Review

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Progress in unraveling the genetic etiology of Parkinson disease in a genomic era

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Parkinson disease (PD) and Parkinson-plus syndromes are genetically heterogeneous neurological diseases. Initial studies into the genetic causes of PD relied on classical molecular genetic approaches in well-documented case families. More recently, these approaches have been combined with exome sequencing and together have identified 15 causal genes. Additionally, genome-wide association studies (GWASs) have discovered over 25 genetic risk factors. Elucidation of the genetic architecture of sporadic and familial parkinsonism, however, has lagged behind that of simple Mendelian conditions, suggesting the existence of features confounding genetic data interpretation. Here we discuss the successes and potential pitfalls of gene discovery in PD and related disorders in the post-genomic era. With an estimated 30% of trait variance currently unexplained, tackling current limitations will further expedite gene discovery and lead to increased application of these genetic insights in molecular diagnostics using gene panel and exome sequencing strategies.

PD and related disorders

Parkinsonism refers to a group of neurological syndromes presenting with bradykinesia, muscle rigidity, resting tremor, and postural instability. PD, the most common form of parkinsonism, is clinically characterized by these four cardinal motor symptoms as well as a good response to levodopa therapy [1]. Various nonmotor symptoms may present, including depression, sleep disturbances, constipation, orthostatic hypertension, and, in later disease stages, dementia [2]. Neuropathological hallmarks comprise neuronal loss in the substantia nigra pars compacta and Lewy body inclusions, of which the primary structural component is the protein α-synuclein (SNCA) in the surviving neurons [3]. However, Lewy body pathology is neither exclusive to the disease nor common to all clinical PD patients. Depending on the underlying genetic defect, variable degrees of SNCA accumulation have been observed in PD cases (Table 1).

Parkinson-plus syndromes include multiple system atrophy (MSA), dementia with Lewy bodies (DLB), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and juvenile-onset pallidopyramidal syndromes. Their diagnosis is based on the Parkinsonian motor symptoms in addition to atypical features serving as PD exclusion criteria, such as early dementia, hallucinations, dysautonomia, ophthalmoparesis, ataxia, dystonia, and poor levodopa response. Neuropathologically, MSA and

Glossary

Anticipation: progressively earlier appearance and increased disease severity in successive family generations, often present in families segregating STR expansions.
Candidate gene association studies: a case-control study in which genetic variation within a prespecified gene of interest is statistically compared.
Epigenetics: transient gene expression controllers that enable rapid biological adaptation, including CpG DNA methylation, post-translational histone modifications, and RNA-associated silencing.
Gene panel sequencing: a multiplex sequencing approach designed to simultaneously analyze multiple genes of interest. This approach is situated between sequencing a single gene that is certainly involved in disease risk and sequencing every gene in the genome.
Genetic heterogeneity: a single disorder caused by a variety of mutated genes or mutation mechanisms.
Genetic phenocopies: an individual whose phenotype mimics that of other affected family members but who does not carry the same genetic defect.
Genome-wide association study (GWAS): a case-control study in which genome-wide genetic variation is statistically compared.
Genome-wide linkage (GWL) studies: a powerful gene-hunting strategy that aims to identify the chromosomal location of a disease-causing variation in meiotic informative families by calculating coinheritance of genetic markers (SNPs or STRs) with disease.
Homozygosity mapping: an efficient gene mapping method to identify homozygous gene defects applicable to rare recessive disorders. The strategy assumes that, because of linkage disequilibrium, genetic markers (SNPs or STRs) surrounding the disease locus will tend to be homozygous in affected individuals.
Massive parallel sequencing (MPS): high-throughput DNA sequencing that generates millions of independent sequence reads in a single run and encompasses genome, exome, and gene panel sequencing.
Penetrance: the proportion of individuals carrying a pathological genetic defect that develops the associated disorder.
Pleomorphic locus: a disease locus that harbors both common and rare risk variants.
Whole-exome sequencing (WES): selective enrichment and subsequent sequencing of the coding (and direct flanking regulatory) regions of the genome.
Whole-genome sequencing (WGS): a sequencing process that provides the most comprehensive collection of an individual's genetic variation.

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Table 1. Overview of the characteristics of causal PD and Parkinson-plus genes

Gene	Inheritance	Identification	Clinical phenotype	Mutation spectrum	Pathological brain accumulation	Refs
SNCA	AD	Linkage analysis (mLOD 6.0)	EO–LO Fast progression	Missense/gene dosage	Synucleinopathy	[126]
PARK2	AR	Linkage analysis (mLOD 7.7)	EO Slow progression	Missense/truncating/ gene dosage	Synucleinopathy (occasionally)	[127]
PARK7	AR	Homozygosity mapping (mLOD 4.2)	EO Slow progression	Missense/gene dosage	NA	[128]
LRRK2	AD	Linkage analysis (mLOD 24.9)	LO Classical	Missense	Synucleinopathy/ tauopathy/TDP-43	[129]
PINK1	AR	Homozygosity mapping (mLOD 4.0)	EO Slow progression	Missense/truncating/ gene dosage	Synucleinopathy (occasionally)	[130]
ATP13A2	AR	Linkage analysis (mLOD 3.7)	JO Atypical	Truncating	Iron	[114]
FBXO7	AR	Linkage analysis (mLOD 4.2)	JO Atypical	Truncating	NA	[131]
PLA2G6	AR	Homozygosity mapping (mLOD NA)[b]	JO Atypical	Missense	Iron	[132]
VPS35	AD	WES Linkage analysis (mLOD 1.4)[b]	LO Classical	Missense	NA	[15,16]
EIF4G1[c]	AD	Linkage analysis (mLOD 3.0)	LO Mild or Atypical	Missense	Synucleinopathy/ tauopathy/Aβ	[9]
DNAJC6	AR	WES Homozygosity mapping (mLOD NA)[b]	JO Atypical	Splice site/truncating	NA	[18]
ATP6AP2[a]	X linked	WES Linkage analysis (mLOD 2.1)[b]	JO or EO Classical or atypical	Synonymous	Tauopathy	[19]
COQ2[c]	AR	WGS Linkage analysis (mLOD 1.9)[b]	LO Atypical (MSA)	Missense	Synucleinopathy	[22]
SYNJ1	AR	WES Homozygosity mapping (mLOD 2.0)[b]	JO Atypical	Missense	NA	[20,21]
DNAJC13[a]	AD	WES Linkage analysis (mLOD 5.3)	LO Classical or atypical	Missense	Synucleinopathy	[17]

AD, autosomal dominant; AR, autosomal recessive; JO, juvenile onset; EO, early onset; LO, late onset; NA, unknown; mLOD, maximum logarithm of odds.

[a]Further replication is warranted.

[b]mLOD scores are either unknown or do not reach the LOD threshold for conclusive linkage.

[c]At the time of writing, many replication studies have failed.

DLB are synucleinopathies whereas PSP and CBD present with tau inclusions [3].

Disentangling the genetic etiology

For many years, PD was considered a nongenetic disorder caused by synergistic environmental factors. A study estimating the heritability of PD risk in over 500 nuclear families revealed, however, that in up to 60% of idiopathic PD patients the phenotype could be explained by genetic factors [4]. Furthermore, this genetic etiology was shown to be significantly heterogeneous [5]. Currently, PD is considered a multifactorial disease involving numerous genetic and environmental factors.

Gene identification studies in monogenic families

Although families with inherited PD are rare (10–15% of all PD patients), they have been instrumental in dissecting the genetic etiology of PD. Genome-wide linkage (GWL) analyses or homozygosity mapping using highly polymorphic DNA markers followed by positional cloning identified five causal PD genes: *SNCA*, *leucine-rich repeat kinase 2* (*LRRK2*), *parkin RBR E3 ubiquitin protein ligase* (*PARK2*), *PTEN-induced putative kinase 1* (*PINK1*), and *parkinson protein 7* (*PARK7*) (see Glossary) (Table 1 and Figure 1) [6]. However, less than 10% of familial PD is explained by a mutation in these genes [7]. Although mutations were also identified in *eukaryotic translation initiation factor 4 gamma 1* (*EIF4G1*), its role in PD etiology is heavily debated as a remarkably high number of nonpenetrant *EIF4G1* mutation carriers have been reported (Table 1) [8–13]. Comparable family-based studies have also successfully identified genes for Parkinson-plus syndromes, including *ATPase type 13A2* (*ATP13A2*), *phospholipase A2 group 6* (*PLA2G6*), and *F-box protein 7* (*FBXO7*) (Table 1 and Figure 1) [14].

The pace of gene identification accelerated rapidly with the availability of massive parallel sequencing (MPS) technologies. Combining MPS with homozygosity mapping in consanguineous families or with GWL in extended pedigrees resulted in the identification of six more genes for PD and Parkinson-plus syndromes [*vacuolar protein sorting 35 homolog* (*VPS35*), *dnaJ homolog subfamily C member 13* (*DNAJC13*), *dnaJ homolog subfamily C member 6* (*DNAJC6*), *ATPase H^+ transporting lysosomal accessory protein 2* (*ATP6AP2*), *synaptojanin 1* (*SYNJ1*), and *coenzyme Q2 4-hydroxybenzoate polyprenyltransferase*

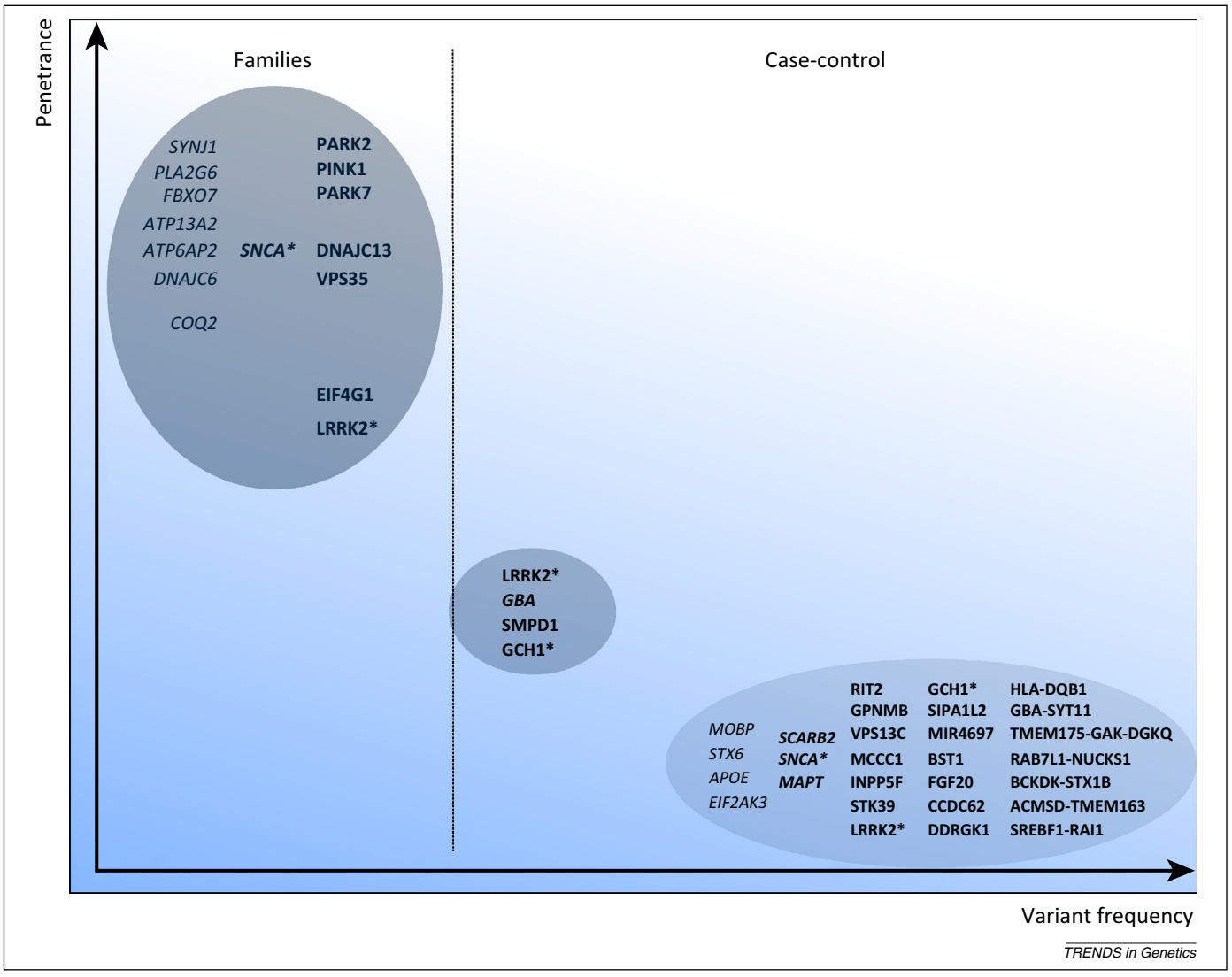

Figure 1. Schematic overview of the penetrance and variant frequencies of the Parkinson disease (PD) and Parkinson-plus genes. An asterisk denotes pleomorphic loci. PD genes are represented in bold; Parkinson-plus genes are shown in italics. Adapted from [133].

(*COQ*2)] (Table 1 and Figure 1) [15–22]. In VPS35, the mutation p.D620N was identified in late-onset PD (Table 1) [15,16]. *VPS35* encodes a key subunit of the retromer cargo-recognition complex, which, on interaction with sorting nexins and the WASH complex, mediates transmembrane protein trafficking between endosomes and either the trans-Golgi network or the plasma membrane [23]. Cellular overexpression of the mutant allele showed destabilization of the retromer–WASH interaction leading to impaired autophagosome formation and clearance [24,25]. Moreover, the occurrence of enlarged endosomes in the perinuclear subcellular region, highlighting a defect in endosomal maturation, was suggested though not replicated [24,26,27].

In DNAJC13, the mutation p.N855S was identified in an extended Saskatchewan kindred segregating PD in an autosomal-dominant manner (Table 1) [17]. Like VPS35, DNAJC13 has an important role in retromer-mediated endosomal protein sorting [28]. On interaction with sorting nexin 1 (SNX-1), DNAJC13 controls the tubulation of endosomal membranes. Cellular overexpression of p.N855S showed disturbances in endocytic processes [17]. Both VPS35 and DNAJC13 interact with the WASH complex and regulate membrane protein sorting, suggesting a significant role for retromer/WASH dysfunction in PD pathogenesis [28,29]. Further, immunofluorescence and coimmunoprecipitation experiments in mouse cortical neurons revealed a direct VPS35–DNAJC13 interaction [17].

Homozygous loss-of-function mutations in DNAJC6 were identified in consanguineous kindreds with juvenile parkinsonism (Table 1) [18,30]. *DNAJC6* encodes a cochaperone protein that recruits heat-shock cognate 70 (Hsc70) to clathrin-coated vesicles after detachment from the donor membrane and promotes clathrin disassembly in an ATP-dependent process [31]. Clathrin-mediated endocytosis is essential for vesicular protein transport between the cell membrane, the trans-Golgi network, endosomes or lysosomes. Dysfunction of protein transport may affect neuronal signaling causing neuronal death. A canonical splice

acceptor site mutation (c.801-2 A>G) was shown to produce two unstable transcripts due to either exon skipping or partial intron retention [18].

A rare synonymous variant, p.S115, in *ATP6AP2* has been linked to parkinsonism (Table 1) [19]. The ATP6AP2 protein is involved in a wide range of processes including autophagy, early endosome maturation, lysosomal degradation, and WNT signaling [19,32–34]. Gene expression analysis in lymphoblast cells of mutation carriers showed a 90-fold increase in *ATP6AP2* cDNA levels of a minor splice isoform [19]. Comparative immunohistochemistry experiments revealed substantial p62 protein accumulation in the striatum, indicative of autophagy dysfunction.

The p.R258Q hotspot mutation in *SYNJ1* was linked to early-onset Parkinson-plus disease in three unrelated families (Table 1) [20,21,35]. Like DNAJC6, SYNJ1 regulates disassembly of clathrin-coated vesicles on transit to their target sites [36]. Whereas DNAJC6 and Hsc70 mediate clathrin disassembly, SYNJ1 dephosphorylates phosphoinositides resulting in detachment of adaptor proteins from the membrane. Membrane attachment of the adaptor proteins is necessary for proper clathrin recruitment. *In vitro* studies showed that p.R258Q caused severe impairment of the protein's phosphatase activity [20].

Mutations in *COQ2* were linked to late-onset MSA (Table 1) [22]. *COQ2* encodes an enzyme involved in the biosynthesis of coenzyme Q10, which acts as a small mobile redox carrier. Hence, reduced COQ2 activity might impair the mitochondrial respiratory chain and increase vulnerability to oxidative stress. COQ2 activity and coenzyme Q10 levels were significantly reduced in lymphoblast cells of homozygous or compound heterozygous mutation carriers [22].

Genetic association studies of candidate genes

Candidate gene association studies focus on selected genes that are genetically, clinically, or functionally linked to a particular disease or disease spectrum. As such, genetic variants in known causal PD or neurodegenerative brain disease genes have been identified as PD risk alleles. Genetic variability in the regulatory region 10 kb upstream of *SNCA* increased PD risk by a factor of 1.4 [37–42]. Many coding variants in *LRRK2* were shown to influence PD susceptibility either as risk or protective variants or haplotypes, although with considerable variability in their risk effects between populations [43–45]. Additionally, the H1 haplotype of *microtubule-associated protein tau* (*MAPT*) has been identified as a risk factor for idiopathic PD [46–48].

Clinical observations led to the identification of *beta acid glucosidase* (*GBA*) and *GTP cyclohydrolase 1* (*GCH1*) (Figure 1). Parkinsonian symptoms were observed in numerous patients with Gaucher disease (GD), an autosomal-recessive lysosomal storage disorder [49–51]. In GD families, relatives carrying heterozygous *GBA* variants have an increased incidence of PD [52,53]. A multicenter study confirmed the genetic association and calculated a fivefold increase in PD risk [54]. Although PD patients carrying a *GBA* mutation present with typical neuropathological hallmarks of idiopathic PD, they clinically present with a high prevalence of dementia and earlier onset age [55,56]. Parkinsonian symptoms were also described in patients with other lysosomal storage disorders [57]. Variability in *sphingomyelin phosphodiesterase 1* (*SMPD1*) was associated with a ninefold increase in PD risk, although larger studies are needed to assess the role and contribution of *SMPD1* genetic variability to PD risk [58,59]. Mutations in *GCH1* cause dopa-responsive dystonia (DRD), a rare childhood-onset dystonic disorder [60]. However, as Parkinsonian features are frequently observed in DRD patients and *GCH1* mutation carriers sporadically develop adult-onset parkinsonism in the absence of dystonia, genetic variability in *GCH1* might contribute to PD etiology [61]. A large association study in 1318 PD patients and 5935 control individuals revealed a sevenfold increase in PD risk for *GCH1* mutation carriers [62]. Association studies of functionally relevant candidate genes have also been performed, although often with conflicting or population-specific outcomes.

Candidate gene association studies also identified risk genes for the Parkinson-plus syndromes. Genetic variability in *GBA*, *SNCA*, *apolipoprotein E* (*APOE*), and *scavenger receptor class B member 2* (*SCARB2*) were reported to influence DLB risk [63,64]. *SNCA* was associated with risk for MSA and the *MAPT* H1 haplotype significantly increased risk for PSP and CBD [65,66].

Risk gene identification by GWASs

The first GWASs confirmed the causal genes *SNCA*, *PARK16*, *LRRK2*, and *MAPT* as risk genes for PD and identified one new gene, *bone marrow stromal cell antigen 1* (*BST1*) [67,68]. Subsequent GWASs, in increasingly larger patient-control cohorts, and meta-analyses not only confirmed candidate gene-based and former GWAS associations but revealed additional risk genes, although with decreasing risk effect sizes (Table 2) [69–76]. The largest meta-analysis pooled SNP data from 15 European GWASs, including 13 708 PD patients and 95 282 control individuals, and identified 28 PD-associated variants cross 24 chromosomal loci [77]. Although most of these risk loci represented small effect sizes, pooling of risk alleles for genetic risk profiling resulted in a threefold increased PD risk for carriers of the highest risk quintile. For Parkinson-plus syndromes, the one published GWAS compared over 2000 PSP cases, of which 1000 were autopsy confirmed, with around 6800 control subjects [78]. Genome-wide significance was obtained for *MAPT*, *eukaryotic translation initiation factor 2-alpha kinase 3* (*EIF2AK3*), *syntaxin 6* (*STX6*), and *myelin-associated oligodendrocyte basic protein* (*MOBP*) (Table 2).

A key feature of GWASs is the association based on linkage disequilibrium enabling a subset of SNPs to serve as proxies. Hence, the next step is the identification of the relevant variants underlying the GWAS associations. Because many of the associated GWAS SNPs reside in non-coding regions and large numbers of individuals need to be analyzed, MPS-based targeted resequencing is used to search for the true functional variants. For future studies in complex disorders, one can expect that this two-step approach will be replaced by whole-genome sequencing (WGS) as soon as the cost becomes affordable. Hence, a single genetic experiment will allow SNP data extraction

Table 2. Overview of the characteristics of PD and Parkinson-plus gene loci identified by GWASs[a]

Locus	Clinical phenotype	P value	Odds ratio
SNCA	PD	4.2×10^{-73}	0.76
BST1	PD	9.4×10^{-18}	1.13
LRRK2	PD	5.2×10^{-14}	1.15
scavenger receptor class B member 2 (*FAM47E-SCARB2*)	PD	2.95×10^{-11}	0.91
ras-like without CAAX 2 (*RIT2*)	PD	7.74×10^{-12}	0.90
glycoprotein (transmembrane) nmb (*GPNMB*)	PD	1.18×10^{-12}	1.11
vacuolar protein sorting 13 homolog C (*VPS13C*)	PD	1.23×10^{-11}	1.11
methylcrotonoyl-CoA carboxylase 1 (alpha) (*MCCC1*)	PD	2.14×10^{-21}	0.84
inositol polyphosphate-5-phosphatase F (*INPP5F*)	PD	4.34×10^{-13}	1.62
serine threonine kinase 39 (*STK39*)	PD	1.15×10^{-20}	1.21
GCH1	PD	5.85×10^{-11}	0.90
signal-induced proliferation-associated 1 like 2 (*SIPA1L2*)	PD	4.87×10^{-10}	1.13
microRNA 4697 (*MIR4697*)	PD	9.83×10^{-12}	1.10
fibroblast growth factor 20 (*FGF20*)	PD	6.68×10^{-8}	0.92
coiled-coil domain containing 62 (*CCDC62*)	PD	6.02×10^{-12}	1.10
DDRGK domain containing 1 (*DDRGK1*)	PD	3.04×10^{-11}	1.11
major histocompatibility complex, class II, DQ beta 1 (*HLA-DQB1*)	PD	1.19×10^{-12}	0.83
beta acid glucosidase-synaptotagmin XI (*GBA-SYT11*)	PD	1.37×10^{-29}	1.82
transmembrane protein 175-cyclin G associated kinase-theta diacylglycerol kinase (*TMEM175-GAK-DGKQ*)	PD	1.02×10^{-43}	0.79
RAB29 member RAS oncogene family-nuclear casein kinase and cyclin-dependent kinase substrate 1 (*RAB7L1-NUCKS*)	PD	1.66×10^{-16}	1.12
branched chain ketoacid dehydrogenase kinase-syntaxin 1B (*BCKDK-STX1B*)	PD	2.43×10^{-12}	1.10
aminocarboxymuconate semialdehyde decarboxylase-transmembrane protein 163 (*ACMSD-TMEM163*)	PD	9.13×10^{-20}	0.87
sterol regulatory element binding transcription factor 1-retinoic acid induced 1 (*SREBF1-RAI1*)	PD	5.98×10^{-5}	0.94
MAPT	PD PSP PSP	2.4×10^{-48} 1.5×10^{-116} 4.2×10^{-70}	0.77 5.46 0.51
MOBP	PSP	1.0×10^{-16}	0.72
STX6	PSP	2.3×10^{-10}	0.79
EIF2AK3	PSP	3.2×10^{-13}	0.75

[a]PD data were based on the meta-GWAS of Nalls *et al.* [77], whereas for PSP the study of Höglinger *et al.* [78] was used.

for GWASs, identification of underlying functional variants in candidate loci and identification of novel parkinsonism genes as well as replication of newly identified disease genes. Integrative approaches that combine DNA sequencing with gene expression and methylome data will support and accelerate the identification and functional characterization of the biological variants and disease genes [77].

Risk gene identification by whole-exome sequencing (WES) and WGS

WES and WGS allow the hypothesis-free identification of rare or low-frequency risk variants exhibiting moderate effect sizes that are generally not detected using GWAS or GWL analyses. These approaches, however, require careful experimental design. For PD and related disorders, only one such study has been reported so far. WES was performed in 100 unrelated PD patients from Sardinia, followed by genetic association analyses of variants shared by at least five patients in replication cohorts, but it failed to identify variants significantly associated with PD risk [79]. This example highlights potential pitfalls with this method, particularly for genetically heterogeneous disorders such as PD. The use of larger and more homogeneous cohorts (e.g., endophenotype oriented), even within fairly isolated study populations, will increase the chances of identifying intermediate-risk variants in future WES/WGS studies.

Confounding factors in gene identification

Although substantial progress has been made toward the elucidation of the complex genetic etiology of PD and related disorders, it has been noticeably slow compared with pure Mendelian diseases. There are many confounding factors that contribute to this relatively slower rate of gene identification. We discuss below two classes of factors – technical and systemic – that present challenges for PD researchers.

Technical limitations and their relevance for PD and related disorders

Compared with WGS, WES is faster, cheaper, and less challenging to interpret from a bioinformatics and biological perspective. Despite potential experimental design challenges, WES has become the preferred tool to identify mutations in inherited forms of complex diseases including PD. Genomic regions targeted by the most advanced WES enrichment solutions include untranslated regions (UTRs) and miRNA genes and the examination of off-target exome sequencing reads allows variant detection in direct exon-flanking regions [80]. However, many noncoding variants

that might affect gene expression will still be missed. This is of particular interest for PD, as specific polymorphisms in both 5′ and 3′ *SNCA* regulatory regions are associated with PD risk [39,81]. Moreover, as most of the GWAS SNPs are noncoding and often located far from genes, it is conceivable that the biologically important variants are also noncoding.

Furthermore, in 80% of the well-known PD genes, pathogenic exon and gene multiplications or deletions have been identified (http://www.molgen.vib-ua.be/PDMutDB/) [6]. Hence, it is likely that dosage mutations in as-yet-unidentified genes might also contribute to PD etiology. At present, copy number variation (CNV) analysis from WES data is not trivial [82]. The combinatorial use of multiple CNV-detecting algorithms may increase call reliability, but sequencing improvements in terms of read length, depth, and assembly are needed to enable confident MPS-based CNV identification. Due to the size and repetitive nature of short tandem repeats (STRs), the detection of PD-related repeat expansions would also benefit from longer sequencing reads. Onset age anticipation has been observed in genetically unresolved PD pedigrees [83,84]. Although this might be partially explained by referral biases and earlier recognition of the symptoms in an affected family, unstable STRs may underlie PD susceptibility [85]. Clinically, a subset of patients with permutations in *fragile X mental retardation 1* (*FMR1*) and *ataxin 3* (*ATXN3*) or *chromosome 9 open reading frame 72* (*C9orf72*) repeat expansions present with Parkinsonian features [86–88]. Additionally, pure *ataxin 2* (*ATXN2*) repeat expansions cause ataxia-predominant phenotypes, whereas interrupted repeat expansions have been observed in typical PD patients [89–92]. A clear and definite causal relationship between increased repeat lengths and PD, however, remains elusive.

Importantly, multiple studies showed a direct link between hypomethylation of CpG islands in the regulatory region of *SNCA* and increased SNCA levels in the brain and leukocytes of sporadic PD patients [93–95]. *MAPT* has also been found to be differentially methylated [96]. Furthermore, significant associations were observed between SNPs of six meta-GWA loci and CpG methylation of nearby genes [77]. Similarly to CNVs, such epigenetic alterations may lead to aberrant protein levels causing neuronal death. Obviously, because the DNA code remains unchanged, epigenetic marks cannot be detected using standard WES or WGS approaches. Various high-throughput sequencing approaches for epigenomic variation analysis exist, such as whole-genome bisulfite sequencing for the detection of DNA methylation, ChIP-seq for the assessment of histone modifications, and long-read single-molecule real-time (SMRT) sequencing for the detection of multiple types of nucleotide modification based on DNA polymerase kinetics. So far, the application of such approaches to PD patient biosamples has not been reported.

Challenges inherent to PD and Parkinson-plus syndromes

In neurodegenerative brain disorders, the clinical penetrance of a particular mutation often varies considerably with increasing age. Among carriers of the pathogenic *LRRK2* mutation p.G2019S, disease penetrance increased from 10% in carriers below 50 years old to 67% in carriers below 94 years [97]. This phenomenon explains the occasional occurrence of PD-causing mutations in healthy individuals. Additionally, it highlights the importance of inclusion of aged control cohorts when selecting MPS-derived variants for follow-up studies. Importantly, genetic modifiers may partially account for differences in disease onset age or progression, making them valuable targets for the development of disease-modifying therapies. In the case of PD, both onset age and disease progression have been associated with *SNCA* variability [98,99]. Additionally, onset age is influenced by *GBA* mutations [100].

Currently, 15 genes are known to cause Mendelian forms of PD or Parkinson-plus syndromes, which highlights the high genetic heterogeneity of these diseases. For such disorders, family-based gene identification approaches have been of paramount importance to success. Nonetheless, a systematic assessment of PD families segregating a pathological mutation in *SNCA*, *LRRK2*, *PARK2*, or *PINK1* ($N = 160$) revealed that in 14.4% of the families genetic phenocopies were present (i.e., patients that carry a mutation in a PD gene other than the segregating gene) [101]. As family-based studies start from the assumption that all affected members share the same genetic defect, the occurrence of genetic phenocopies might cause omission of the pathological variant. In part, this can be prevented by alternating exclusion of one of the patients when prioritizing the MPS variants. Another approach involves the identification and exclusion of family members carrying known parkinsonism-causing mutations before sequencing or novel variant prioritization. Genetic heterogeneity is also expected to complicate disease gene identification in groups of unrelated patients. In the Flanders–Belgian PD cohort, *LRRK2* mutations were observed in 4.8% of PD patients, indicating that WES or WGS data from about 50 unrelated patients are needed to identify two patients carrying a *LRRK2* mutation [102]. In the case of infrequently mutated genes like *VPS35*, MPS data from thousands of patients are needed. Selecting well-defined disease endophenotypes might increase the genetic homogeneity of the study cohort and increase the probability of finding multiple patients with mutations in the same gene. Hence, deep and accurate patient phenotyping is clearly of major importance for the success of gene identification in heterogeneous diseases.

Key molecular processes and therapeutic implementations

The identification of parkinsonism genes has been followed by intense molecular research indicating that neuronal death may originate from the interconnection of various processes including endosomal protein sorting and recycling, synaptic transmission, mitochondrial quality control, and lysosome-mediated autophagy. As discussed above, most of the recent gene discoveries converge on abnormal endocytosis and endosome trafficking as key pathomechanisms for PD and related disorders. Genetic findings in disease models also implicated SNCA and LRRK2 in this process. At the presynaptic terminal, SNCA regulates membrane curvature, which is critical for neurotransmitter

uptake and release [103,104]. LRRK2 mediates synaptic vesicle formation, synaptic growth, and chaperone-mediated autophagy [105–107]. PARK2, PINK1, PARK7, and FBXO7, by contrast, jointly drive mitophagy of depolarized mitochondria [108–110]. Neurons, which are postmitotic and have high energy demands, might particularly suffer from cellular accumulation of damaged mitochondria. ATP13A2, a lysosomal membrane protein, has been implicated in mitochondrial maintenance, lysosome-mediated clearance of autophagosomes, and lysosomal membrane stability and acidification [111,112]. Moreover, ATP13A2 mutations cause not only atypical parkinsonism but also neuronal ceroid lipofuscinosis, a lysosomal storage disorder [113,114]. GBA has also been linked to lysosomal activity and mutant forms were shown to increase SNCA aggregation [115].

Dopamine replacement therapies in addition to deep-brain stimulation temporarily improve PD symptoms, yet they fail to halt or slow disease progression. Furthermore, in Parkinson-plus patients they may induce severe side-effects. Targeted disease-modifying therapies are therefore a major unmet need in the treatment of PD and related disorders. Each key molecular pathway is actively being investigated in model systems, aiming to identify novel therapeutic targets [116]. For example, work in *Drosophila* has shown that flies deficient for PARK2 and PINK1 exhibit an alleviated phenotype on vitamin K2 administration, which restores mitochondrial energy production [117]. Research in rats on the transcription factor EB suggests that high levels of EB rescue defects in lysosome-mediated autophagy, enhance clearance of SNCA oligomers, and reduce neurodegeneration in an SNCA overexpression model [118]. Disturbances in endocytosis and vesicular transport were only recently firmly associated with Parkinsonian disorders. Hitherto, no PD-oriented endocytic targets have been reported. This opens another avenue for potential therapies that is actively being pursued.

Translating genetic findings to the clinic

Molecular diagnostic testing encompasses systematic screening of patients and at-risk individuals for the presence of pathogenic, so far mostly exonic, variants in known causal genes. Diagnosing PD is not an exact science, as diagnostic accuracy rates at best reach 88% [119]. Molecular diagnoses may complement clinical diagnoses and consequently improve both diagnostic sensitivity and specificity, reducing the diagnostic uncertainty. Furthermore, it enables early diagnosis as well as prenatal testing and improves prognostic predictions. Beyond improving diagnoses, patients' genetic information can contribute to enhanced clinical trial design by establishing more homogeneous patient subgroups. Ultimately, genetic classification will pave the way for personalized medicine, which aims to improve treatment efficacy by delivering the most beneficial therapy to a patient [120]. Additionally, families segregating a pathological mutation can be followed longitudinally for translational clinical and therapeutic research, which can be of major importance for the discovery of disease modifiers or biomarkers. MPS technologies allow simultaneous mutation analyses of all known PD and Parkinson-plus genes in a cost- and time-efficient manner. Several molecular diagnostic laboratories are using disease-targeting gene panels. Advantages of this approach include flexibility concerning assay design and very high read coverage, also allowing the identification of somatic mutations. Only genes related to the phenotype are screened, avoiding complex ethical discussions [121]. Because additional PD and Parkinson-plus genes are expected to be discovered in the coming years, the gene panel approach will require continuous updating of the target sequences of existing assays.

With the declining cost of sequencing technologies, many clinical laboratories have started to use WES for molecular diagnostic purposes in common and rare disorders [122]. The use of WES in molecular diagnostics requires deeper sequencing to obtain on-target read depths comparable with gene panel sequencing. Other drawbacks include incomplete exome enrichment due to sequence capture failure or probe/primer design restrictions and inability to detect noncoding variation. Additionally, current bioinformatics analysis strategies for both WES and gene panel sequence datasets still have difficulties in reliably detecting CNVs or structural variants, highlighting the importance of performing complementary dosage analyses of the known PD and Parkinson-plus genes in a molecular diagnostic setting [82]. The abovementioned limitations would be partially overcome by using WGS, a virtually complete and one-time genetic test that can provide the basis for lifelong medical follow-up [123]. However, WGS is more challenging from a bioinformatics perspective and generates lower read coverage for the same cost. Furthermore, it will identify more variants of unknown clinical significance, as the post-genomic consequences of noncoding variations are less well understood. The latter hampers the feedback of correctly interpreted results to patients.

Concluding remarks

The pathological and genetic overlap between PD and Parkinson-plus syndromes suggests that expanding our knowledge of one disease entity might shed light on the other members of this disease continuum. Genetic commonalities have been observed, with *MAPT*, *SNCA*, and *GBA* variability contributing to susceptibility to different Parkinsonian disorders [39,46,47,54,63–66]. However, family-based gene identification studies and subsequent functional characterization of the encoded proteins have revealed differences in the molecular mechanisms underlying early-onset compared with late-onset parkinsonism. Whereas impaired mitophagy and lysosomal dysfunction seem to be common denominators for early-onset disease, deficiency of synaptic transmission and vesicular recycling have been linked to late-onset forms. Moreover, as the underlying genetic defects correlate with pathological events, we hypothesize that classification of patients based on genetic etiology will be crucial for future clinical trials and personalized therapy (Table 1).

The advent of MPS technologies has radically changed the biomedical research field, enabling fast and cost-effective analysis of the entire exome or genome. Family-based MPS approaches successfully identified six PD and Parkinson-plus genes, although three of these (*DNAJC13*,

ATP6AP2, and *COQ2*) await replication in independent cohorts (Table 1). The mutation frequencies of these genes, however, are low, and a substantial fraction of heritability in familial patients remains to be explained. Additionally, the GWAS risk factors have marginal effects, together leaving ±30% of trait variance unexplained and suggesting the existence of substantially more PD and Parkinson-plus genes [4,124]. If future study designs anticipate the potential confounding aspects, we expect many more PD and Parkinson-plus genes to be identified in the coming years.

As MPS costs continue to decrease, WGS will become the standard approach to identify novel causal or risk genes for PD and related disorders. Multiple candidate gene association studies have shown that noncoding variation impinges on parkinsonism risk. WGS, however, will allow hypothesis-free examination of the role of noncoding variants in the development of PD or related disorders. With WGS comes longer lists of candidate variants, making it difficult to pinpoint variants that truly contribute to disease. Concurrent investigation of (epi)genomic alterations and their post-genomic consequences, such as transcriptome and/or proteome analyses, may provide first-line insights into the aberrant cellular processes, thereby accelerating gene discovery. Given the high genetic heterogeneity and low mutation frequency of the known PD and Parkinson-plus genes, the field would benefit from the establishment of a worldwide MPS PD database. Such a database would facilitate acquisition of supporting evidence for putative causal genes by enabling the identification of additional mutation carriers in a very large patient cohort.

Identification of additional PD and Parkinson-plus genes will enable further elucidation of the disease mechanisms and the development of disease-modifying therapeutic strategies. This is of particular interest in PD, as current therapeutic agents provide only symptomatic relief and often have severe side effects. Without the development of therapies slowing or halting the disease process, the number of PD patients will double in the next 15 years [125].

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