively correlated with disease severity, motor symptoms, and degree of cognitive damage (2).

NSE, BDNF, and NGEF are well-established biomarkers of cognitive impairment in PD patients, indicating that they may play a pathogenic role (20). In this study, plasma NGF and BDNF levels in PD patients were significantly lower, whereas NSE levels were significantly higher than those in the healthy controls. NSE is an acidic protease that is unique to neurons and neuroendocrine cells. When neurons in the brain are damaged in PD, a large amount of NSE enters the blood circulation; thus, the elevated level of NSE in plasma is related to disease severity. Brain tissue BDNF and NGF are neurotrophic factors (nutrients required for neuronal differentiation and development in the brain) that play roles in repairing damaged neurons and regulating synaptic functions, and participate in memory and learning; therefore, their deficiency is also closely related to the progression of neurodegenerative diseases and the occurrence of cognitive dysfunction (21, 22). We found that the H&Y stage and NMSS score were negatively correlated with NGF and BDNF levels, and were positively correlated with NSE levels. By contrast, MMSE and MoCA scores in PD patients were positively correlated with NGF and BDNF levels and negatively correlated with NSE levels, confirming these close associations with PD severity and cognitive impairment.

To our knowledge, this is the first clinical study to explore the expression of lncRNA *MEG3* in patients with PD. Methylation is one of the most well-studied epigenetic changes in degenerative diseases of the nervous system. Methylation is a dynamic process that regulates gene expression: normal cells tend to be hypomethylated, whereas brain tissues exhibit a higher level of methylation than other tissues, especially in genome repeat regions. DNA methylation typically occurs at cytosine-guanosine dinucleotide (CpG) sites with a GC content >55%. Methylation can bind transcriptional activators to DNA to inhibit gene expression or cause conformational changes in chromosomes

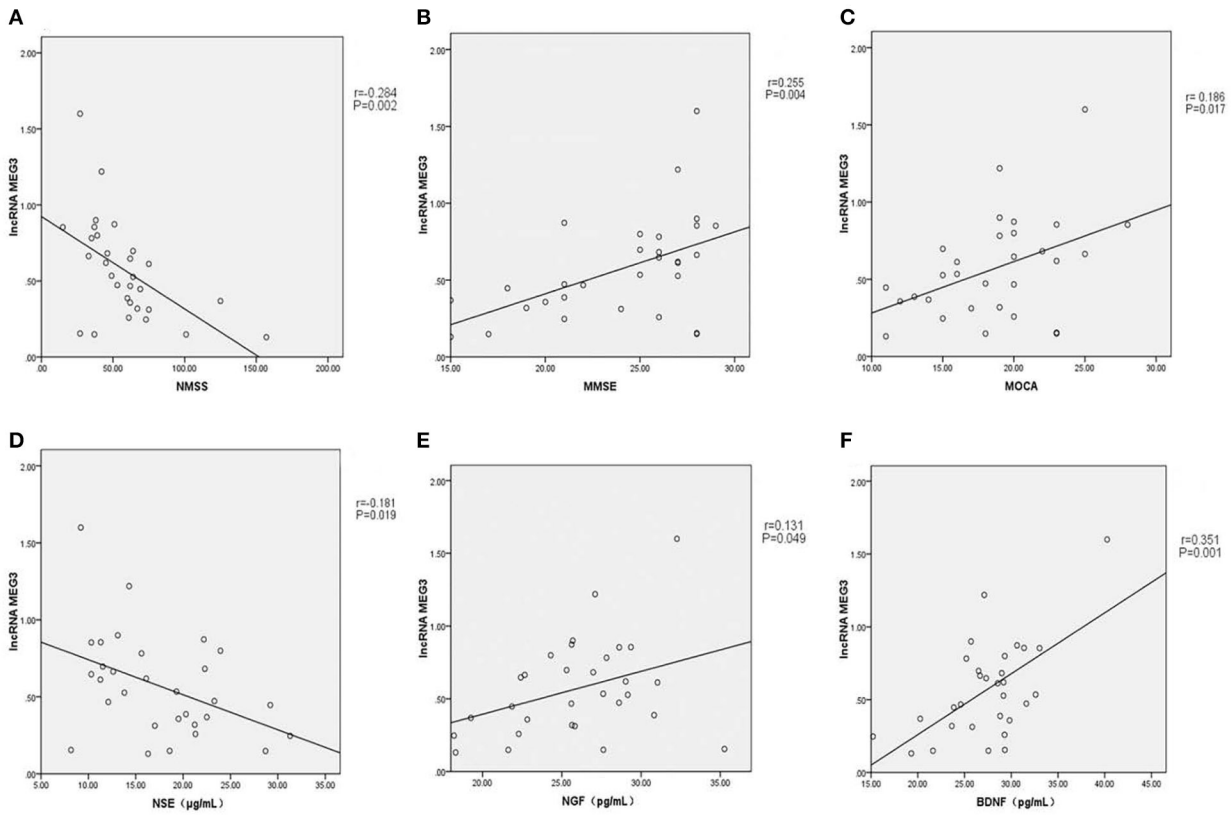

FIGURE 1 | (A) Plasma lncRNA *MEG3* level in PD patients was negatively correlated with NMSS score ($r = -0.284$, $P = 0.002$). **(B)** Plasma lncRNA *MEG3* level in PD patients was positively correlated with MMSE score ($r = 0.255$, $P = 0.004$). **(C)** Plasma lncRNA *MEG3* level in PD patients was positively correlated with MOCA score ($r = 0.186$, $P = 0.017$). **(D)** Plasma lncRNA *MEG3* level in PD patients was negatively correlated with NSE levels ($r = -0.181$, $P = 0.019$). **(E)** Plasma lncRNA *MEG3* level in PD patients was positively correlated with NGF levels ($r = 0.131$, $P = 0.049$). **(F)** Plasma lncRNA *MEG3* level in PD patients was positively correlated with BDNF levels ($r = 0.351$, $P = 0.001$).

leading to gene silencing. The 5′-end of lncRNA *MEG3* is rich in CpG dinucleotides, in which a large amount of DNA is methylated, and DNA methylation in the functional region of *MEG3* may lead to expression silencing (23). Since the brain tissue and peripheral blood show highly similar methylation modes, methylation levels in the peripheral blood can reflect those in the brain tissue. Tan et al. (24) suggested that the *SNCA* level in peripheral blood leukocytes and low *LRRK2* methylation levels can be used as potential biomarkers for PD. In addition, the levels of peripheral blood free small RNAs such as microRNAs can be used for the diagnosis of PD (25). Despite the accuracy of this approach, there are also some problems to overcome. For example, the transcription levels in the blood do not entirely reflect the local levels in the brain tissue, and expression levels can vary in different brain regions at the same time and in different conditions. In PD, the dopaminergic neurons are lost in the substantia nigra, and the blood–brain barrier can lead to low permeability. Therefore, further clinical samples from PD patients are needed to verify the free lncRNA *MEG3* expression levels (26).

We found that the relative expression level of plasma lncRNA *MEG3* was negatively correlated with the H&Y stage and NMSS score, but positively correlated with MMSE and MoCA scores, indicating that the downregulation of lncRNA *MEG3* expression may be one of the possible pathogenic mechanisms of PD. Moreover, the positive correlations between lncRNA *MEG3* and NGF or BDNF levels, and negative correlations with NSE levels suggest synergistic effects with nerve-related factors in the development and progression of PD. However, this study can provide only a preliminary discussion on the association of plasma lncRNA *MEG3* with PD, and the mechanism remains to be further elucidated with animal model experiments. In addition, due to the limitation of the number of samples, we did not rule out monogenic forms of PD, which also brings a little regret for the final result. We look forward to increasing the sample size in the later experimental verification stage and to evaluate whether different PD subtypes show different biomarker signals.

In summary, the expression of lncRNA *MEG3* in the plasma of PD patients was downregulated compared to that of healthy control subjects, and its expression level was closely related to the aggravation of non-motor symptoms, cognitive decline, and PD stage. These associations may reflect the synergism of the increase of NSE and decrease of NGF and BDNF levels, highlighting plasma lncRNA *MEG3* as a new candidate biomarker of PD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Beijing Xuanwu hospital ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YQ had full access to all of the study data, takes responsibility for the integrity, and accuracy of the data analysis. YQ and JW: study concept, design, and statistical analysis. SW and JZ: study supervision. All authors: critical revision of the manuscript for important intellectual content.

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