Applying genomics to stratify Parkinson's disease: from REM sleep behavior disorder to therapy

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ABSTRACT

REM sleep behavior disorder (RBD) is a sleep disturbance that, in its idiopathic form (iRBD), is considered a prodromal stage of synucleinopathies, such as Parkinson's disease (PD). PD with RBD, in particular, is more often in comorbidity with neuropsychiatric symptoms than PD without RBD, suggesting that the presence of RBD might define a specific PD subtype with a distinctive clinical presentation. PD and RBD in part share risk variants, such as *GBA1* variants, but show also some divergences, for example, *LRRK2* variants are associated with increased risk for PD but with reduced frequency of RBD. Despite the opposite effect of *GBA1* and *LRRK2* variants in RBD, they are moth common genetic risk factors in PD. New drugs targeting *GBA1* and *LRRK2* pathways are being investigated in clinical trials. Currently, however, PD therapy is still symptomatic and one of the main medications used to treat PD symptoms is levodopa. One of the most frequent and debilitating adverse effects of this drug is levodopa-induced dyskinesia (LID), characterized by involuntary and uncontrolled movements. The genetics of LID is still unclear and previous studies show contradicting results.

In Chapter 2, I performed Optimized Sequence Kernel Association Test to evaluate the role in iRBD of rare variants in *PSAP*, a gene encoding a coactivator of glucocerebrosidase, the enzyme encoded by *GBA1.* I found a nominal enrichment of rare loss of function (lof) *PSAP* variants in iRBD and also observed that all these variants were absent in the healthy control group. Furthermore, carriers of two of these variants were also carriers of a *GBA1* variant.

In Chapter 3, I investigated the genetic difference between PD with and without RBD performing GWAS. I found that the top *SNCA* variant was associated with PD with RBD. Conversely, previous independent variants associated with increased risk for PD in the *LRRK2* and *SNCA* regions were associated with PD without RBD. In addition, I assessed the

potential genetic correlation between RBD and neuropsychiatric manifestations in PD performing linkage disequilibrium score regression. I identified a nominally significant correlation between PD with RBD and attention deficit and hyperactivity disorder (ADHD), not sustained after correction for multiple comparisons.

In Chapter 4, I performed linear regression to investigate the relationship between *LRRK2* variants and the activity of glucocerebrosidase. I found that the p.G2019S and p.M1646T *LRRK2* variants are associated with increased glucocerebrosidase activity. In addition, p.M1646T, a less characterized variant in PD, was nominally associated with a mild increase of PD risk in the most recent PD GWAS in Europeans.

In Chapter 5, I investigated the genetic bases of LID performing GWAS and focusing on specific genes and gene sets. I demonstrated that *GBA1* variants are associated with increased risk for LID while *LRRK2* variants with reduced time to development of LID. Additionally, PD risk variant-based polygenic risk score (PRS) was associated with increased LID risk and dopaminergic transmission pathway PRS with reduced time to LID.

This thesis uncovers the genetic underpinnings of PD biological and clinical phenotypes and contributes to patient stratification, essential for clinical trials and to enhance future precision medicine in PD.

RESUMÉ

Le trouble du comportement en sommeil paradoxal (RBD) est un trouble du sommeil qui, sous sa forme idiopathique (iRBD), est considéré comme un stade prodromique des synucléinopathies, telles que la maladie de Parkinson (MP). La MP avec RBD, en particulier, est plus souvent associée à des symptômes neuropsychiatriques que la MP sans RBD, ce qui suggère que la présence de RBD pourrait définir un sous-type spécifique de MP avec une présentation clinique distinctive. PD et RBD partagent en partie des variantes de risque, telles que les variantes GBA1, mais présentent également certaines divergences, par exemple, les variantes LRRK2 sont associées à un risque accru de MP mais à une fréquence réduite de RBD. Malgré l'effet opposé des variantes GBA1 et LRRK2 dans la RBD, ce sont des facteurs de risque génétiques courants dans la MP. De nouveaux médicaments ciblant les voies GBA1 et LRRK2 sont étudiés dans le cadre d'essais cliniques. Cependant, à l'heure actuelle, le traitement de la MP est toujours symptomatique et l'un des principaux médicaments utilisés pour traiter les symptômes de la MP est la lévodopa. L'un des effets indésirables les plus fréquents et les plus débilitants de ce médicament est la dyskinésie induite par la lévodopa (LID), caractérisée par des mouvements involontaires et incontrôlés. La génétique du LID est encore floue et des études antérieures montrent des résultats contradictoires.

Au chapitre 2, j'ai effectué un test d'association de noyau de séquence optimisé pour évaluer le rôle dans l'iRBD de variantes rares du PSAP, un gène codant pour un coactivateur de la glucocérébrosidase, l'enzyme codée par GBA1. J'ai trouvé un enrichissement nominal de rares variantes PSAP avec perte de fonction (lof) dans l'iRBD et j'ai également observé que toutes ces variantes étaient absentes dans le groupe témoin sain. De plus, les porteurs de deux de ces variants étaient également porteurs d'un variant GBA1.

Au chapitre 3, j'ai étudié la différence génétique entre la MP avec et sans RBD exécutant GWAS. J'ai découvert que la principale variante SNCA était associée à la PD avec

RBD. À l’inverse, les variantes indépendantes précédentes associées à un risque accru de MP dans les régions LRRK2 et SNCA étaient associées à la MP sans RBD. De plus, j'ai évalué la corrélation génétique potentielle entre le RBD et les manifestations neuropsychiatriques dans la MP en effectuant une régression du score de déséquilibre de liaison. J'ai identifié une corrélation nominalement significative entre la MP avec RBD et le trouble de déficit de l'attention et d'hyperactivité (TDAH), non maintenue après correction pour des comparaisons multiples.

Au chapitre 4, j'ai effectué une régression linéaire pour étudier la relation entre les variants de LRRK2 et l'activité de la glucocérébrosidase. J'ai découvert que les variants p.G2019S et p.M1646T LRRK2 sont associés à une activité glucocérébrosidase accrue. De plus, p.M1646T, une variante moins caractérisée de la MP, était nominalement associée à une légère augmentation du risque de MP dans le plus récent PD GWAS chez les Européens.

Dans le chapitre 5, j'ai étudié les bases génétiques du LID exécutant GWAS et en me concentrant sur des gènes et des ensembles de gènes spécifiques. J'ai démontré que les variants GBA1 sont associés à un risque accru de LID, tandis que les variants LRRK2 sont associés à un délai de développement du LID réduit. De plus, le score de risque polygénique (PRS) basé sur les variantes de risque de MP était associé à un risque accru de LID et à une voie de transmission dopaminergique PRS avec un délai réduit jusqu'à LID.

Cette thèse découvre les fondements génétiques des phénotypes biologiques et cliniques de la MP et contribue à la stratification des patients, essentielle pour les essais cliniques et pour améliorer la future médecine de précision dans la MP.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis presents the following original contributions to scientific knowledge:

CHAPTER 2

- Finding of rare loss of function *PSAP* variants in REM sleep behavior disorder (RBD).
- Report of a coinheritance of *PSAP* and *GBA1* variant in 0.2% of the population, suggesting a possible modifier effect of *PSAP* variants on *GBA1*.

CHAPTER 3

- Nomination of *SNCA* variant associated with increased risk for RBD in Parkinson's disease (PD). Nomination of two *SNCA* variants and one *LRRK2* variant associated with PD without RBD.
- Potential genetic correlation between PD with RBD and attention deficit hyperactivity disorder.

CHAPTER 4

- Association between *GBA1* variants and increased risk for levodopa-induced dyskinesia (LID)
- Association between *LRRK2* variants and accelerated progression to LID development
- Association between PD polygenic risk score and increased LID risk
- Association between dopaminergic transmission pathway and accelerated progression to LID

CHAPTER 5

- Demonstration of an association between *LRRK2* variants and increased glucocerebrosidase (GCase) activity, with important implication for therapies targeted towards *LRRK2* and GCase pathways

CONTRIBUTION OF AUTHORS

Chapter 2

- YLS: conception and design of the study, manuscript drafting, analysis
- EY, MAE, LK, KM, UR, JAR, FA, SBL, DS, JFT, TGQ, NO, IA, JYM, JFG, AD, YD, GLG, MV, FJ, AB, KS, DK, WO, AJ, GP, EA, FB, MF, MP, BM, CT, FSD, VCDC, CCM, AH, LDS, FD, MV, BA, BFB, RBP, GAR, AI, AS, BH, MTMH, ZGO: data acquisition/analysis
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- LK: conception of the study, analysis, manuscript review
- ZGO: conception and design of the study, manuscript review

Chapter 4

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- ZGO: conception and design of the study, manuscript drafting, manuscript review

Chapter 5

- YLS: conception and design of the study, manuscript drafting, analysis
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- ZGO: conception and design of the study, manuscript drafting, manuscript review
- KS: conception and design of the study, manuscript review, analysis

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LIST OF ABBREVIATIONS

18F-fluorodeoxyglucose (18F-FDG)

Age at onset (AAO)

Alzheimer's disease (AD)

Antisense oligonucleotides (ASO)

Attention and hyperactivity deficit disorder (ADHD)

Autophagy lysosomal pathway (ALP)

Body mass index (BMI)

Catechol-O-methyltransferase (COMT)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Copy number variation (CNV)

Dementia with Lewy body (DLB)

Dopaminergic replacement therapy (DRT)

Early-onset Parkinson's Disease (EOPD)

Electroencephalography (EEG)

Gaucher's disease (GD)

Genome-wide association study (GWAS)

Glucocerebrosidase (GCase)

Human leukocyte antigens (HLA)

Idiopathic Parkinson’s disease (iPD)

Induced pluripotent stem cells (iPSC)

Idiopathic REM sleep behavior disorder (iRBD)

Levodopa-induced-dyskinesia (LID)

Monoamine oxidase- (MAO)-B

Multiple systems atrophy (MSA)

Parkinson’s disease (PD)

Polygenic risk score (PRS)

Rapid eye movement (REM)

RBD single-question screen (RBD1Q)

RBD screening questionnaire (RBDSQ)

REM sleep behavior disorder (RBD)

REM sleep without atonia (RSWA)

Probable REM sleep behavior disorder (pRBD)

Untranslated region (UTR)

Video polysomnography (vPSG)

Ventromedial medulla (VMM)

FORMAT OF THE THESIS

The thesis uses a manuscript-based format. It contains two manuscripts published in peer-reviewed journals (Chapters 2,5) and two published as preprints and submitted to peer-reviewed journals (Chapters 3,4).

Chapter 2. Rare PSAP variants and possible interaction with GBA in REM sleep behavior disorder. Published on *Journal of Parkinson's disease*, J Parkinsons Dis. 2022;12(1):333-340.

Chapter 3. Genome-wide association study of REM sleep behavior disorder in Parkinson's disease. Published as a pre-print on MedRxiv (https://www.medrxiv.org/content/10.1101/2023.05.24.23289628v1) and submitted to *Npj Parkinson's disease.*

Chapter 4. Dopamine transmission pathway and Parkinson's disease risk variants are associated with risk and time to develop levodopa-induced dyskinesia. Published as a pre-print on MedRxiv (https://www.medrxiv.org/content/10.1101/2023.08.28.23294610v1) and submitted to *Movement Disorders*.

Chapter 5. LRRK2 p.M1646T is associated with glucocerebrosidase activity and with Parkinson's disease. Published on *Neurobiology of Aging*, Neurobiol Aging. 2021 Jul;103:142.e1-142.e5

Supplementary files: Thesis_YLS_Supplementary material.zip

CHAPTER 1: INTRODUCTION

Features of Parkinson's disease

Epidemiology

Parkinson's disease (PD) is a common neurodegenerative disease affecting 1-2% of individuals over 60 years of age and displaying a lifetime risk of around 6-7%.[1, 2] This disorder produces a substantial psychological, social and economic impact on society. In 2016 approximately 6.1 million individuals were affected worldwide and, for reasons not completely understood, its prevalence has increased dramatically in the past two decades[3] and is expected to double by 2030.[4] The projected economic burden of PD in 2037 is $79 billion only in the US.[5] PD onset is age-related and commonly diagnosed between the ages 55 and 65.[6] However, 5-10% of patients shows early onset PD (EOPD), manifesting before the age of 50.[7, 8] Men are more affected than women, but the latter suffer more often from motor fluctuations and dyskinesia in response to levodopa, urinary complaints and depression.[9]

Etiopathogenesis

Development of PD is arguably a result of the interplay of multiple mechanisms, including the deposition of aberrant alpha-synuclein, dysfunction of mitochondria and vesicle transport, disruption of synaptic transmission, oxidative stress, lysosomal dyshomeostasis and neuroinflammation.[10, 11] The consequence of these aberrancies is progressive neuronal degeneration with a distinctive involvement of dopaminergic neurons in the substantia nigra.[12] This loss is responsible for an imbalance between the direct and indirect pathways of the basal ganglia, which causes dysregulation of the motor impulses from the motor cortex, causing the typical motor symptoms of PD.[13]

PD heritability is estimated to be around 20-30%.[14, 15] A minority of PD cases are monogenic, caused by mutations in genes including *LRRK2, PRKN, PINK1* and *PARK7*.[16]

More frequently, however, PD is sporadic, with several genetic and environmental risk factors implicated. Among the genetic risk factors, variants in the *SNCA,*[17] *GBA1*[18] and *LRRK2*[19] genes are the most common but many others have been nominated in genome-wide association studies (GWAS).[20] They will be discussed more in-depth in the following chapters.

The role of environmental risk factors in PD is still not fully understood and many discoveries failed to be replicated. In a meta-analysis, 11 environmental risk factors were identified. Some of them could be related to each other, such as rural living, agricultural occupation and pesticide exposure. The latter, in particular, is one of the most widely confirmed environmental risk factors for PD. Another confirmed environmental driver for PD is represented by head injuries, observed, for example, in former professional soccer players.[21] One of the main environmental contributors to PD development is age.[22] Part of the reasons for this association can be found in alterations connected with aging such as disruption of lysosomal and mitochondrial activity, as well as increased overall oxidative stress.[23] The female sex is also considered a protective factor for PD, an effect suggested to be mediated by estrogens and removed after ovariectomy.[24, 25] Other protective environmental factors have also been suggested, including smoking, coffee, calcium channel blockers, alcohol consumption and higher levels of urate.[21] However, their role is still to be confirmed and could be subject to confounders. For example, smoking can be associated with overall higher levels of dopamine which would be protective against PD manifestation and make smoking a consequence more than a risk factor of PD.[9]

Clinical presentation

According to the International Parkinson and Movement Disorder Society (MDS), PD is clinically defined by the presence of bradykinesia in conjunction with at least one additional cardinal motor symptom, including rigidity or rest tremor. Other criteria are also included in the definition, including supporting (such as responsiveness to levodopa) or exclusionary

criteria (such as stronger clinical/imaging evidence suggesting an alternative diagnosis).[26] Bradykinesia is defined as a slowness of movement which encompasses impaired planning, initiation and execution of movements, especially when performing sequential or simultaneous tasks.[27] It is dependent on the emotional state of the patient. Excitement, for example, could prompt them to have a rapid response such as in catching a ball, suggesting an intact motor program but hindered access to it without an external trigger.[28] Postural tremor can be present in PD, but the peculiar Parkinsonian tremor is rest tremor, unilateral and at a frequency between 4 and 6 Hz, predominantly involving the distal portion of an extremity, with progression of disease rest tremor may spreads to involve the other side and becomes bilateral. Rigidity is characterized by increased resistance and generally manifests the "cogwheel" phenomenon, with movements in small increments, similar to how gears move.[28] Postural instability gait difficulty (PIGD) is another important PD motor manifestation, characterized by stooped posture, reduced arm swing, and shuffling gait, owing to a loss of postural reflexes and usually emerging at advanced stages of the disease.[28, 29] One of the most disabling motor symptoms in PD is freezing, appearing in 47% of the patients, which typically manifests as a sudden and transient motor block.[30, 31]

While the motor features are hard to miss, PD also presents a rich variety of sometimes more subtle non-motor symptoms, some of which can precede motor manifestations. They are, however, more frequently non-specific and underdiagnosed.[32, 33] Constipation is an example of these symptoms, which can occur even years or decades before PD motor disturbances but is highly non-specific and insufficient in isolation to raise suspicion of PD.[34] Virtually any other autonomic function can be impaired in PD. Other gastrointestinal disorders include gastroparesis, small intestinal bacterial overgrowth and dysphagia. The latter, in particular, can be responsible for severe complications like aspiration.[35] Orthostatic hypotension shows a prevalence of 60%, however, most of the patients can be asymptomatic.[36] Urinary disturbances are reported in 25-50% of the cases and include urinary urgency, frequency, nocturia and

incontinence. More rarely, dysfunctions in sphincter relaxation and detrusor contractility can also occur.[37, 38] Sexual dysfunctions are also observed in men and women with PD. In the latter, they typically affect libido and orgasm. In addition to that, men also suffer from erectile dysfunction, which may precede motor symptoms.[39] Hyposmia is present in up to 90% of PD patients and represents another frequent prodromal symptom of PD. However, less than 30% of them are estimated to be aware of their olfactory impairment.[40] Another important prodromal manifestation of PD is represented by depression, which can occur at any moment of the disease progression, both before and after motor symptoms. 17% of individuals with PD are affected by major depressive disorder, 22% by minor depression and 13% by dysthymia.[41] Other neuropsychiatric symptoms are also extremely common. Cognitive impairment is observed in around 80% of the cases, especially in the more advanced stages of the disease,[42] anxiety in 25-40% of the patients and can also occur in the prodromal period, apathy in approximately 40% of the individuals with PD,[43] showing a correlation with disrupted executive functions, specifically difficulty with initiation.[44, 45] Hallucinations are reported in around 40% of the cases, associated with severe cognitive impairment, disease duration and excessive daytime sleepiness. They typically emerge as side-effects of dopaminergic drugs but they are individually not sufficient to explain visual hallucinations.[46] Sleep disorders are also highly prevalent in PD, affecting up to 90% of the patients. The most common one is represented by sleep fragmentation, causing insomnia, plausibly a consequence of many factors, including motor symptoms, nocturia, medication effects and periodic limb movements. Another sleep disorder in PD often occurring before motor symptoms is REM sleep behavior disorder (RBD), which, differently from other prodromal clinical biomarkers shows a larger specificity in predicting PD and other synucleinopathies. RBD will be discussed later in more extensive detail.[34, 47] Despite the greater importance of motor symptoms to diagnostically define PD, non-motor symptoms have demonstrated a greater impact on PD quality of life.[28, 48]

Different scales have been used to characterize PD motor and non-motor symptoms, as well as the degree of disability. The Unified Parkinson's Disease Rating scale (UPDRS) and the more recent Movement Disorder Society-sponsored revision of the UPDRS (MDS-UPDRS) are probably the most popular ones.[49] This scale is divided into four parts, Part I focuses on the "nonmotor experiences of daily living", Part II on the "motor experiences of daily living", Part III concerns the "motor examination" and finally Part IV the "motor complications" of dopaminergic drugs, including motor fluctuations, dystonia and dyskinesia.[50] The Hoehn and Yahr (H&Y) is another widely used scale, predominantly focused on motor manifestations and disability, with the purpose of providing an assessment of the general disease progression. It ranges from stage 0, when the individual shows no sign of the disease, to stage 5, when the patient is wheelchair-bound or bedridden unless assisted.[51]

Neuropathology

Macroscopically, the PD brain is often unremarkable, especially before the less advanced stages, with minimal cortical atrophy and ventricular dilation. The predominant alterations in PD neuropathology in these stages involve the peripheral nervous system, the brainstem and the subcortical regions. The pathologic hallmark of PD is the deposition of alpha-synuclein in cytoplasmatic aggregates called Lewy Bodies (LBs) in the substantia nigra, pars compacta.[52, 53] The cells affected the most are the neuromelanin-containing neurons, with dopaminergic activity.[12] The selective vulnerability of these neurons is probably due to the frequent intracellular calcium transient coupled with insufficient calcium buffering, which exposes them more extensively to stress and potential death. Along with LBs, alpha-synuclein frequently accumulates also in axons, giving rise to dystrophic neurites (Lewy neurites).[52, 54] Alpha-synuclein inclusions in the cytoplasm are per se aberrant since this protein is normally enriched in its physiologic conformation in the presynaptic terminals, where it regulates synaptic vesicle release. The deposition of alpha-synuclein is the result of an alteration in the

conformation of this protein ("amyloid-like") that promotes aggregation, but also multiple post-translational changes, including truncation, phosphorylation and oxidation.[54]

The principal staging system of LB pathology in PD was introduced in 2003 by Braak and colleagues.[55] According to this system, in the brain LBs spread in a caudorostral direction. At Braak stages I and II the regions where LBs are observed include the dorsal motor nucleus of the vagus nerve and the anterior olfactory nucleus. At these stages, PD individuals are typically asymptomatic or in a prodromal phase. For example, they could start developing olfactory disturbances and autonomic symptoms such as constipation.[32, 56] Even though the pathophysiology of the autonomic manifestations in PD is still unclear, the LBs lesions identified in the dorsal motor nucleus and along with the alpha-synuclein observed peripherally in the sympathetic ganglia, cardiovascular and gastrointestinal systems are plausibly involved.[32, 56, 57] In a successive revision to the Braak system, a "dual-hit hypothesis" suggested that the pathology started in the nasal and intestinal mucosal regions.[58] Full vagotomy is associated with reduced risk of PD, suggesting a gut-brain diffusion of PD neuropathology.[59, 60] In Braak stage II LBs accumulate also in the sublaterodorsal nucleus and Subcoeruleus/Pre-Locus Coeruleus complex (SubC/PC), arguably involved in RBD development.[57] In Braak stages III LBs reach the substantia nigra, which correlates with the appearance of the motor symptoms.[53] More specifically, around 50% of dopaminergic cells are lost at that time.[61] The pathology reaches the locus coeruleus and the amygdala, extending to the transentorhinal region at Stage IV. Finally, at Stages V and VI LBs deposit in the neocortex, involving the prefrontal, primary sensory and motor cortices. From a clinical perspective, this may translate into cognitive deficiency and more severe motor disorders such as PIGD.[53]

An overlap between PD pathology and pathologies traditionally attributed to other diseases can also be observed. For example, tau protein which, in its abnormally hyperphosphorylated form, aggregates into neurofibrillary tangles (NFTs), is characteristic of tauopathies, like Alzheimer's disease (AD). However, postmortem studies identified

hyperphosphorylated tau in PD brains.[53, 62] And GWAS identified an association between the H1 haplotype of the *MAPT* gene, encoding tau protein, and increased risk for PD.[63] In addition, both NFTs and amyloid-beta senile plaques, another pathologic hallmark of AD, were reported in almost all postmortem brains of PD patients who develop some degree of cognitive decline, suggesting that the convergence of AD pathology with PD pathology contributes to the development of dementia in PD.[64]

GBA1

GBA1 (previously *GBA*), encodes glucocerebrosidase (GCase), a lysosomal enzyme that degrades glucosylceramide (or glucocerebroside) and glucosylsphingosine.[65] In homozygous carriers, *GBA1* mutations cause a lysosomal storage disorder, called Gaucher Disease (GD), divided into different types depending on the severity, going from the milder form, non-neuronopathic, GD type I, to type II and type III, severe and neuronopathic.[66] Clinical observation of PD features in GD individuals led to the discovery of the association between *GBA1* variants and PD risk.[67-69] Albeit some variants are shared between the two disorders, other ones such as p.E326K and p.T369M are unique to PD.[70, 71] Penetrance of heterozygous carriers of *GBA1* is incomplete in PD, ranging between 10% and 30%.[72-74] However, *GBA1* variants are among the most common genetic risk factors for PD, with 5-20% of carriers among PD patients.[66] The prevalence of *GBA1* variants in PD varies across populations. For example, Ashkenazi Jewish individuals with PD carry *GBA1* variants in 20% of the cases.[66, 75] In contrast, carriers of *GBA1* variants account for 5.4-8.4% of the Chinese population affected by PD.[76-78] Similarly, the type of *GBA1* variants is differently distributed between different ancestries. PD in Ashkenazi Jews is most frequently associated with the p.N370S mutation, in Europeans the p.E326K and p.T369M variants are the most prevalent, whereas in the Asian population it is the p.L444P variant.[66, 79] There is also a difference between these variants in terms of their risk for GD and PD. In particular, p.N370S is associated with

GD I and with a mild increase in the risk for PD, similar to the p.E326K and p.T369M variants which, however, are not risk factors for GD. In contrast, the p.L444P variant is associated with the severe forms of GD and with increased PD risk by around 10-fold.[79] In addition, a subset of PD patients shows dampened GCase activity in the absence of an underlying *GBA1* mutation.[80, 81]

The mechanisms at the basis of the association between *GBA1* and PD are still not fully understood. One of the main mechanisms where GCase has been implicated is the autophagy-lysosomal pathway (ALP).[82] One hypothesis is that deficiency in GCase changes the composition of the lysosomal membrane, disrupting the lysosomal ability to degrade alpha-synuclein and leading to a progressive accumulation of this substrate.[83] An interaction between the GCase pathway and alpha-synuclein has also been demonstrated. In particular, the accumulation of glucosylceramide in the lysosome can interact with alpha-synuclein. Increased levels of alpha-synuclein would in turn inhibit the transport of GCase to the lysosome, triggering a vicious cycle.[84] Normally, GCase is synthesized in the endoplasmic reticulum (ER), processed in the Golgi and then transported to the lysosomal membrane, where it degrades glycolipids.[85] Another hypothesis would, therefore, involve a misfolding of GCase in the ER, causing ER stress and cell death.[86] This mechanism can contribute to PD pathogenesis, but is plausibly not necessary to PD development, as *GBA1* null mutations (such as c.84GG) are associated with PD also in the absence of GCase and c.84GG is also associated with a higher risk for PD compared to other mutations that do not affect ER retention like p.N370S.[82] On the other hand, only a minority of GD patients, with minimal GCase activity, will develop PD, suggesting a more complex mechanism than reduction of GCase levels at the basis of PD pathogenesis.[85, 87] Oxidative stress was also demonstrated to be an earlier driver of PD development. Reduced GCase activity is associated with prolonged mitochondrial oxidant stress, which results in impaired lysosomal function. Conversely, mitochondrial antioxidants are associated with reduced levels of alpha-synuclein. *GBA1* variants might therefore reduce

the threshold that produces mitochondrial oxidant stress.[85] Finally, carriers of *GBA1* mutations show an elevation in cytokines. Since lysosomes participate in antigen processing and presentation, it is possible that *GBA1* mutations alter these processes and facilitate neuroinflammation, one of the mechanisms suggested to be involved in PD pathogenesis.[82]

Clinical presentation in *GBA1* variant-associated PD is more malignant than idiopathic PD (iPD), not associated with any known genetic mutation. *GBA1*-PD shows an earlier onset and faster progression. The worsening of motor symptoms to H&Y stage three is accelerated and the life expectancy is lower, with approximately a twofold increase in mortality risk,[88, 89] compared to iPD. In addition, *GBA1*-PD patients manifest more often non-motor symptoms, including olfactory dysfunction, RBD, and neuropsychiatric disturbances, showing lower quality-of-life scores.[90] Depression, anxiety and hallucinations are all highly prevalent in *GBA1*-PD compared to iPD, but the most significant difference is represented by the burden of cognitive impairment.[85] The rate of progression to cognitive decline is increased and the prevalence at the age of 65 ranges between 24-31%. At age 70, 56% of *GBA1*-PD patients presented with dementia compared to 15% of patients with iPD.[85, 89] Depression and RBD are also more common in carriers of *GBA1* variants without a diagnosis of PD, arguably reflecting a prodromal stage of the disease.[90]

PSAP

GCase is co-activated by saposin C (sapC), one of the four active domains of prosaposin, encoded by the gene *PSAP.* After its synthesis, prosaposin is cleaved by cathepsin D into its domains, saposin A, B, C and D.[91, 92] Saposins are cofactors that act at the lysosomal level facilitating sphingolipid degradation. Mutations in *PSAP* have been linked to a number of lysosomal storage disorders. In particular, Gaucher disease, typically caused by biallelic mutations in *GBA1,* is more rarely also associated with mutations of *PSAP* in the sapC domain.[92, 93]

The role of *PSAP* n PD is not fully understood and shows divergences between populations. More specifically, *PSAP* variants were associated with PD in multiple studies in Asians[94-96] but this association failed to be replicated in Europeans.[97, 98] A study measuring the activity of lysosomal enzymes linked to the *GBA1* pathway in PD demonstrated that sapC activity was significantly decreased in PD patients compared to controls and correlated with increased levels of alpha-synuclein, suggesting a role of sapC in the GCase-mediated accumulation of alpha-synuclein.[99]

LRRK2

The LRRK2 protein is a multidomain enzyme constituted by a catalytic kinase, armadillo, ankyrin leucine-rich repeats, WD40 and GTPase domain. The p.G2019S variant, located in the kinase domain, is the most common among different PD populations. It is reported in 28% of Ashkenazi Jews and 38% of North African Berbers with PD and is common also in Europeans. Conversely, it is rarely found in East Asians, where the p.R1628P and p.G2385R variants, located respectively in the GTPase and WD40 domains, are more frequent.[19] Another variant that shows specificity for a certain ancestry is p.M1646T, a GTPase domain variant, which was reported to be associated with PD in Europeans but not in Asians or Arab-Berbers. *LRRK2* p.N551K-p.R1398H-p.K1423K is a haplotype associated with reduced risk in PD.[100]

LRRK2 protein has been suggested to be involved in multiple mechanisms in the context of PD pathophysiology, which are illustrated in Figure 1. *LRRK2* mutations are typically associated with increased LRRK2 kinase activity, inducing phosphorylation of its substrates. One of the main targets are the Rab 8 and Rab 10 proteins, which are activated by LRRK2 in response to stress and under normal condition maintain lysosomal homeostasis. *LRRK2* mutations have been associated with abnormalities in the lysosomal morphology, localization, pH and function, with processes that might involve Rab8/10.[19] Similar to GCase,

LRRK2 kinase has a role in the ALP and variants in the *LRRK2* gene are associated with the accumulation of alpha-synuclein and other ALP substrates.[82] LRRK2-mediated hyperactivation of Rab10 enhances kinesin activity and interferes with the normal trafficking of intracellular vesicles, disrupting ALP and synaptic transmission. Abnormal activation of Rab8/10 in the presence of *LRRK2* mutations was also shown to influence centromere cohesion and ciliogenesis, processes that were suggested to affect the transmission of neuroprotective agents to dopaminergic neurons of the substantia nigra.[101] *LRRK2* variants have also been associated with calcium dyshomeostasis, ER stress and disruption of mitophagy.[19] Multiple lines of evidence also found a relationship between LRRK2 and inflammation. For example, the p.G2019S mutation is associated with increased microglial activity and cytokine levels in response to interferon-γ.[102] In addition, compared to controls, monocytes of PD patients show increased expression of LRRK2, which correlates with increased production of cytokines.[103] Another link between LRRK2 and inflammation is the implication of LRRK2 in inflammatory bowel disease, including Chron's disease and ulcerative colitis, which are also associated with 20-90% increased risk for PD.[104] The *LRRK2* p.N2081D variant, in particular, which correlates with enhanced kinase activity, is associated with Chron's disease and with a mild increase in PD risk. Conversely, the *LRRK2* p.N551K-p.R1398H-p.K1423K haplotype is associated with reduced kinase activity and is protective for both diseases.[100, 105]

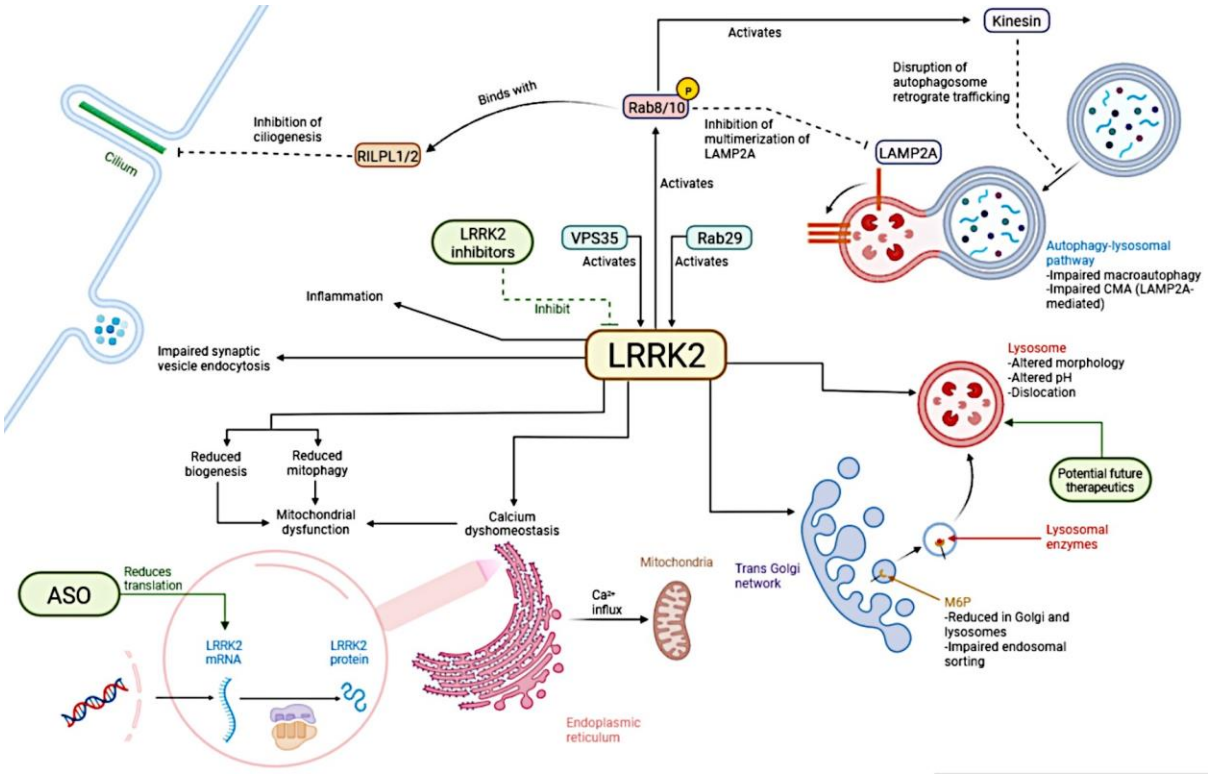

Figure 1: Principal mechanisms where LRRK2 has been implicated in Parkinson's disease and therapeutic targets.

ASO: Antisense oligonucleotides; CMA: Chaperon-mediated autophagy; M6P: mannose-6-phosphate, deputed to transfer of lysosomal enzymes from the Golgi to the lysosome. Created with Biorender. Adapted from Sosero YL & Gan-Or Z, 2023.[19]

The pathology of *LRRK2*-PD diverges in part from iPD, since it does not present the typical LB pathology in about 60% of the cases. This difference is even more exacerbated if we consider the non-p.G2019S *LRRK2* variants, associated with LBs in only around 40% of the individuals. In contrast, AD pathology is prominent in *LRRK2*-PD, with approximately 70% of the patients showing tau deposits, rising to 90% if we consider only carriers of the p.G2019S variant.[19] A study found tau inclusions in 100% of the *LRRK2*-PD cases and through antibodies demonstrated that the deposits were AD-like.[106] More rarely, *LRRK2*-PD can also show

alternative neuropathologic features, such as ubiquitin-positive inclusions or TAR DNA-binding protein 43 (TDP-43) deposits, typical of frontotemporal dementia,[107] as well as pure nigrostriatal degeneration.[108]

From the clinical point of view, *LRRK2*-PD, on average, shows again distinctive features, compared to iPD and even more to other types of PD with a known genetic basis, like the aforementioned *GBA1*-PD or *SNCA*-PD, discussed later, with an overall more benign phenotype characterized by less frequent non-motor symptoms,[19] which as previously mentioned, strongly affect the quality of life of the patients. *LRRK2*-PD progresses slower and cognitive functions are more conserved compared to iPD. In particular, *LRRK2*-PD patients show better performance in attention, executive functions and language test, cognitive decline generally manifests at the more advanced phases of the disease and dementia is less prevalent.[109, 110] Moreover, hyposmia and autonomic dysfunctions are rarer than iPD,[111, 112] showing a lower frequency of orthostatic hypotension,[113] gastrointestinal disturbances[114] and greater cardiac [123I]metaiodobenzylguanidine uptake on scintigraphy.[112] RBD is also rare, displayed in only 0-15% of PD carriers of the p.G2019S variant.[19, 115, 116] Despite the overall benign presentation, some other features also characterize a more malignant outlook of *LRRK2*-PD, including more frequent PIGD and slightly earlier age at the onset (AAO) of PD. In addition, *LRRK2*-PD patients rarely but more often manifest atypical phenotypes, such as tauopathy-like symptoms, including progressive aphasia and choreoathetosis.[19] Finally, in *LRRK2*-PD the predominance of males over females typical of iPD is absent.[117, 118]

SNCA

SNCA encodes alpha-synuclein and, while the function of this protein currently remains elusive, its overexpression is associated with increased aggregation and anomalies in neuronal function. Suggested mechanisms for alpha-synuclein include synaptic transmission, including dopamine release and transport, regulation of mitochondrial function, ALP and proteolysis.[119]

SNCA copy number variations (CNV) and some missense mutations, such as p.A53T, p.A30P, p.E46K, and p.G51D, cause autosomal dominant PD, with a more malignant PD phenotype compared to iPD, including earlier AAO, faster disease progression and severe fluctuations of cognitive functions.[120] They can also be associated with atypical features, including pyramidal symptoms, myoclonus and seizures.[18] Triplications are associated with a worse clinical presentation compared to duplications.[120] GWAS identified additional *SNCA* variants associated with sporadic PD, including the rs356182 variant, located in the 3' untranslated region (UTR), and a secondary signal, rs7681154, located in the promoter region.[20]

The predominant pathological model for PD suggests that alpha-synuclein monomers assemble into oligomers, which in turn constitute fibrils. Alpha-synuclein proteins and aggregates would then spread trans-synaptically across neural networks in a prion protein-like fashion. Fibrils are the main component of Lewy bodies and neurites.[121] It is controversial, however, whether Lewy bodies and neurites represent the drivers of neurodegeneration in PD or are rather a collateral epiphenomenon or again a compensatory mechanism consisting in the storage of the actual toxic aggregates.[18, 121]

GWAS signals in PD

Numerous GWAS have been performed in PD. The most recent GWAS in Europeans included 37,688 PD patients, 18,618 proxy cases (healthy individuals having first-degree relatives with PD), and 1.4 million healthy controls.[20] This study nominated 90 different signals, including the aforementioned rs356182 and rs7681154 *SNCA* variants, the *LRRK2* p.G2019S variant and the *GBA1* p.N370S variant. Other genes that have been nominated include *VPS13C,* involved in mitochondrial function, *TMEM175,* encoding a potassium or proton channel in the lysosomes, *GCH1,*[122] implicated in dopamine synthesis, *HLA,* encoding proteins with the function of antigen presentation,[123] *MAPT*, encoding tau.[124] [20]

More recently, GWAS in non-European populations identified some loci shared also with the GWAS in Europeans, but also novel signals. A large GWAS in Asians identified two signals that were absent in the European GWAS, including the *SV2C* and *WBSCR17* loci.[125] In a GWAS in Latinos, only the *SNCA* rs356182 variant reached genome-wide significance, but this result arguably depends on the smaller sample size (1,497 participants in total) and insufficient power of this study. However, in the admixture mapping analysis the *STXBP6*, achieved significance in a joint test of ancestries and in the Native American single-ancestry test, whereas the *RPS6KA2* locus was significant in the African single-ancestry test.[126] A separate, larger, GWAS in Africans and African admixed populations has been recently performed, nominating a novel *GBA1* variant, rs3115534, associated with different PD risk and AAO. This variant was suggested to affect *GBA1* expression and, as a consequence, GCase levels.[127] The presence of different loci depending on the populations included in the GWAS highlights some degree of genetic differences between different ancestries at the basis of PD development.

REM sleep behavior disorder

Epidemiology, etiopathology, conversion

REM sleep behavior disorder (RBD) is a sleep disorder characterized by a loss of normal atonia during the REM phases of sleep and the enactment of the content of dreams.[128] The main pathophysiologic model suggests that RBD is a consequence of a dysfunction in the circuit involving SubC/PC, which normally activates the ventromedial medulla (VMM) and glycinergic neurons of the spinal anterior horn, which in turn inhibit the spinal motor neurons. This process results in the physiologic muscle atonia that we observe in patients during the REM phases.[129, 130] Other neurotransmitters have also been proposed to participate in the pathogenesis of RBD, such as acetylcholine, noradrenaline, serotonin and dopamine.[131]

RBD is distinguished into secondary RBD, when there is a known underlying cause, and idiopathic RBD (iRBD), when no clear cause is identified. Secondary RBD can be a consequence of the use of the anti-depressants SSRI, by increasing the serotonergic tone and thus interfering with the normal REM atonia. Other causes include neurologic affections including narcolepsy and multiple sclerosis as well as lesions such as meningiomas and pontine lymphoma.[132]

IRBD, on the other side, is typically associated with neurodegeneration. It is estimated to convert into an alpha-synucleinopathy in up to 90% of the cases within 10-20 years, including PD, Dementia with Lewy Bodies (DLB) or, more rarely, Multiple System Atrophy (MSA),[133] a parkinsonism characterized by more specific disturbances in the autonomic and cerebellar functions.[134] DLB is diagnosed like PD and shares a similar clinical presentation, with the only difference being that cognitive symptoms appear before or within 12 months from the manifestation of motor symptoms and are generally more prominent.[135] On average, RBD occurs 8 years before the manifestation of motor and cognitive symptoms used to diagnose PD and DLB.[136] It is therefore currently considered in most cases as a prodromal stage of synucleinopathies, even though RBD can also occur after the occurrence of the clinically manifest disease.[137] It is not uncommon to observe in these patients the presence of other prodromal synucleinopathy symptoms, including hyposmia, constipation, erectile dysfunction and subtle motor/cognitive deficits.[136] Alpha-synuclein deposits are detected in autopsies as well as skin or submandibular gland biopsies in most RBD patients.[138-140] Environmental risk factors for PD are also shared with RBD patients, including head injuries, pesticides and farming.[141, 142]

RBD prevalence is around 1-2% in the general population and is more frequent in males. iRBD typically manifests after the 6th decade of life, whereas secondary RBD usually occurs in younger individuals.[132, 143] In PD, RBD generally correlates with a more malignant

phenotype, including more frequent neuropsychiatric symptoms[144, 145] and levodopa-induced dyskinesia.[146]

Diagnosis

The gold standard for RBD diagnosis is video-polysomnography (vPSG), which detects movements during sleep and several electric activities, including electroencephalography (EEG), electromyography, heart rate and other parameters. RBD diagnosis requires the presence of REM sleep without atonia (RSWA) in association with sleep-related disruptive behaviors that suggest dream enactment, in the absence of EEG epileptiform activity in the REM phases.[147] Alternatively, when there is suspicion of RBD, this disorder can also be diagnosed using only screening questionnaires, including the RBD screening questionnaire (RBDSQ) and the RBD single-question screen (RBD1Q). These questionnaires have limited accuracy in the general population but showed elevated sensitivity and specificity in patients having already a diagnosed synucleinopathy, defining the so-called probable RBD (pRBD).[148]

Genetic risk factors

We experienced relatively recent advances in the understanding of RBD. Candidate gene studies showed that variants previously associated with PD, including those in *GBA1, TMEM175* and *SNCA* genes, were also associated with iRBD.[149-151] A recent RBD GWAS[152] identified 5 loci associated with increased risk for RBD, including the aforementioned genes and the novel *INPP5F* and *SCARB2* loci. Expression analyses showed differential expression of *SNCA-AS1* and *SCARB2* between different brain regions in RBD patients. Polygenic risk score (PRS) analyses using the RBD GWAS of the same study showed a good ability to differentiate PD with RBD from PD without RBD, with an area under the curve of 0.61. While some loci are shared between RBD and synucleinopathies, especially PD, others are different. The most notable difference is shown in candidate genes studies, which demonstrated no

association between PD risk variants in the *LRRK2* gene and RBD.[19, 115] Also, while the 5' *SNCA* rs10005233 variant was associated with iRBD, the 3' *SNCA* rs356182 variant, a risk factor for PD identified in the most recent GWAS in Europeans,[20] was not associated with iRBD.[151] The *MAPT* H1 haplotype, implicated in PD risk[20] and suggested to be associated also with DLB and more frequent and severe cognitive symptoms in PD,[153, 154] showed no association with RBD.[152] The *APOE* ε4 allele, which is not a risk factor for PD but is associated with DLB and increased risk for cognitive decline in PD, is also found not to be associated with RBD.[152, 155]

Parkinson's disease therapy

Dopaminergic therapy

Currently, there is no disease-modifying treatment for PD, therefore, therapy is still symptomatic. The cornerstone of PD therapy is represented by dopaminergic replacement therapy (DRT).[156] Dopamine is a charged molecule and is therefore unable to surpass the blood-brain barrier. Therefore other alternatives are used to enhance dopaminergic transmission.[157] One of the main drugs used to treat motor symptoms in PD, levodopa, was introduced more than 50 years ago. This drug is a precursor of dopamine and is able to surpass the blood-brain barrier.[157] In the central nervous system (CNS), levodopa is then converted into dopamine by the dopa decarboxylase, thus repleting the storage of dopamine in the dopaminergic cells of the substantia nigra. Dopa decarboxylases are present also in the periphery, where the metabolization of levodopa into dopamine produces adverse effects such as nausea and vomiting.[158] Therefore, levodopa is typically combined with a peripheral decarboxylase inhibitor, including benserazide and carbidopa, which also increases the availability of dopamine in the CNS.[159] However, one of the main problems with long-term levodopa treatment is the emergence of alterations in the response to levodopa as the disease progresses, including LID (discussed later) and motor fluctuations.[160] These include the wearing-off effect,

i.e., a gradual shrinking of the effectiveness window of levodopa which conserves greater predictability based on the therapeutic pattern, and the "on-off" effect, which represents sudden and unpredictable switches between periods of positive responses to levodopa ("on") and periods in which parkinsonism reappears ("off").[160, 161]

Other drugs acting on dopamine metabolism can also be used in PD, independently or in combination with levodopa, including monoamine oxidase- (MAO)-B inhibitors and Catechol-O-methyltransferase (COMT) inhibitors. MAO-B is an enzyme that specifically degrades dopamine, so MAO-B inhibitors prolong dopamine availability in the synaptic cleft. COMT catalyzes levodopa and dopamine methylation, and its inhibition leads to increased dopamine half-life.[162]

Dopamine agonists are another valid option for the DRT, acting on more dopamine receptors, with the D2 receptor being considered to be one of the most relevant to obtain an antiparkinsonian response. They are also associated with LID but are less likely to induce it compared with levodopa. However, they are also less effective than levodopa in determining motor improvements and more often provoke other adverse effects, including impulse control disorder, orthostatic hypotension, nausea and edema.[163-165] Dopamine agonists are therefore usually preferred over levodopa in the earlier stages of the disease and in younger people, to delay the manifestation of motor fluctuations/LID and since older people show worse responses to these medications.[9, 166]

Levodopa-induced dyskinesia

Levodopa-induced dyskinesia (LID) is a common adverse effect of DRT, and especially levodopa. It affects around 40-50% of PD patients after 5 years of levodopa treatment[167, 168] and shows a wide variability in the time of its development,[169] suggesting that each individual has its own distinct predisposition to a certain risk and time to LID development. Multiple environmental risk factors have been identified, the most important of which are represented

by PD AAO and disease duration. Lower body mass index (BMI) has also been suggested as a potential risk factor, probably due to pharmacokinetic differences, if not taken into account. Female patients are more at risk than male patients, this could be in part linked to the generally lower BMI in females compared to males, but it was also shown that female sex represents an independent risk factor for LID. Factors related to levodopa therapy have also been associated with LID, including longer therapy duration and higher levodopa dosage or levodopa equivalent daily dose.[170-173]

There are three main types of LID. Peak-dose dyskinesia is by far the most common (75-80% of the cases) and it occurs when the levodopa plasmatic levels reach their peak ("on" phase). It is mainly characterized by choreiform movements, but can manifest in other forms, such as dystonia, myoclonus or ballism. In contrast, off-period dyskinesia occurs when the levodopa reaches its lowest concentrations ("off"), usually in the early morning, and most often manifests as dystonia. Finally, diphasic dyskinesia is observed when levodopa concentrations are falling ("on" to "off") and rising ("off" to "on"). While we refer to these disturbances with the umbrella term "dyskinesia" they may arise from different anomalies. Most of the etiopathologic information we have up-to-date, however, refers particularly to the most common subtype of LID, peak-dose dyskinesia.[174]

According to the main pathophysiologic hypothesis behind LID development, the short half-life of levodopa in conjunction with the presynaptic nigrostriatal degeneration would determine an aberrant pulsatile stimulation of dopamine receptors.[175] This overstimulation, in line with the classic pathophysiologic model of PD, would lead to abnormal activation of the direct pathway.[175, 176] The loss of dopaminergic neurons would result in a limitation in the dopamine storage capacity, leading to a radical increase in the dopamine release for each dose of levodopa.[177]

Multiple genetic risk factors have been proposed to intervene in LID development, but many of these failed to be replicated in other studies. Most genes implicated in LID belong to dopaminergic pathways. Variants in genes belonging to dopaminergic transmission are among the most widely reported, they are the genes encoding the dopamine receptors DRD2 and DRD3,[178-181] as well as *SLC6A3,* encoding the dopamine transporter DAT.[182, 183] Variants in genes belonging to the dopamine metabolism pathway, including *MAOB* and *COMT,* have a controversial role in LID, with studies that did not find any evidence supporting their involvement in LID development.[184] *ADORA2A*, encoding the adenosine receptor, is also indirectly implicated in the dopaminergic pathways, as adenosine modulates the activation of the glutamatergic system enacted by dopamine.[185] Variants in the gene *GRIN2A,* encoding a glutamate receptor, were also associated with LID.[186] Another gene with evolving evidence of its role in LID is *BDNF,* which modulates multiple neural circuits, including the dopaminergic, GABAergic and glutamatergic systems.[178, 187] Interestingly, *GBA1* and *LRRK2* variants have also been reported as potential risk factors for LID,[188-193] with conflicting results,[194, 195] probably due to insufficient power.

Numerous therapeutic approaches have been proposed to manage LID. One of the most used approaches is aiming at continuous dopaminergic stimulation to attenuate the pulsatile stimulation of dopamine receptors. This is achieved in several ways, including an increase in the frequency of levodopa administration, association of levodopa with COMT or MAO-B inhibitors, and levodopa intestinal gel infusion. The most widely used pharmacologic treatment for LID is represented by amantadine, a drug that is typically used in the earlier stages of PD in alternative to levodopa, with a mechanism that probably involves an increase in GABAergic transmission. More invasive techniques to treat LID, used also in advanced stages of PD to control motor symptoms, include deep brain stimulation and transcranial magnetic stimulation.[196-198]

GBA1 pathway-targeted therapy

GCase activity is reduced in multiple brain areas and particularly in the substantia nigra. This dysfunction is associated with PD both in individuals with and without *GBA1* variants.[80] Gene therapy has been proposed to increase levels of GCase and intravenous injection of adeno-associated virus expressing *GBA1* in rodent models carrying the A53T-*SNCA* mutation restored previously altered GCase levels, reduced alpha-synuclein deposit in the substantia nigra and striatum and prevented neurodegeneration of nigrostriatal dopaminergic neurons.[199, 200] PRV-PD101 (https://clinicaltrials.gov/, ID:NCT04127578, PROPEL Study) is a gene-therapy acting with the aforementioned mechanism and is currently at phase 1/2a of clinical trial.[201]

Another attempt to target *GBA1* pathway was made using venglustat, an inhibitor of glucosylceramide synthase, the enzyme that produces GCase substrate, with the hypothesis that its accumulation was associated with PD pathogenesis. However, despite the initial promising results in terms of safety and target engagement, not only venglustat failed to demonstrate any disease-modifying effects, but it also led to earlier worsening of motor symptoms and has been interrupted at phase 2.[201, 202]

One of the most promising therapeutics in *GBA1*-targeted therapy is Ambroxol. This over-the-counter mucolytic was shown to act as a chaperone for GCase.[203] The effects at the biological levels were multiple, Ambroxol increases GCase levels, reduces oxidative stress, reduces the accumulation of alpha-synuclein and impacts also other cellular mechanisms, such as mitochondrial homeostasis and lysosomal biogenesis.[201] Phase 2 is currently completed (https://clinicaltrials.gov/, ID: NCT02941822) and showed optimal safety outcomes and a decrease in the MDS-UPDRS part III score, indicating an improvement in motor symptomatology.[204]

LRRK2 pathway-targeted therapy

LRRK2 inhibitors represent the main therapeutic options for the LRRK2 pathway, supported by the notion that *LRRK2* deleterious variants in PD are associated with increased LRRK2 kinase activity. Two LRRK2 inhibitors are currently tested in clinical trials, DNL201, which completed Phase Ib, and DNL151, which completed Phase II (https://clinicaltrials.gov ,ID: NCT05348785; https://www.denalitherapeutics.com, 2021) and is currently in Phase III, with previous positive safety outcomes.[205] Another mechanism that has been targeted is the modulation of LRRK2 expression. Antisense oligonucleotides (ASO) are RNA molecules that directly reduce LRRK2 expression or alter its splicing. In mice with PD with and without the p.G2019S mutation, they were demonstrated to reduce LRRK2 levels, alpha-synuclein accumulation and dopaminergic loss in the substantia nigra.[206] In human iPSC-derived neurons with the p.G2019S mutation, ASO restored ER calcium homeostasis and physiologic mitophagy.[207] They are currently in phase I. (https://clinicaltrials.gov , ID: NCT03976349)

Hypothesis

I hypothesize that Parkinson's disease is not a single entity and that can be stratified based on underlying genetic determinants.

Objectives

Chapter 2: Rare *PSAP* Variants and Possible Interaction with *GBA1* in REM Sleep Behavior Disorder. I aimed to investigate the role of rare *PSAP* variants in iRBD. Additionally, I evaluated the carrier status of *GBA1* variants to assess a potential interaction between *PSAP* and *GBA1* variants affecting iRBD risk. Understanding the genetic underpinnings of RBD has potential to further our understanding of this disorder and nominate potential targets for neuroprotective trials.

Chapter 3: Genome-wide association study of REM sleep behavior disorder in Parkinson's disease: in this chapter, I aimed at studying the different genetic profile between PD with and without RBD and study potential correlation and causative associations between PD with RBD and neuropsychiatric traits. Uncovering the genetic basis for the manifestation of RBD in PD will allow us to better define the determinants of these two PD subtypes, which share also different clinical correlates.

Chapter 4: LRRK2 p.M1646T is associated with glucocerebrosidase activity and with Parkinson's disease: I aimed to evaluate the association between *LRRK2* variants and GCase activity. Defining the nature of this association will be essential for therapies currently tested in clinical trials that target the *LRRK2* pathway, accounting for the collateral changes they might produce on GCase activity and potential implications this might cause on the disease course.

Chapter 5: Dopamine transmission pathway and Parkinson's disease risk variants are associated with risk and time to develop levodopa-induced dyskinesia: in this chapter, I aimed to investigate the genetic underpinnings of risk and time to develop LID. Unraveling the genetic factors that modify the vulnerability of PD patients taking levodopa for this cumbersome adverse effect could contribute to modifying the therapeutic management, nominating potential therapeutic targets and improving the quality of life of PD patients taking levodopa.

PREFACE TO CHAPTER 2

In this chapter, I will focus on analyzing the burden of rare *PSAP* variant in iRBD. I analyze 1,113 iRBD patients and 2,324 controls and demonstrate that around 0.3% of iRBD patients, and no controls, carry loss of function *PSAP* mutations compared to about 0.007% of the general population. In addition, I report that approximately 0.2% of the iRBD patients carry both *PSAP* loss of function mutation and a *GBA1* variants, compared to about 0.000035% of the general population. These results show a possible interaction between *PSAP* and *GBA1* in the risk for RBD and suggest a potential additional biomarker to use in neuroprotective clinical trials in RBD.

CHAPTER 2: RARE *PSAP* VARIANTS AND POSSIBLE INTERACTION WITH *GBA* IN REM SLEEP BEHAVIOR DISORDER

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Abstract

PSAP encodes saposin C, the co-activator of glucocerebrosidase, encoded by *GBA*. Since *GBA* mutations are associated with idiopathic/isolated REM sleep behavior disorder (iRBD), a prodromal stage of synucleinopathy, we examined the role of *PSAP* mutations in iRBD. We fully sequenced *PSAP* and performed Optimized Sequence Kernel Association Test in 1,113 iRBD patients and 2,324 controls. We identified loss-of-function (LoF) mutations, which are very rare in *PSAP*, in three iRBD patients and none in controls (uncorrected p=0.018). Two variants were stop mutations, p.Gln260Ter p.Glu166Ter, and one was an in-frame deletion, p.332_333del. All three mutations have a deleterious effect on saposin C, based on *in silico* analysis. In addition, the two carriers of p.Glu166Ter and p.332_333del mutations also carried a *GBA* variant, p.Arg349Ter and p.Glu326Lys, respectively. The co-occurrence of these extremely rare *PSAP* LoF mutations in two (0.2%) *GBA* variant carriers in the iRBD cohort, is unlikely to occur by chance (estimated co-occurrence in the general population based on gnomAD data is 0.00035%). Although none of the three iRBD patients with *PSAP* LoF mutations have phenoconverted to an overt synucleinopathy at their last follow-up, all manifested initial signs suggestive of motor dysfunction, two were diagnosed with mild cognitive impairment and all showed prodromal clinical markers other than RBD. Their probability of prodromal PD, according to the Movement Disorder Society research criteria was 98% or more. These results suggest a possible role of *PSAP* variants in iRBD and potential genetic interaction with *GBA*, which requires additional studies.

Introduction

Rapid eye movement (REM) sleep behavior disorder (RBD) is characterized by the enactment of dreams during the REM phase of sleep [1]. In its idiopathic/isolated form (iRBD, presenting before the clinical diagnosis of a neurodegenerative disease), it represents a common prodromal stage of synucleinopathies, including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [1, 2]. Notably, over 80% of iRBD cases convert to a synucleinopathy within 10-15 years [2, 3]. In line with their clinical overlap, iRBD and overt synucleinopathies also share some of their genetic risk factors. For example, iRBD and PD are both associated with *GBA* variants, which represent one of the most common genetic risk factors for both diseases [4, 5]. *GBA* variants display an incomplete penetrance in iRBD as well as in PD [4, 5], suggesting that other factors, genetic and/or environmental, contribute to the development of these disorders among *GBA* carriers.

GBA encodes glucocerebrosidase (GCase), a lysosomal hydrolase whose main function is the degradation of glucocerebrosides into ceramide and glucose, although it has additional substrates [5]. To properly function, GCase requires a co-activator, saposin C (sapC) [6]. This protein is one of the four active domains of a protein precursor, prosaposin, encoded by the *PSAP* gene. After its synthesis, prosaposin is cleaved by cathepsin D (CTSD) into its functional proteins: saposins A, B, C and D [7, 8]. Saposins are lysosomal cofactors that activate enzymes degrading sphingolipids. Mutations in *PSAP* have been associated with the accumulation of sphingolipids and with different lysosomal storage disorders (LSD). For example, Gaucher's disease, an LSD that is typically caused by biallelic mutations in *GBA*, is also rarely caused by biallelic mutations in the sapC domain of *PSAP* [7, 9].

Whereas the association of *GBA* variants with PD is widely accepted, the role played by *PSAP* in general and sapC specifically in PD remains controversial. Studies in Asian populations suggested an association between *PSAP* variants and PD [10-14], yet these results did

not replicate in Europeans [15-17]. These conflicting results may suggest a possible role played by ethnic differences and/or by the extreme rarity of deleterious *PSAP* variants, reducing their detection in PD. Despite the clinical, biological, and, possibly, genetic links of *PSAP* with *GBA* and PD, the role of *PSAP* in iRBD has not been investigated. Herein, we analyzed a multi-center cohort of 1,113 iRBD patients and 2,324 healthy controls to evaluate a possible association between rare *PSAP* variants and iRBD.

Methods

Population

The current study included 1,113 unrelated iRBD patients and 2,324 unrelated healthy controls of European descent. Details on the cohorts and their recruitment have been previously published [4]. RBD was diagnosed with video polysomnography (vPSG) according to the International Classification of Sleep Disorders, version 2/3 criteria [18, 19]. About 81% of the iRBD patients were males (N=897) and their mean age at the time of the sampling was 68 ± 9.4 (age range 18-93 years). Among the controls, 48% of the participants were males (N=1,122) and their mean age was 48 ± 16.7 (age range 19-93 years).

Standard Protocol Approvals, Registrations, and Patient Consents

All patients signed an informed consent form before entering the study, and the study protocol was approved by the institutional review boards.

Genetic analysis

The *PSAP* coding regions were fully sequenced using Molecular Inversion Probes (MIPs) as previously described [15, 20]. A detailed description of the MIPs library and protocols is available online (https://github.com/gan-orlab/MIP_protocol). Variant annotation was performed with ANNOVAR [21]. The frequency of each variant was extracted from the Genome Aggregation

Database (gnomAD) [22]. Post-alignment quality control and variant calling were done using the Genome Analysis Toolkit (GATK, v3.8) [23] as previously described [24]. Full code is available at https://github.com/gan-orlab/MIPVar/.

In silico structural analysis

The impact of the rare variants on the structure and function of the saposin chains was investigated with *in silico* structural analyses. The atomic coordinates of the human saposin chains B and C were downloaded from the Protein Data Bank [25](ID 1n69 and 1m12, respectively). Images were generated using PyMol v. 2.4.0.

Statistical analysis

Rare *PSAP* variants were filtered using a minor allele frequency (MAF) threshold of < 0.01. To test for rare *PSAP* variants enrichment in iRBD patients we performed optimized sequence Kernel association test (SKAT-O) for all rare variants and subsets of rare variants. These subsets included nonsynonymous, regulatory, potentially functional (nonsynonymous, frameshift, stop-gain and splicing) and loss-of-function (frameshift, stop-gain and splicing) rare variants. A further subset consisted of variants predicted to have a high deleteriousness probability based on a Combined Annotation Dependent Depletion (CADD) score ≥ 12.37. SKAT-O analysis was performed using SKAT package in R 3.5.2 [26]. False discovery rate (FDR) correction was applied to correct for multiple comparisons, using Benjamini-Hochberg method with stats package in R 4.0.2.

Results

We identified 59 rare variants within the *PSAP* region, of which 15 were nonsynonymous and 3 were loss of function (LoF) variants (Supplementary Table 1). The mean coverage was 568X, and a minimum threshold of 30X was applied for variant quality control. To evaluate if rare

PSAP variants are associated with iRBD, we performed SKAT-O comparing iRBD patients and healthy controls. There was a nominally significant enrichment of rare *PSAP* LoF variants (p=0.018, Q=1255) in iRBD patients. However, after FDR correction, the results lost statistical significance (p=0.1, Table 1). Three out of 1,113 iRBD patients (0.3%) carried a rare *PSAP* LoF variant, while no carriers of LoF variants were found among the controls (0/2324, Supplementary Table 1). In particular, p.Gln260Ter and p.Glu166Ter are both stop variants located, respectively, within the sapB and between sapA and sapB domains, therefore the sapC domain is not translated. The p.332_333del mutation is an in-frame deletion located within the sapC domain.

Table 1 - Optimized sequence Kernel association test (SKAT-O) for *PSAP* rare variants

Rare variant subset	P.value	P.adj
CADD	0.034792187	0.1043766
Encode	0.286264029	0.3021955
Func	0.302195501	0.3021955
LoF	0.017929809	0.1043766
NS	0.052448772	0.1048975
All	0.246565484	0.3021955

CADD: Variants selected based on a Combined Annotation Dependent Depletion threshold >12.37; Encode: variants in regulatory elements; Func: potentially functional variants; LoF; loss of function variants; NS: nonsynonymous; All: all rare variants; P.adj: corrected p-value for multiple comparisons using false discovery rate

Given the interplay between sapC and GCase, we examined whether any of these three iRBD patients with PSAP LoF mutations also carry a GBA variant. Furthermore, we tested the presence of GBA copy number variants (CNVs), as was done previously [27]. We found that two of the patients, carrying the p.Glu166Ter and p.332_333del variants, also carried a GBA variant: p.Arg349Ter and p.Glu326Lys, respectively. None carried GBA CNVs. All PSAP and GBA variants were confirmed by Sanger sequencing.

We further examined the frequency of *PSAP* LoF variants on gnomAD database v2.11 (https://gnomad.broadinstitute.org). None of the LoF variants found in this study have been reported in gnomAD, and the overall frequency of *PSAP* LoF variants was extremely low, with a total allele count of high-quality LoF variants of 10 in 141,456 individuals (~0.007%, compared to ~0.3% in the iRBD cohort). With a frequency of ~5% in the general European population for *GBA* variants (based on gnomAD data), the estimated combined carrier frequency of both LoF *PSAP* variants and *GBA* variants is 0.00035%, compared to 0.2% observed in the iRBD cohort, more than a 500-fold difference.

In silico structural analyses

To evaluate the impact of the three iRBD-associated variants on the structure and function of the saposin chains we performed *in silico* analyses. The p.Glu166Ter variant, located between saposin chains A and B, would result in the termination of expression for chains B-D. The p.Gln260Ter variant is located towards the C-terminus of the sapB domain and would result in the deletion of its C-terminal helix (Figure 1A), as well as in the termination of sapC and sapD translation. This deletion would also unfold sapB and prevent its dimerization, which is critical for binding lipids [28]. Finally, the variant p.332_333del is located in a linker between helices 1 and 2 of sapC (Figure 1B) [29]. This shortened linker would prevent the formation of stabilizing contacts between these helices and thus interfere with its ability to bind membranes and GCase. Therefore, all three variants result in a loss of function of the sapC chain.

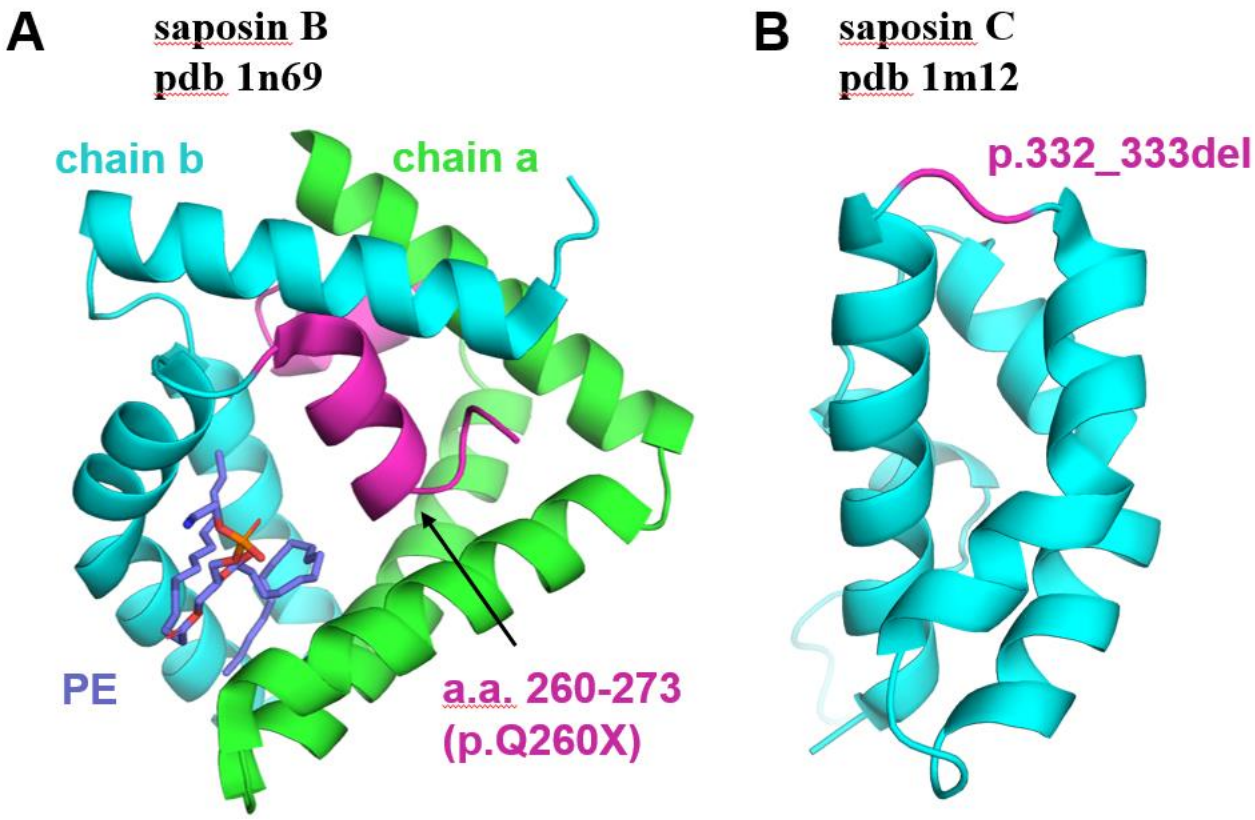

Figure 1: Structural analysis of the saposin B and C domains

(**A**) Crystal structure of the saposin B dimer (green and cyan, pdb 1n69). A bound phosphatidylethanolamine (PE) is shown in violet. The C-terminal helix (a.a. 260-273) deleted in the p.Gln260Ter variant is shown in magenta. (**B**) Solution NMR structure of the human saposin-C domain (cyan, pdb 1m12). In-frame deletion of amino acids Asn332 and Lys333 is shown in magenta.

Clinical presentation of the iRBD patients with PSAP LoF variants

The iRBD patient with the p.332_333del *PSAP* variant was a male in the age range 75-79 who showed minor gait impairment, not quite erect posture, slight global slowness and poverty of spontaneous movements on the neurological examination. His Unified Parkinson's Disease

Rating Scale (UPDRS) III [30] score at last follow-up was 3. No cognitive deficits were present (Montreal Cognitive Assessment (MoCA) = 29/30), yet the patient manifested autonomic symptoms associated with prodromal PD, including constipation, erectile dysfunction and orthostatic hypotension. The risk of prodromal PD according to the Movement Disorder Society (MDS) research criteria [31] at the last follow-up was 1.000 (LR = 37452.7, Table 2).

Table 2 – Clinical data at last follow-up of iRBD patients carrying *PSAP* rare variants

Center	**Innsbruck (Austria)**	**Oxford (UK)**	**Oxford (UK)**
***PSAP* LoF rare variants**	p.332_333del	p.Glu166Ter	p.Gln260Ter
***GBA* variants**	p.Glu326Lys	p.Arg349Ter	No
Sex	Male	Male	Male
AAD range	75-79	80-84	60-64
Disease duration	>13 years	>2 years	>5 years
Tremor	No	No	No
Hypokinesia	No	Initial signs	No
Bradykinesia	No	Initial signs	Initial signs
Postural instability	No	No	No
Cognitive symptoms	No	Yes	Yes
Psychiatric symptoms	No	No	No
Hyposmia	No	Yes	Yes
Orthostatic hypotension	Yes	Yes	Dizziness standing up (negative tilt test)
Constipation	Yes	No	No
Urinary dysfunction	No	No	No
Erectile dysfunction	Yes	No	No
Imaging signs	Substantia nigra hyperechogenicity on the right side	/	/
Risk prodromal PD	0.98 (288) – 1 (37458)	0.96 (551) - 0.99 (25600)	0.53 (88) - 0.98 (4072)
MDS-UPDRS III	3	4	3
Smoker	Yes (ex-smoker)	Yes (ex-smoker)	Yes (ex-smoker)

LoF: loss of function variant; AAD range: age at diagnosis range; Disease duration: disease duration from age at diagnosis to last follow-up; Risk prodromal PD: risk for prodromal Parkinson's disease according to the Movement Disorder Society (MDS) criteria, not considering iRBD (values on the left) and considering iRBD (values on the right). Values in parentheses indicate the likelihood ratios; MDS-UPDRS: Movement Disorder Society - Unified Parkinson's Disease Rating Scale

The iRBD patient with the *PSAP* p.Glu166Ter variant was a male in the age range 80-84 displaying initial PD motor symptoms, including mild right leg rigidity, slight bilateral slowing of finger tapping movements and stooped posture, with a UPDRS III score of 4. He was also diagnosed with mild cognitive impairment (MCI, MoCA = 23/30). Furthermore, the patient had some non-motor PD-related symptoms, including significant hyposmia and orthostatic hypotension. His risk for prodromal PD was 0.99 (LR=25600, Table 2).

Finally, the iRBD patient with the *PSAP* p.Gln260Ter variant was a male in the age range 60-64 showing some signs of motor impairment, including mild asymmetric finger tapping and top tapping bradykinesia. His UPDRS III score was 3 and he was diagnosed with MCI (MoCA = 26/30). He displayed severe hyposmia, while no autonomic symptoms were present. His risk of prodromal PD at his last follow-up was 0.98 (LR=4072, Table 2).

Discussion

In this study, we found three iRBD patients with extremely rare *PSAP* LoF variants, not reported on gnomAD, while no controls were found with LoF variants. Interestingly, two of the three *PSAP* LoF variant carriers also carried a *GBA* variant. While the enrichment of rare *PSAP* LoF variants in iRBD was only nominally significant, given their rarity it is plausible that this reflects a real association. Furthermore, assuming that in the general European population the carrier frequency of *GBA* variants is about 5%, and the carrier frequency of LoF variants (based on gnomAD) is about 0.007%, the probability to carry both a *GBA* variant and a *PSAP* LoF variant is 0.00035%. In the iRBD cohort, the carrier frequency of both was ~0.2%, suggesting that this is likely not due to chance alone. The deleteriousness of the three *PSAP* LoF variants was further exemplified by structural analyses (Figure 1A and 1B). All iRBD patients met the MDS criteria for probable prodromal PD (Table 2).

Although the role of *PSAP* in iRBD and in synucleinopathies in general is still controversial, this study provides the first evidence for a possible role of *PSAP* variants in

iRBD. The lack of a statistically significant enrichment in iRBD patients after correction for multiple comparisons can be explained by the extreme rarity of *PSAP* variants, resulting in insufficient power. The Residual Variation Intolerance Score (RVIS) of *PSAP* is -1, putting it in the top 8.47% of genes in the human genome which are intolerant to genetic variance, especially for LoF variance (FDR corrected p=0.00037 for the observed vs. expected number of LoF variants - http://genic-intolerance.org/Search?query=psap).

Two iRBD carriers of *PSAP* LoF variants were also carriers of a *GBA* variant. Given the incomplete penetrance of *GBA* in iRBD, the presence of potentially pathogenic variants in *PSAP* among *GBA* carriers may suggest oligogenic inheritance and that *PSAP* variants might act as genetic modifiers of risk in *GBA*-iRBD. This is in line with the biological link between sapC and GCase [6, 9]. In particular, it is possible that an impairment of the sapC-mediated activation of GCase contributes to an increased risk to develop iRBD in *GBA* variant carriers. These hypotheses require additional genetic and functional studies. We cannot rule out that the co-occurrence of *GBA* and *PSAP* variants is a coincidence, due to chance alone. However, the fact that two out of three extremely rare *PSAP* LoF variant carriers also carried a *GBA* mutation makes a coincidental association less likely.

It is still unclear whether *PSAP* mutations alone can increase the risk of iRBD or PD. It is possible that LoF of sapC, as seen in our patient with the p.332_333del mutation, will result in reduced activation of GCase and be an independent risk factor. On the other hand, it is also possible that *PSAP* variants might lead to iRBD through mechanisms independent of *GBA*. A possible mechanism can be due to an impairment of CTSD and progranulin (PGRN) activity, as previously hypothesized in PD [8]. PSAP, CTSD and PGRN interact in a network involved in lysosomal homeostasis and clearance of alpha-synuclein. PSAP dysfunction might lead to decreased transport of PGRN into the lysosome, reduction of the pro-CTSD conversion into active CTSD, and consequently to impaired lysosomal trafficking and degradation of deleterious or overrepresented proteins, such as alpha-synuclein [8].

This study has several limitations. Age and sex differed between patients and controls. However, this difference would generally lead to false negative results (as young mutation carriers still would not develop the disease), and is, therefore, less likely to affect our results, as no carriers were found in the controls. Although this study was performed in the largest genetic cohort of iRBD patients worldwide, the sample size may still be insufficient to detect extremely rare variants in *PSAP*. Finally, we were able to find *PSAP* LoF variants, different from each other, in only 3 iRBD patients. However, the absence of such variants in the ~twofold larger control group and in the ~140-fold larger gnomAD control population suggests that this finding might not be random.

Further studies in larger cohorts and functional analyses will be required to clarify the role of *PSAP* variants in iRBD and alpha-synuclein physiopathology. In addition, studies in other populations, such as East Asians, where *PSAP* variants have already been proposed as PD risk factors [10-14], will be necessary to further explore differences in the genetic underpinnings of synucleinopathies between different ethnic groups.

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PREFACE TO CHAPTER 3

In the previous chapter, I investigated the role of rare *PSAP* variants in iRBD and reported that rare loss of function *PSAP* mutations are present in 0.3% of iRBD patients and 0.2% of iRBD patients carry also a *GBA1* variant. These variants were absent in more than 2,000 healthy controls. These results suggest a possible modifier effect of *PSAP* mutations on *GBA1* variants, adding an important piece of the puzzle of RBD genetic background. Since *PSAP* variants have also been suggested to play a role in PD, this finding might also contribute to predicting iRBD conversion and stratifying RBD for potential future neuroprotective trials.

Since iRBD can convert in multiple synucleinopathies, in the following chapter I specifically focus on the genetics of RBD in the context of PD. In particular, I compare the genetic background of PD with and without RBD to assess if genetics play a role in differentiating these two subtypes. Given the comorbidity between RBD and neuropsychiatric manifestations in PD, I also evaluate potential genetic correlations and causative associations between the presence of RBD in PD and multiple neuropsychiatric traits. Defining the genetic background of PD with RBD can help to define a PD subtype characterized by an overall greater malignity, with important implications for prognosis, inclusion criteria for clinical trials and therapeutic management of PD patients.

CHAPTER 3: GENOME-WIDE ASSOCIATION STUDY OF REM SLEEP BEHAVIOR DISORDER IN PARKINSON'S DISEASE

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Keywords: REM sleep behavior disorder, Parkinson's disease, genetics, *SNCA*, *LRRK2*, cognitive decline

Abstract

Objective: REM sleep behavior disorder (RBD) is a prodromal synucleinopathy, reported in a subset of Parkinson's disease (PD) patients, and associated with neuropsychiatric symptoms in PD. We aimed to compare the genetic background of PD patients with probable RBD (PD+RBD) and PD patients without probable RBD (PD-RBD). Furthermore, we examined genetic correlations and potential causal associations between multiple neuropsychiatric traits and PD+RBD.

Methods: We performed a genome-wide association study (GWAS) including 5,403 PD+RBD and 13,020 PD-RBD. To test for genetic correlations and potential causal associations between neuropsychiatric traits and PD+RBD, we used linkage disequilibrium score regression and Mendelian randomization.

Results: The *SNCA* locus was associated with PD+RBD compared to PD-RBD (rs10005233, OR=1.21, 95% CI=1.16-1.27, *p*=1.81e-15). Further examination of known genetic loci associated with PD from the most recent PD GWAS in Europeans and Asians identified additional variants associated with reduced risk for PD+RBD: two in the *SNCA* locus (rs5019538-G, OR=0.85, 95% CI=0.81-0.89, *p*=2.46E-10; rs356182-G, OR=0.89, 95% CI=0.84-0.95, *p*=0.0001), and one in the *LRRK2* locus (rs34637584, p.G2019S, OR=0.41, 95% CI=0.28-0.61, *p*=1.04E-5). We found a potential genetic correlation between attention deficit hyperactivity disorder (ADHD) and PD+RBD, which was not statistically significant after correction for multiple comparisons. No causative association emerged between PD and neuropsychiatric traits.

Interpretation: Genetic variants contribute to the occurrence of RBD in PD, further distinguishing between the PD+RBD and PD-RBD subtypes. Understanding the mechanisms underlying these genetic associations could contribute to the development of subtype-specific treatments.

Introduction

Rapid-eye-movement (REM) sleep behavior disorder (RBD) is a parasomnia characterized by the absence of muscle atonia during REM sleep and dreams enactment. [1] When no neurological conditions or other concomitant factors are identified, it is referred to as isolated/idiopathic RBD (iRBD). [2] iRBD is typically considered a prodromal stage of synucleinopathies, as about 80%-90% of the cases convert to either Parkinson's disease (PD), dementia with Lewy bodies (DLB) or, more rarely, multiple system atrophy (MSA). [3,4] These disorders are all characterized by the accumulation of alpha-synuclein, encoded by the *SNCA* gene. [5] RBD is therefore a key prodromal clinical marker of synucleinopathies, and its presence is also associated with a distinctive, more severe clinical presentation. In PD patients with RBD (up to 52% of cases), [6] RBD is associated with a more malignant phenotype, characterized by faster progression[7] and greater frequency and/or severity of neuropsychiatric manifestations, including cognitive decline, hallucinations, depression, anxiety and apathy. [8-11]

In recent years, it was shown that the genetic background of iRBD only partially overlaps with that of PD or DLB. Genes such as *GBA1*, [12] *TMEM175*[13] and *SNCA*[14] are important across all conditions, [15,16] whereas other genes including *LRRK2*, [17] *APOE*[18] and familial PD genes, [19] seem to not have a major role in iRBD. A recent RBD genome-wide association study (GWAS) identified 5 risk loci associated with RBD, [20] namely *GBA1*, *TMEM175*, *INPPSF*, *SNCA* and *SCARB2*. Notably, the variants associated with RBD in the *SNCA* and *SCARB2* regions were different and independent to those associated with PD, [15,20] supporting RBD as a distinctive subtype, with specific genetic and clinical correlates.

In the current study, we aimed to examine whether there are differences in the genetics of PD with probable RBD (PD+RBD) compared to PD without RBD (PD-RBD). For this purpose, we performed a GWAS including 18,423 patients, composed of PD+RBD patients (N=5,403), and PD-RBD patients (N=13,020). To further explore the relationships between

RBD and neuropsychiatric traits, we performed genetic correlation and Mendelian randomization (MR) analyses using the GWAS summary statistics of the current study and multiple neuropsychiatric conditions.

Methods

Population

The study population included 18,423 PD patients (detailed in Table 1), of whom 5,403 had probable RBD (PD+RBD) and were treated as cases, whereas 13,020 did not (PD-RBD) and were treated as controls. Probable RBD was defined using either the RBD single-question screen (RBD1Q) [21] or the RBD screening questionnaire (RBDSQ), [22] both of which show high sensitivity and specificity in PD patients. [23] We refer to iRBD when RBD occurs prior to the neurodegeneration and to RBD for subjects with RBD regardless of the time of onset of neurodegeneration. PD was diagnosed by movement disorder specialists according to the UK Brain Bank[24] or International Parkinson Disease and Movement Disorders Society criteria. [25] The 23andMe cohort had self-reported a diagnosis of PD as well as RBD and/or dream re-enactment behaviors.

The participants were of European ancestry and their clinical and genetic data were collected from 15 different cohorts (Table 1), 11 of which are from the International Parkinson's Disease Genomics Consortium (IPDGC), three cohorts are from the Accelerating Medicines Partnership Parkinson's disease (AMP-PD, https://amp-pd.org/) and one cohort was collected and analyzed by 23andMe Inc. (https://www.23andme.com/research/). The Central European Group on Genetics of Movement Disorders (CEGEMOD) contributed to the Kosice cohort.

Table 1

Demographic characteristics of PD patients in the individual cohorts

Center	PD+RBD, n	Age PD+RBD (SD)	%Fem PD+RBD	PD-RBD, n	Age PD-RBD (SD)	%Fem PD-RBD	Total
Oslo	130	65.6 (8.8)	25%	180	66.1 (10)	44%	310
Lund	365	71.4 (7)	39%	555	70.8 (9.2)	33%	920
McGill	285	67.4 (9.2)	31%	217	67 (8.8)	49%	502
AMP-PD	111	66.4 (9.4)	69%	269	65.5 (10.3)	62%	380
Sydney	105	59 (10.8)	32%	125	60 (10.7)	38%	230
Tuebingen	453	68.8 (9.1)	33%	659	67.7 (10.2)	38%	1,112
Barcelona	133	69.5 (9.8)	44%	71	74.3 (10)	66%	204
PRoBaND	585	67.3 (8.9)	30%	1,134	67.7 (9.3)	38%	1,719
PFP	257	62.4 (12.5)	37%	339	61.4 (13)	47%	596
OPDC	274	67.3 (9.3)	27%	539	67.4 (9.6)	38%	813
Kosice	102	71.4 (8.3)	33%	225	69.2 (10.7)	42%	327

23andMe	2,603	NA*	38%	8,707	NA*	45%	11,310
TOTAL	5,403	/	/	13,020	/	/	18,423

PD+RBD, n: Number of Parkinson's disease patients with REM sleep behavior disorder; PD-RBD, n: Number of Parkinson's disease patients without REM sleep behavior disorder; Age: mean age in the group; SD: standard deviation; %Fem: percentage of females; Tot: total number of PD patients in the cohort; Oslo: Oslo University Hospital; Lund: Lund University; McGill: McGill University; AMP-PD: Accelerating Medicines Partnership Parkinson's disease, including the New Discovery of Biomarkers (BioFIND), the Harvard Biomarker Study (HBS) and the Parkinson's Disease Biomarkers Program (PDBP) cohorts; Sydney: University of Sydney; Tuebingen: University of Tuebingen; Barcelona: Hospital Universitari Mutua de Terrassa; PRoBaND: Parkinson's repository of biosamples and networked datasets; PFP: Parkinson's Families Project; OPDC: Oxford Parkinson's Disease Centre; Kosice: Pavol Jozef Šafárik University in Kosice.

*23andMe only provides age ranges (i.e., in cases: 5 individuals <30 years of age, 49 individuals 30-45, 346 individuals 45-60, 2203 individuals >60; in controls: 11 individuals <30, 146 individuals 30-45, 1302 individuals 45-60, 7248 individuals >60)

Genetic analysis

23andMe

Participants provided informed consent and volunteered to participate in the research online, under a protocol approved by the external AAHRPP-accredited IRB, Ethical & Independent (E&I) Review Services. As of 2022, E&I Review Services is part of Salus IRB (https://www.versiticlinicaltrials.org/salusirb). DNA extraction and genotyping were performed on saliva samples by National Genetics Institute (NGI), a CLIA-licensed clinical laboratory and a subsidiary of Laboratory Corporation of America. Samples were genotyped

on one of five genotyping platforms. The v1 and v2 platforms were variants of the Illumina HumanHap550 + BeadChip, including about 25,000 custom single nucleotide polymorphisms (SNPs) selected by 23andMe, with a total of about 560,000 SNPs. The v3 platform was based on the Illumina OmniExpress+ BeadChip, with custom content to improve the overlap with the 23andMe v2 array, with a total of about 950,000 SNPs. The v4 platform was a fully customized array, including a lower redundancy subset of v2 and v3 SNPs with additional coverage of lower-frequency coding variation, and about 570,000 SNPs. The v5 platform, in current use, is an Illumina Infinium Global Screening Array (~640,000 SNPs) supplemented with ~50,000 SNPs of custom content. This array was specifically designed to better capture global genetic diversity and to help standardize the platform for genetic research. Samples that failed to reach 98.5% call rate were re-analyzed. Individuals whose analyses failed repeatedly were re-contacted by 23andMe customer service to provide additional samples.

Participants were restricted to European ancestry through an analysis of local ancestry. [26] A support vector machine (SVM) to classify individual haplotypes into one of 31 reference populations was used (https://www.23andme.com/ancestry-composition-guide/). The SVM classifications are then fed into a hidden Markov model (HMM) that accounts for switch errors and incorrect assignments, and gives probabilities for each reference population in each window. Finally, we used simulated admixed individuals to recalibrate the HMM probabilities so that the reported assignments are consistent with the simulated admixture proportions. A maximal set of unrelated individuals was chosen for each analysis using a segmental identity-by-descent (IBD) estimation algorithm. [27]

We phased participant data using either an internally-developed tool, Finch (V1-V4 genotyping arrays) or Eagle2 (V5 genotyping array). [28] Finch implements the Beagle haplotype graph-based phasing algorithm, modified to separate the haplotype graph construction and phasing steps. [29] It extends the Beagle model to accommodate genotyping error and

recombination, to handle cases where there are no consistent paths through the haplotype graph for the individual being phased. We constructed haplotype graphs for European and non-European samples on each 23andMe genotyping platform from a representative sample of genotyped individuals, and then performed out-of-sample phasing of all genotyped individuals against the appropriate graph. For the X-chromosome, we built separate haplotype graphs for the non-pseudoautosomal region and each pseudoautosomal region, and these regions were phased separately.

Imputation panels created by combining multiple smaller panels have been shown to give better imputation performance than the individual constituent panels alone. [30] To that end, we combined the May 2015 release of the 1000 Genomes Phase 3 haplotypes with the UK10K imputation reference panel to create a single unified imputation reference panel. [31,32] Multiallelic sites with N alternate alleles were split into N separate biallelic sites. We then removed any site whose minor allele appeared in only one sample. For each chromosome, we used Minimac3 to impute the reference panels against each other, reporting the most probable genotype at each site. [33] This gave us calls for all samples over a single unified set of variants. We then joined these together to get, for each chromosome, a single VCF with phased calls at every site for 6,285 samples.

In preparation for imputation, we split each chromosome of the reference panel into chunks of no more than 300,000 variants, with overlaps of 10,000 variants on each side. We used a single batch of 10,000 individuals to estimate Minimac3 imputation model parameters for each chunk. [33] We imputed phased participant data against the chunked merged reference panel using Minimac3, treating males as homozygous pseudo-diploids for the non-pseudoautosomal region.

We excluded SNPs that: 1) had a call rate<90%, 2) had a Hardy-Weinberg p<10–20 in people with European ancestry, 3) were only genotyped on the V1 and/or V2 platforms, 4)

were found on the mitochondrial chromosome or the Y- chromosome, 5) failed a test for parent-offspring transmission (specifically, we regressed the child's allele count against the mean parental allele count and excluded SNPs with fitted <0.6 and p<10–20 for a test of <1), 6) had an association with genotype date (p<10–50 by ANOVA of SNP genotypes against a factor dividing genotyping date into 20 roughly equal-sized buckets), 7) had a large sex effect (ANOVA of SNP genotypes, r2>0.1), or 8) had probes matching multiple genomic positions in the reference genome.

We excluded SNPs with imputed r2<0.3, as well as SNPs that had strong evidence of a platform batch effect. For each SNP we identified the largest sub- set of the data passing other quality control criteria based on their original genotyping platform – either v2+v3+v4+v5, v4+v5, v4, or v5 only – and computed association test results for the largest passing set. The batch effect test is an F test from an ANOVA of the SNP dosages against a factor representing the V4 or V5 platform; we excluded results with p<10–50 .

Across both genotyped and imputed GWAS results, we excluded SNPs that had sample size of less than 20% of the total GWAS sample size. We also removed SNPs that did not converge during logistic regression, as identified by abs (effect)>10 or stderr>10 on the log-odds scale. If SNPs were both genotyped and imputed, and they passed QC for both, we used results from the imputed analysis. After quality control, we had analysed 904,040 genotyped SNPs and 25,208,208 imputed SNPs.

GWAS was performed using logistic regression adjusted for age, sex, top five principal components as well as the genotype platform to account for genotype batch effects. The significance threshold was set at $p<5x10E-8$.

Other cohorts

Genotyping in the different centers was performed using the OmniExpress, NeuroX or Global Screening (GSA) GWAS array according to the manufacturer's instructions (Illumina Inc.). Parkinson's Families Project (PFP) was genotyped with NeuroChip, Parkinson's repository of biosamples and networked datasets (PRoBaND) with HumanCoreExome array, and Oxford Parkinson's Disease Centre (OPDC) with either HumanCoreExome-12 v.1.1 or Infinium HumanCoreExome-24v.1.1 arrays. Quality control was performed following standard pipelines (detailed in https://github.com/neurogenetics/GWAS-pipeline) using plink 1.9. [34] In brief, we filtered out heterozygosity outliers using an F-statistic cut-off of<-0.15 or >0.15. Individuals with a variant call rate<95% and sex mismatch were excluded. Variants missing in >5% of the participants, with disparate missingness between cases and controls (p<1E-04), or significantly deviating from the Hardy-Weinberg equilibrium in controls (p<1E-04) were also removed. We used GCTA to check for relatedness closer than first cousins between participants (pihat>0.125). We performed imputation using the Michigan imputation server (https://imputationserver.sph.umich.edu/index.html#) with the Haplotype Reference Consortium reference panel r1.1 2016 under default settings. Ancestry outliers were detected using HapMap3 principal component analysis (PCA) data in R version 4.0.1. After imputation, we selected variants with r2>0.8 and a minor allele frequency (MAF)>0.01, while retaining variants that have strong pathogenic implications in PD (i.e., the LRRK2 p.G2019S variant and the GBA1 p.N370S, p.E326K and p.T369M variants).

To test for genetic associations to RBD in PD, we performed GWAS using logistic regression comparing PD+RBD and PD-RBD adjusted for age at RBD questionnaire administration, sex and principal components. The significance threshold was set at p<5x10E-8. The analyses were performed separately in each cohort and the results were then meta-analyzed with a fixed-effect model using METAL (https://genome.sph.umich.edu/wiki/METAL_Documentation).[35] To identify any possible

secondary associations hidden by the principal signals of the GWAS, we also performed Conditional and Joint - Genome-wide Complex Trait Analysis (COJO-GCTA), a method that harnesses a conditional stepwise regression approach to identify independent associations (https://yanglab.westlake.edu.cn/software/gcta/#Overview).[36]

Genetic correlation

To investigate the potential genetic correlation between the presence of RBD in PD and known neuropsychiatric conditions we used linkage-disequilibrium score regression (LDSC) on LDHub (http://ldsc.broadinstitute.org/ldhub/).[37] The neuropsychiatric traits we analyzed include epilepsy, headache, amyotrophic lateral sclerosis, cognitive decline, Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, alcohol dependence, cannabis dependence, attention deficit hyperactivity disorder, Tourette syndrome, anorexia nervosa, post-traumatic syndrome, schizophrenia, bipolar disorder, obsessive-compulsive disorder, autism spectrum disorder and major depressive disorder. Summary statistics for the compared traits were accessed through the LDHub platform or downloaded from publicly available sources, then formatted and analyzed using LDHub python v2.7 scripts (https://github.com/bulik/ldsc/wiki/). Positive correlation indicates association with PD+RBD, and negative correlation indicates association with PD-RBD.

Mendelian randomization

To assess any possible causal association between neuropsychiatric disorders and the presence of RBD in PD we performed Mendelian randomization (MR). [38] In brief, this method harnesses summary statistics from an exposure (the neuropsychiatric traits, in this case) and an outcome (the presence of RBD in PD) and uses the statistically significant variants from the former as instrumental variables (IVs) to infer a potential causative association with the latter. This approach mimics randomized control trials, since genetics is randomly assigned at conception

and unaffected by the environment. [39] The neuropsychiatric traits for this analysis were selected based on relevance to RBD comorbidities, known neuropsychiatric manifestations in PD or with clinical relevance to PD. They include Alzheimer's disease, dementia with Lewy Bodies, schizophrenia, major depressive disorder and bipolar disorder. We used the TwoSampleMR R package (https://mrcieu.github.io/TwoSampleMR/)[40] to perform MR analyses, including sensitivity analyses, tests assessing pleiotropy and heterogeneity between IVs, in R version 4.0.1 according to protocols previously established. [41] Sensitivity analyses included MR Egger, inverse variance weighted (IVW), weighted median, simple mode and weighted mode. Steiger filtering was also performed to check for reverse causality. Summary statistics were downloaded by the MRBase GWAS catalog (http://www.mrbase.org/) and the Psychiatric Genomics Consortium (https://pgc.unc.edu/) publicly available database. To calculate the power to detect an odds ratio=1.2 we used an online Mendelian Randomization power calculation tool (https://sb452.shinyapps.io/power/).[42]

Results

Genome-wide association study identifies the SNCA and LRRK2 loci as modifiers of risk for RBD in PD

To assess whether genetics can affect the risk of RBD in PD we performed GWAS between PD+RBD (N=5,403) and PD-RBD (N=13,020). We evaluated the genomic inflation using quantile-quantile plots (Q-Q plots) and the lambda factor, showing no inflation (lambda=0.994, lambda1000=0.999), (Supplementary Fig 1).

We found that rs10005233, in the 5' region of the *SNCA* locus, was associated with PD+RBD (OR=1.21, 95% CI=1.16-1.27, *p*=1.81E-15, Fig 1). No secondary signal was detected in the GCTA-COJO analysis at a GWAS significance level. We also examined the 92 variants associated with PD in the most recently published GWAS in Europeans[15] and Asians[43] (Table 2, Supplementary Table 1).

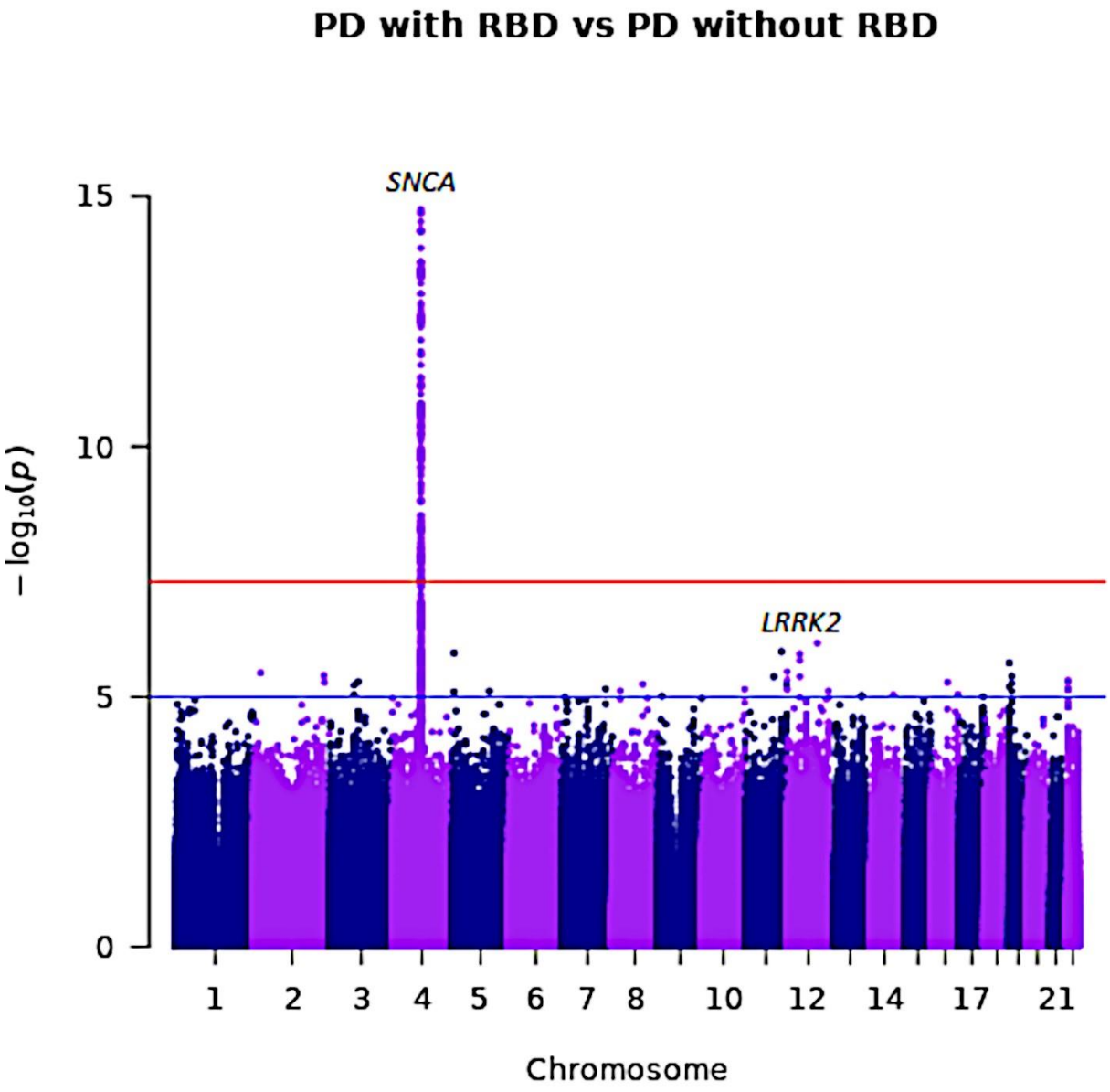

Fig 1: Manhattan plot of PD+RBD vs. PD-RBD

Manhattan plot showing the results of the GWAS meta-analysis, comparing PD+RBD and PD-RBD, highlighting the *SNCA* and *LRRK2* loci. The Y axis represents the negative logarithm of p-value, the X axis represents the chromosomal position of the variants and each dot on the figure represents a SNP. The red line represents the genome-wide Bonferroni-corrected statistical significance threshold ($5x10^{-8}$), whereas the blue line is the suggestive significance threshold ($1x10^{-5}$), as defined by the R qqman package (https://cran.r-project.org/web/packages/qqman/).

Chr: chromosome; PD: Parkinson's disease; RBD: REM sleep behavior disorder.

Table 2

Association of variants from previous PD GWAS with PD+RBD in the current study

		PD with and without RBD		PD[15, 37]	
Variant	**Nearest gene**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
4:90636630	*SNCA*	0.85 (0.81-0.89)	2.46E-10*	1.17 (1.14-1.2)	1.13E-36
12:40734202	*LRRK2*	0.41 (0.28-0.61)	1.04E-05*	11.35 (9.44-13.63)	3.61E-148
4:90626111	*SNCA*	0.89 (0.84-0.95)	0.0001365*	1.32 (1.29-1.35)	3.89E-154
16:30977799	*SETD1A*	0.92 (0.88-0.97)	0.001952	1.09 (1.07-1.12)	5.12E-20
19:2341047	*SPPL2B*	1.09 (1.03-1.15)	0.002506	0.93 (0.91-0.95)	4.18E-10
17:43798308	*CRHR1*	1.19 (1.05-1.36)	0.008267	0.79 (0.75-0.84)	6.71E-16
3:28705690	*LINC00693*	0.95 (0.9-0.99)	0.02552	1.07 (1.05-1.09)	8.09E-12

PD: Parkinson's disease; RBD: REM sleep behavior disorder; OR (95% CI): odds ratio with relative 95% confidence interval.

*variant statistically significant after Bonferroni correction (α/ number of variants=0.00054).

Using Bonferroni correction based on the number of these variants (α/number of variants=0.00054), we identified three associations. Two were variants in the *SNCA* locus,

independent of each other, whose minor alleles were associated with decreased risk for PD+RBD (rs5019538-G, OR=0.85, 95% CI=0.81-0.89, *p*=2.46E-10 and rs356182-G, OR=0.89, 95% CI=0.84-0.95, *p*=0.0001), and one was the LRRK2 p.G2019S variant, also associated with a reduced risk for PD+RBD (rs34637584, OR=0.41, 95% CI=0.28-0.61, *p*=1.04E-5, the carrier frequency for this variant across the different cohorts is detailed in Table 3). These three variants were associated with increased risk for PD in the most recent GWAS.[15,43] *GBA1* variants did not show significant associations with PD+RBD (Supplementary Table 2). Additional potential associations in the *SETD1A*, *SPPL2B*, *CRHR1* and *LINC00693* loci should be further studied (Table 2).

Genetic correlation and causative associations between PD with RBD and neuropsychiatric disorders

To examine potential genetic correlations between the risk of RBD in PD and multiple neuropsychiatric conditions, we performed LDSC (Fig 2, Supplementary Table 3). We found that the PD+RBD trait was mildly correlated with attention deficit hyperactivity disorder (ADHD, rg=0.30, SE=0.14, *p*=0.04). The most recently published European PD GWAS was genetically correlated with PD-RBD (rg=-0.38, SE=0.15, *p*=0.01). However, these correlations were not statistically significant after Bonferroni correction (α=0.0025).

To assess possible causative associations between neuropsychiatric conditions and PD+RBD we performed MR using neuropsychiatric disorders as exposures and PD+RBD as the outcome (Supplementary Fig 2 and 3, Supplementary Tables 4-7). No test showed a statistically significant causative association between neuropsychiatric traits and PD+RBD. However, our power for this analysis was suboptimal (35.7%), therefore there could be associations that we could not detect. We were not able to conduct reverse MR using PD+RBD as the exposure since only one locus passed GWAS significance, preventing us from performing appropriate sensitivity analyses.

Table 3

Carriers of the *LRRK2* p.G2019S variant across different cohorts

Cohort	Non-carriers PD-RBD, N	Carriers PD-RBD, N (%)	Non-carriers PD+RBD, N	Carriers PD+RBD, N (%)
Oslo	156	1 (0.64%)	122	1 (0.81%)
Lund	552	3 (0.54%)	364	1 (0.27%)
McGill	212	5 (2.30%)	282	3 (1.05%)
AMP-PD	258	9 (3.37%)	110	1 (0.90%)
Sydney	115	1 (0.86%)	98	1 (1.01%)
Tuebingen	365	0 (0.00%)	641	0 (0.00%)
Barcelona	45	0 (0.00%)	100	0 (0.00%)
PRoBaND	1132	2 (0.18%)	582	3 (0.51%)
PFP	328	9 (2.67%)	246	6 (2.38%)
OPDC	537	2 (0.37%)	274	0 (0.00%)
Kosice	216	1 (0.46%)	101	0 (0.00%)
23andMe	8459	248 (2.85%)	2584	19 (0.73%)
TOTAL*	**12010**	**281 (2.29%)**	**4863**	**35 (0.71%)**

PD-RBD: participants without REM sleep behavior disorder (RBD); PD+RBD: participants with RBD; N: number of participants; %: percentage of participants; Oslo: Oslo University Hospital; Lund: Lund University; McGill: McGill University; AMP-PD: Accelerating Medicines Partnership Parkinson's disease, including the New Discovery of Biomarkers (BioFIND), the Harvard Biomarker Study (HBS) and the Parkinson's Disease Biomarkers Program (PDBP) cohorts; Sydney: University of Sydney; Tuebingen: University of Tuebingen; Barcelona: Hospital Universitari Mutua de Terrassa; PRoBaND: Parkinson's repository of biosamples and networked datasets; PFP: Parkinson's Families Project; OPDC: Oxford Parkinson's Disease Centre; Kosice: Pavol Jozef Šafárik University in Kosice.

*The total excludes individuals with unknown carrier status for p.G2019S

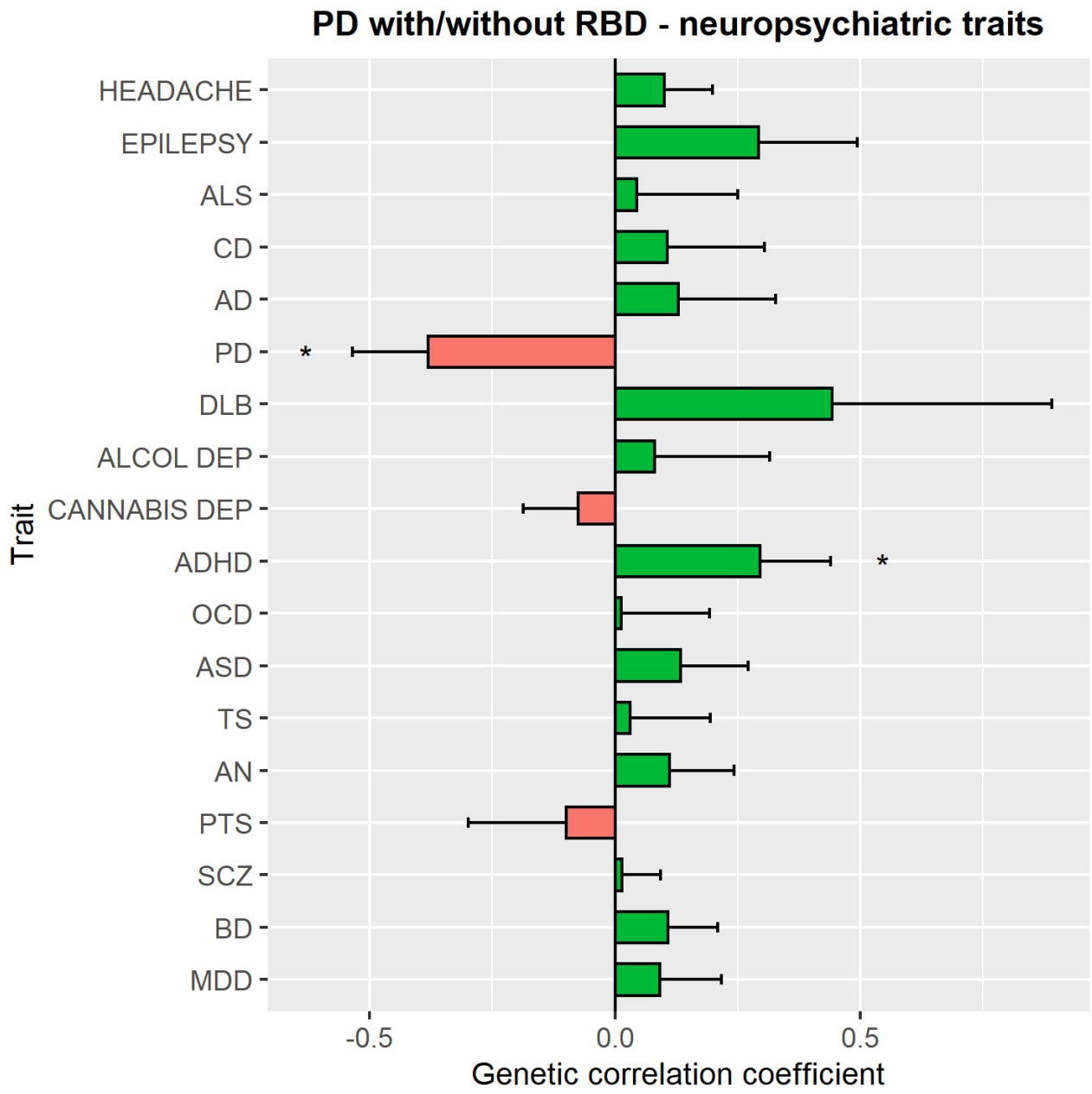

Fig 2: Genetic correlation between PD+RBD and neuropsychiatric traits

The bar plot shows the genetic correlations between PD+RBD and neuropsychiatric traits. The correlation coefficient is illustrated on the X axis. Green bars represent positive correlations whereas red bars negative ones (i.e., a positive correlation of the neuropsychiatric trait with PD-RBD). The asterisks highlight the nominally significant correlations.

ALS: amyotrophic lateral sclerosis; CD: cognitive decline; AD: Alzheimer's disease; PD: Parkinson's disease; DLB: dementia with Lewy bodies; Alcohol dep: alcohol dependence;

cannabis dep: cannabis dependence; ADHD: attention deficit hyperactivity disorder; OCD: obsessive-compulsive disorder; ASD: autism spectrum disorder; TS: Tourette syndrome; AN: anorexia nervosa; PTS: post-traumatic syndrome; scz: schizophrenia; BD: bipolar disorder; MDD: major depressive disorder.

Discussion

In the current GWAS, we found that variants in the *SNCA* and *LRRK2* loci may modify the risk of RBD in PD. Additional loci (*SETD1A*, *SPPL2B*, *CRHR1* and *LINC00693*) require further studies to examine whether they have a role in PD+RBD. The top variant in the *SNCA* locus, rs10005233, was previously reported to be associated with iRBD in a candidate gene study. [14] Another study, using the Oslo and Parkinson's Progression Marker Initiative cohorts, found another variant in the *SNCA* locus associated with PD+RBD (rs3756063), which is in strong linkage disequilibrium (LD) with rs10005233 (D'=0.97, r^2=0.91). [44] Furthermore, rs10005233 is in LD with other 5' region *SNCA* variants associated with synucleinopathies, including rs7681440 (D'=0.99, r^2=0.94), associated with DLB, [45] rs763443 (D'=0.89, r^2=0.78), a secondary PD GWAS signal, [14,15,46] as well as rs2583988 (D'=0.99, r^2=0.40), a variant located in the *SNCA-AS1* region (discussed below) and associated with Lewy body variant of Alzheimer's disease (ADLBV). [47] It is still unclear whether it is a specific variant in the *SNCA* locus or the presence of a specific *SNCA* haplotype that drives these associations with cognitive phenotypes across synucleinopathies. [14] The rs10005233 variant is also in LD (D'=0.97, r^2=0.91) with the top signal of a recently published RBD GWAS, rs3756059, [20] which was associated with reduced expression of *SNCA-AS1,* an antisense RNA molecule that could potentially reduce the translation of alpha-synuclein when it is overexpressed or increase the translation of alpha-synuclein when it is down-regulated. Notably, this reduced expression of *SNCA-AS1* is mainly in cortical areas, [20] thus potentially increasing alpha-synuclein levels and exposing the cerebral cortex to a greater risk of neurodegeneration in carriers of this RBD-

associated variant. The latter hypothesis should be tested in relevant animal models. Altogether, these data suggest that, depending on possible region-specific effects, different *SNCA* variants might play different roles in synucleinopathies, as it has been investigated in previous studies. [14,20,44]

Similar to PD+RBD, other variants could be involved in the development of PD-RBD. We found that three of the 92 PD GWAS signals associated with increased PD risk in Europeans and Asians, [15,43,48] the LRRK2 variant p.G2019S and the *SNCA* variants rs5019538 and rs356182, were all associated with PD-RBD, compared with PD+RBD. The association between p.G2019S and PD-RBD is in line with a previously reported reduced frequency of RBD in PD patients carrying this variant, [49] and with lack of p.G2019S carriers in about 1,000 iRBD patients in another study. [19] In addition to a reduced occurrence of RBD, carriers of the p.G2019S LRRK2 variant also present an overall more benign phenotype, including less frequent and milder cognitive decline. [50,51] These findings, in addition to the nominal correlation between PD+RBD and the most recent PD GWAS in Europeans[15] suggest that such GWAS might explain the genetic background of PD-RBD more than it does for PD+RBD.

These findings further support a pathophysiological relationship between the manifestation of RBD in PD and cognitive decline, which is in line with the comorbidity of these two clinical entities. It was hypothesized that this clinical and pathophysiological correlation could reflect the two alternative directions of alpha-synuclein spreading, body-first or brain-first. [52] In body-first PD, alpha-synuclein pathology may start in the enteric nervous system, whereas in brain-first PD it may arise in the amygdala, entorhinal cortex and substantia nigra. These different neuropathological patterns correspond to two different subgroups of clinical progression. In body-first PD, RBD may manifest before the motor PD symptoms, and cognitive decline occurs faster, whereas in brain-first PD, RBD may occur after the onset of motor PD symptoms, if at all, and cognitive impairment develop more slowly. [52-55] We can

therefore speculate that the *SNCA* rs10005233 variant associated with PD+RBD might also be associated with the body-first subtype of PD, whereas the LRRK2 p.G2019S variant might be associated with the brain-first PD subtype, with less frequent RBD and milder cognitive decline. Since we cannot determine in our data which patients had RBD prior to PD diagnosis and which had it after PD diagnosis, this hypothesis should be studied in future genetic analyses of brain-first vs. body-first PD.

Similar to previous reports in iRBD and PD+RBD, [14,15,44] in this study we did not observe any involvement of *APOE* variants in PD+RBD, suggesting that this gene does not affect RBD risk in PD patients. The rs117615688 variant (chromosomal position 17:43798308) in the *CRHR1* gene, located in the *MAPT* locus, was nominally associated with RBD (OR=1.19, 95% CI=1.05-1.36, *p*=0.008) with an opposite direction of effect to that seen in PD (OR=0.79, 95% CI=0.75-0.84, *p*=6.71E-16) (Table 2).

There are several limitations in this study. All participants were Europeans, therefore our results might not fully apply to other ancestries. In addition, although we included a large number of patients with PD, insufficient power in our analysis might explain the lack of causative associations between PD+RBD and neuropsychiatric traits as well as of genome-wide significance of the LRRK2 p.G2019S and *GBA1* variants. It is possible that *GBA1* variants are strongly implicated also in the PD subtype without RBD, thus counterbalancing their previously reported contribution to RBD risk. [12,20] Another limitation is represented by the inclusion of patients who developed RBD both before and after PD, as they may represent body-first vs. brain-first subtypes of PD as discussed above. Future research with larger sample sizes could investigate possible genetic and biological differences between them and specifically differentiate brain-first and body-first PD in that sense.

In conclusion, in this study we demonstrated that the risk of PD+RBD may be modified by variants in the *SNCA* and *LRRK2* loci, and potentially other loci. These genetic associations

may explain why cognitive decline is more frequently observed in PD+RBD compared to PD-RBD, with possible implications for therapeutic management of PD patients. Future research will need to further explore the relationship between genetics, biology and clinical comorbidities to define PD subtypes and implement a precision medicine guided by early markers.

Competing interests

ZGO has received consulting fees from Lysosomal Therapeutics Inc., Idorsia, Prevail Therapeutics, Denali, Ono Therapeutics, Neuron23, Handl Therapeutics, UBC, Bial Biotech Inc., Bial, Deerfield, Guidepoint, Lighthouse and Inception Sciences (now Ventus). None of these companies were involved in any parts of preparing, drafting and publishing this study. KH, PF, LNK, and the 23andMe Research Team are employed by and hold stock or stock options in 23andMe. HM is employed by UCL. In the last 12 months he reports paid consultancy from Roche, Aprinoia and Amylyx; lecture fees/honoraria - BMJ, Kyowa Kirin, Movement Disorders Society. Research Grants from Parkinson's UK, Cure Parkinson's Trust, PSP Association, Medical Research Council, Michael J Fox Foundation. Dr Morris is a co-applicant on a patent application related to C9ORF72 - Method for diagnosing a neurodegenerative disease (PCT/GB2012/052140)

Remaining authors declare no competing interests.

Data availability

The PD with and without RBD GWAS summary statistics without the 23andMe cohort is publicly available on GWAS catalog (https://www.ebi.ac.uk/gwas/). To access the full summary statistics with 23andMe an application to 23andMe is required. The full GWAS summary statistics for the 23andMe dataset will be made available through 23andMe to qualified researchers under an agreement with 23andMe that protects the privacy of the

23andMe participants. Please visit https://research.23andme.com/collaborate/#dataset-access/ for more information and to apply to access the data. The top 10,000 SNPs GWAS summary statistics including 23andMe is available on https://github.com/gan-orlab. The GWAS summary statistics for the neuropsychiatric traits used in the study are available on the GWAS catalog (https://www.ebi.ac.uk/gwas/) and Psychiatric Genomics Consortium (https://pgc.unc.edu/).

Code availability

The codes used for the analyses are available on https://github.com/daskrohn/RBD_GWAS and https://github.com/gan-orlab.

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Ethical Compliance Statement

IRB Study Number A11-M60-21A (21-11-023) was reviewed and approved by the Research Ethics Offices (REOs). Informed written patient consent was provided in each center before the inclusion of each in the study.

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PREFACE TO CHAPTER 4

In the previous chapter, I demonstrated that PD with and without RBD harbour partially different genetic underpinnings. In particular, I show that one *SNCA* variant is associated with increased risk of RBD in PD, whereas three other variants, two in the *SNCA* locus and one in the *LRRK2* locus, are associated with reduced risk of RBD in PD.

RBD in PD is more frequently associated with LID. However, although *GBA1* variants have been previously suggested as risk factors for both RBD and LID, *LRRK2* variants were suggested to have an opposite direction, being associated with reduced risk for RBD but increased for LID. To further explore the role of these two genes in LID, we tested the association of *GBA1* and *LRRK2* variants with LID risk and time to LID. In addition, to further stratify PD based on the risk and time to LID, we performed GWAS, and evaluated the impact of PD PRS and dopamine transmission pathway in LID development.

Stratifying PD based on their risk and rate of progression to LID development can have a crucial impact on the therapeutic management of symptomatic therapy and also highlight potential genetic targets for PD patients more susceptible to this adverse effect.

CHAPTER 4: DOPAMINE TRANSMISSION PATHWAY AND PARKINSON'S DISEASE RISK VARIANTS ARE ASSOCIATED WITH DYSKINESIA

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Keywords: levodopa-induced dyskinesia, Parkinson’s disease, dopamine, *GBA1*, *LRRK2*

Abstract

Background: Levodopa-induced dyskinesia (LID) is a common adverse effect of levodopa, one of the main therapeutics used to treat the motor symptoms of Parkinson's disease (PD). Previous evidence suggests a connection between LID and a disruption of the dopaminergic system as well as genes implicated in PD, including *GBA1* and *LRRK2*.

Objectives: To investigate the effects of genetic variants on risk and time to LID.

Methods: We performed a genome-wide association study (GWAS) and analyses focused on *GBA1* and *LRRK2* variants. We also calculated polygenic risk scores including risk variants for PD and variants in genes involved in the dopaminergic transmission pathway. To test the influence of genetics on LID risk we used logistic regression, and to examine its impact on time to LID we performed Cox regression including 1,612 PD patients with and 3,175 without LID.

Results: We found that *GBA1* variants were associated with LID risk (OR=1.65, 95% CI=1.21-2.26, p=0.0017) and *LRRK2* variants with reduced time to LID onset (HR=1.42, 95% CI=1.09-1.84, p=0.0098). The fourth quartile of the PD PRS was associated with increased LID risk ($OR_{fourth\_quartile}$=1.27, 95% CI=1.03-1.56, p=0.0210). The third and fourth dopamine pathway PRS quartiles were associated with a reduced time to development of LID ($HR_{third_quartile=}$1.38, 95% CI=1.07-1.79, p=0.0128; $HR_{fourth_quartile=}$1.38, 95% CI=1.06-1.78, p=0.0147).

Conclusions: This study suggests that variants implicated in PD and in the dopaminergic transmission pathway play a role in the risk/time to develop LID. Further studies will be necessary to examine how these findings can inform clinical care.

Introduction

Levodopa is one of the most commonly administered therapies for Parkinson's disease (PD), particularly to treat motor symptoms. [1] However, as the disease progresses and patients are exposed to long-term levodopa therapy, a significant proportion develops levodopa-induced dyskinesia (LID), a debilitating side effect characterized by involuntary, uncontrolled, and often choreiform movements. [2] LID is estimated to affect around 40%-50% of PD patients within 4-6 years of initiating levodopa therapy, [3, 4] however, a subset of them manifests LID also within the first year of the therapy, [5] demonstrating the broad variability of LID risk and onset. The most widely-accepted pathophysiologic hypothesis suggests that LID development is connected with a pulsatile stimulation of the dopamine receptors in the nucleus striatum. [6] This phenomenon occurs due to the progressive dopaminergic loss in PD, resulting in impaired presynaptic storage capacity of dopamine, and is exacerbated by elevated doses of levodopa. [6-8] Other pathways have also been implicated in LID development, including the glutamatergic, serotonergic and noradrenergic neural circuits. [7, 8]

Multiple environmental risk factors affecting LID have been identified, including levodopa dosage and duration of the therapy, use of dopamine agonists, PD age at onset (AAO), disease duration and severity, female sex and lower body mass index (BMI). [9-13] Most of the suggested genetic risk factors for LID are related to the dopamine pathway, including genes encoding the dopamine receptors, especially *DRD2* and *DRD3*, [14-16] the dopamine transporter *SLC6A3*, [17, 18] or enzymes that metabolize dopamine and are targeted by PD therapeutics, [19, 20] catechol-O-methyltransferase (*COMT*) [21-23] and monoamine oxidases A and B (*MAOA*, *MAOB*). [22-24] Interestingly, variants in *GBA1* and *LRRK2,* among the most frequent genetic risk factors for PD, [25, 26] have also been identified as potential risk factors for LID. [27-32] Carriers of *GBA1* and *LRRK2* variants show distinctive clinical presentations in PD, with *GBA1* variants being associated with a more rapidly progressive PD with earlier onset, [33] and *LRRK2* variants with an overall more benign disease course, but with also more frequent

postural instability and gait difficulty as well as slightly earlier AAO compared to sporadic PD. [34] Other variants reported in LID include those in *BDNF,* involved in neural plasticity, [35, 36] *GRIN2A,* encoding a glutamatergic receptor, [37] and *ADORA2A*, encoding the adenosine A2a receptor gene. [38] However, the association between LID and most of the above-mentioned putative genetic risk factors is still controversial, with most findings reported deriving from candidate genes studies that failed to be confirmed in replication studies. [39-44]

Here, we aimed to systematically evaluate how genetics affect the risk and rate of progression to LID including a total of 4,787 PD patients from multiple centers. For this purpose, we performed genome-wide association studies (GWAS) and downstream analyses focused on specific genes previously implicated in LID. In addition, we tested the effect produced by cumulative genetic risk on the occurrence and rate of progression to LID, including risk variants previously associated with PD and variants in genes involved in the dopaminergic transmission pathway.

Methods

Population

The study population included a total of 4,787 PD patients, of which 1,612 with and 3,175 without LID (Table 1). PD was diagnosed by movement disorder specialists according to the UK Brain Bank or International Parkinson Disease and Movement Disorders Society criteria. [45] LID diagnosis was made based on the Unified Parkinson's Disease Rating Scale (UPDRS) part IV and direct clinical evaluation. The participants were of European ancestry and their clinical and genetic data were collected from 15 different cohorts (Table 1), 12 of which were from the International Parkinson's Disease Genomics Consortium (IPDGC) and 3 from the Accelerating Medicines Partnership Parkinson's Disease (AMP-PD, https://amp-pd.org/). The latter includes the Parkinson's Disease Biomarkers Program (PDBP), Parkinson's Progression Markers Initiative (PPMI) and Harvard Biomarker Study (HBS) cohorts. The cohorts were

included in the different analyses depending on data availability. The cohorts included in each analysis are specified in Supplementary Table 1.

Table 1 - Demographic characteristics of PD patients in the individual cohorts

Center	LID-, n	Age LID- (SD)	%Mal LID-	LID+, n	Age LID+ (SD)	%Mal LID+	Tot
Barcelona	103	73.3 (10.9)	50%	48	72.3 (7.7)	40%	151
CORIELL	221	62.7 (8.9)	67%	117	61.7 (9.6)	59%	338
DIGPD	220	67.5 (9.3)	62%	166	63.9 (10.4)	56%	386
LEAP	336	68.9 (8.8)	75%	75	67.7 (8.5)	50%	411
Luxembourg	330	67.8 (11.4)	66%	140	66.2 (10.0)	66%	470
Mayo Clinic Florida	404	75.8 (9.9)	69%	151	72.0 (10.1)	62%	555
McGill	258	63.2 (16.5)	34%	120	61.3 (15.7)	43%	378
Oviedo-HUCA	80	69.8 (8.9)	51%	110	70.4 (10.5)	55%	190
PDBP – PPMI – HBS (AMP-PD)	580	58.4 (12.4)	66%	87	56.1 (12.2)	53%	667
PreCEPT	181	61.6 (8.6)	68%	137	58.5 (9.7)	66%	318
SCOPA	109	59.1 (10.9)	66%	177	60.0 (10.6)	62%	286
Sevilla	180	69.7 (10.9)	61%	252	66.0 (11.1)	57%	432
Tartu	173	73.0 (8.2)	38%	32	72.0 (8.6)	50%	205
TOTAL	3175	67.0 (10.4)	52%	1612	65.2 (10.4)	54%	4787

AMP-PD: Accelerating Medicines Partnership Parkinson's disease, including the Parkinson's Disease Biomarkers Program (PDBP), Parkinson's Progression Markers Initiative (PPMI) and Harvard Biomarker Study (HBS) cohorts; Barcelona: Hospital Universitari Mutua de Terrassa, Spain; CORIELL: NINDS Exploratory Trials in PD Long-Term Study 1 (NET-PD LS1), Coriell Institute for Medical Research, USA; DIGPD: Drug Interaction With Genes in Parkinson's Disease, France; LEAP: Levodopa in Early Parkinson's Disease, Netherlands; Luxemburg: Luxembourg Centre for Systems Biomedicine; Mayo Clinic Florida: Mayo Clinic Florida, USA; McGill: McGill University, Canada; Oviedo: Central University Hospital of Asturias, Spain; PreCEPT: Parkinson Research Examination of CEP-1347 Trial; SCOPA: SCales for Outcomes in PArkinson's disease; Sevillla: Universidad de Sevilla; Tartu: University of Tartu; LID-, n: individuals without levodopa-induced dyskinesia; LID+, n: individuals with levodopa-induced dyskinesia; Age (SD): mean age (standard deviation); %Mal: percentage of males; Tot: total number of individuals per cohort.

Genetic analyses

Excluding the AMP-PD cohorts, with whole genome sequencing (WGS) data, the other centers were genotyped using the OmniExpress, NeuroX, NeuroChip or MegaChip GWAS array according to the manufacturer's instructions (Illumina Inc.). Quality control was performed following standard pipelines (detailed in https://github.com/neurogenetics/GWAS-pipeline) using plink 1.9. [46] In brief, we filtered out heterozygosity outliers using an F-statistic cut-off of <-0.15 or >0.15. Individuals with a variant call rate <95% and sex mismatch were excluded. Variants missing in >5% of the participants, with disparate missingness between cases and controls (p<1E-04), or significantly deviating from Hardy-Weinberg equilibrium in controls (p<1E-04) were also removed. We used GCTA to check for relatedness closer than first cousins between participants (pihat>0.125). We performed imputation using the Michigan imputation server (https://imputationserver.sph.umich.edu/index.html#) with the Haplotype Reference Consortium reference panel r1.1 2016 under default settings. Ancestry outliers were detected using HapMap3 principal component analysis (PCA) data in R version 4.0.1.

After imputation, we selected variants with r2>0.8 and a minor allele frequency (MAF)>0.05, while retaining risk variants in the *GBA1* (p.N370S, p.E326K and p.T369M) and *LRRK2* (p.G2019S, p.M1646T and p.R1441G/C) regions, to perform specific analyses on these variants (detailed below). These genes were specifically selected given their importance in PD etiology[25, 26] and recent clinical trials[47] as well as their previously suggested association with LID. [27-32] The carrier status of *GBA1/LRRK2* risk variants in individuals with and without LID is detailed in Table 2 and Table 3.

Table 2 - Carriers of *GBA1* variants across different cohorts

Center	*GBA1* carriers LID-, N/tot	*GBA1* carriers LID-, %	*GBA1* carriers LID+, N/tot	*GBA1* carriers LID+, %
Barcelona	3/103	2.90%	1/48	2.10%
CORIELL	29/221	13.10%	20/117	17.10%
DIGPD	3/220	1.40%	7/166	4.20%
LEAP	38/336	11.30%	12/75	16.00%
Luxembourg	6/330	1.80%	4/140	2.90%
Mayo Clinic Florida	6/404	1.50%	2/151	1.30%
McGill	18/258	7.00%	15/120	12.50%
Oviedo-HUCA	2/80	2.50%	2/110	1.80%
PDBP - PPMI - HBS (AMP-PD)	34/580	5.90%	7/87	8.00%
PreCEPT	17/181	9.40%	12/137	8.80%
SCOPA	14/109	12.80%	32/177	18.10%
Sevilla	7/180	3.90%	25/252	9.90%
Tartu	7/173	4.00%	3/32	9.40%
TOTAL	184/3175	5.80%	142/1612	8.80%

Carrier status for *GBA1* variants p.N370S, p.E326K and p.T369M. *GBA1* carriers LID-, N/tot: carriers of *GBA1* variants without LID out of all patients without LID; *GBA1* carriers LID+, N/tot: carriers of *GBA1* variants with LID out of all the patients with LID; *GBA1* carriers LID-, %: percentage of carriers of *GBA1* variants without LID; *GBA1* carriers LID+ , %: percentage of carriers of *GBA1* variants with LID

Table 3 – Carriers of *LRRK2* variants across different cohorts

Center	*LRRK2* carriers LID-, N/tot	*LRRK2* carriers LID-, %	*LRRK2* carriers LID+, N/tot	*LRRK2* carriers LID+, %
Barcelona	2/103	1.90%	0/48	0.00%
CORIELL	12/221	5.40%	12/117	10.30%
DIGPD	10/220	4.50%	13/166	7.80%
LEAP	16/336	4.80%	3/75	4.00%
Luxembourg	21/330	6.40%	13/140	9.30%
Mayo Clinic Florida	22/404	5.40%	5/151	3.30%
McGill	14/258	5.40%	9/120	7.50%
Oviedo-HUCA	4/80	5.00%	8/110	7.30%
PDBP – PPMI – HBS (AMP-PD)	21/580	3.60%	2/87	2.30%
PreCEPT	11/181	6.10%	8/137	5.80%
SCOPA	7/109	6.40%	8/177	4.50%
Sevilla	11/180	6.10%	27/252	10.70%
Tartu	6/173	3.50%	0/32	0.00%
TOTAL	157/3175	4.90%	108/1612	6.70%

Carrier status for *LRRK2* variants p.G2019S, p.M1646T and p.R1441G/C. *LRRK2* carriers LID- , N/tot: carriers of *LRRK2* variants without LID out of all patients without LID; *LRRK2* carriers LID+ , N/tot: carriers of *LRRK2* variants with LID out of all patients with LID; *LRRK2* carriers LID- , %: percentage of carriers of *LRRK2* variants without LID; *LRRK2* carriers LID+ , %: percentage of carriers of *LRRK2* variants with LID

To examine the association between the *GBA1* and *LRRK2* risk variant carrier status and LID occurrence we performed logistic regression, and to evaluate the association between the carrier status and time to LID onset we performed Cox regression using the R package "survival" (https://cran.r-project.org/web/packages/survival/). The time to LID variable included in the Cox regression was defined as the period between the start of levodopa therapy and LID onset, as previously done. [48] When LID did not manifest, this parameter was right-censored at the last follow-up. We adjusted the analyses by multiple covariates including principal components (PCs), PD AAO, sex, levodopa dosage, levodopa equivalent daily dose (LEDD), [49, 50] dopamine agonist use, BMI, Hoehn and Yahr score (HY) and, exclusively for

logistic regression, disease duration. In logistic regression, we included the cumulative levodopa dosage and LEDD starting from the baseline (i.e., levodopa initiation) to the last time point (i.e., LID onset or last follow-up when LID was not present). In Cox regression, to avoid collinearity with the time to LID onset dependent variable, we replaced cumulative doses with doses at the last time point. All the covariates were selected using an Akaike Information Criterion (AIC)-based stepwise regression approach, which evaluated the model goodness of fit and selected the most appropriate covariates to include in the model. We performed the analyses separately in each cohort and then meta-analyzed the results using the R package "metafor" (https://cran.r-project.org/web/packages/metafor/index.html). Since variants in these genes have been previously associated with LID, we used a significance threshold of α=0.05.

Similar to the analyses on specific genes, to investigate the overall impact of genetics on LID risk and time to onset we also performed GWAS with, respectively, logistic and Cox regression adjusted for the above-specified covariates. Cox regression was performed using the SurvivalGWAS_SV software (https://www.liverpool.ac.uk/population-health/research/groups/statistical-genetics/survival-gwas-sv/).[51] We conducted the analyses in each cohort separately, and then meta-analyzed the results using METAL software (https://genome.sph.umich.edu/wiki/METAL_Documentation) with a fixed effects model weighted by β coefficients and the inverse of the standard errors.

PD risk variant-based polygenic risk score

To assess the impact on LID of the cumulative genetic risk for PD we calculated polygenic risk score (PRS) for each PD patient including the 90 variants associated with PD in the most recent GWAS in Europeans.[52] PRS calculation was performed based on the weighted allele dose as implemented in PRSice2 (https://choishingwan.github.io/PRSice/).[53] To investigate the association between the PRS and LID risk we performed logistic regression, while to evaluate

the association between PRS and progression to LID we performed Cox regression. The analyses were adjusted for PCs, PD AAO, sex, HY and levodopa dosage, cumulative in logistic regression and at the last time point in Cox regression. These analyses were repeated using PRS as a continuous variable and then as a discrete variable by dividing the PRS into quartiles. For the analysis using PRS quartiles, we separately compared the association of individual membership to the second, third and fourth quartiles vs the first quartile with LID risk/progression.

Dopamine pathway polygenic risk score

To assess the impact of genes involved in the dopaminergic transmission pathway we also constructed a pathway polygenic risk score, or polygenic effect score (PES) [54] using the PRSet feature of PRSice2 (https://choishingwan.github.io/PRSice/prset_detail/). Genes involved in this pathway were obtained from Explore the Molecular Signatures Database (MSigDB), a collection of annotated gene sets for use with Gene Set Enrichment Analysis (GSEA) software (https://www.gsea-msigdb.org/gsea/msigdb/). These genes included *CDK5, FLOT1, PARK7, CHRNB2, ADORA2A, CRH, CRHBP, DRD1, DRD2, DRD3, DRD4, DRD5, TOR1A, RASD2, PNKD, GDNF, ARRB2, PRKN, PTGS2, RAB3B, PINK1, SLC6A2, SLC, 6A3, SLC6A4, SNCA, TH, CNTNAP4* (detailed at http://www.gsea-msigdb.org/gsea/msigdb/human/geneset/GOBP_SYNAPTIC_TRANSMISSION_DOPAMINERGIC). To select the variants in each of those genes to include in the analyses we used the LID GWAS meta-analysis summary statistics, filtering variants with a p-value less than or equal to 0.05. In addition, we performed linkage disequilibrium (LD) clumping using the default r2=0.1 and selecting variants at 250 Kb of distance from the pathway-related genes. 1000 permutations were implemented to generate the empirical p-value corresponding to the optimized PES prediction of the dependent variable in the target cohort. We then calculated individual PES for each target cohort. To avoid potential inflation due to the presence of the

target cohort in the meta-analysis summary statistics, each time we calculated the PES for a target cohort we excluded such cohort from the meta-analysis using a leave-one-out approach. To investigate the association between the dopamine pathway PES and LID risk we performed logistic regression, while to evaluate the association between the PES and progression to LID we performed Cox regression, as specified above for the PRS analyses.

Results

GBA1 and LRRK2 variants show significant associations with LID risk and time to LID

Analyses focusing on *GBA1* showed that *GBA1* variants were significantly associated with LID risk (OR=1.65, 95% CI=1.21-2.26, p=0.0017, Fig. 1A). No association was found with time to LID (HR=1.25, 95% CI=0.99-1.58, p=0.0635, Fig. 1B). In contrast, *LRRK2* variants showed no association with LID risk (OR=1.18, 95% CI=0.84-1.67, p=0.3484, Fig. 2A) but were significantly associated with reduced time to development of LID (HR=1.42, 95% CI=1.09-1.84, p=0.0098, Fig. 2B)

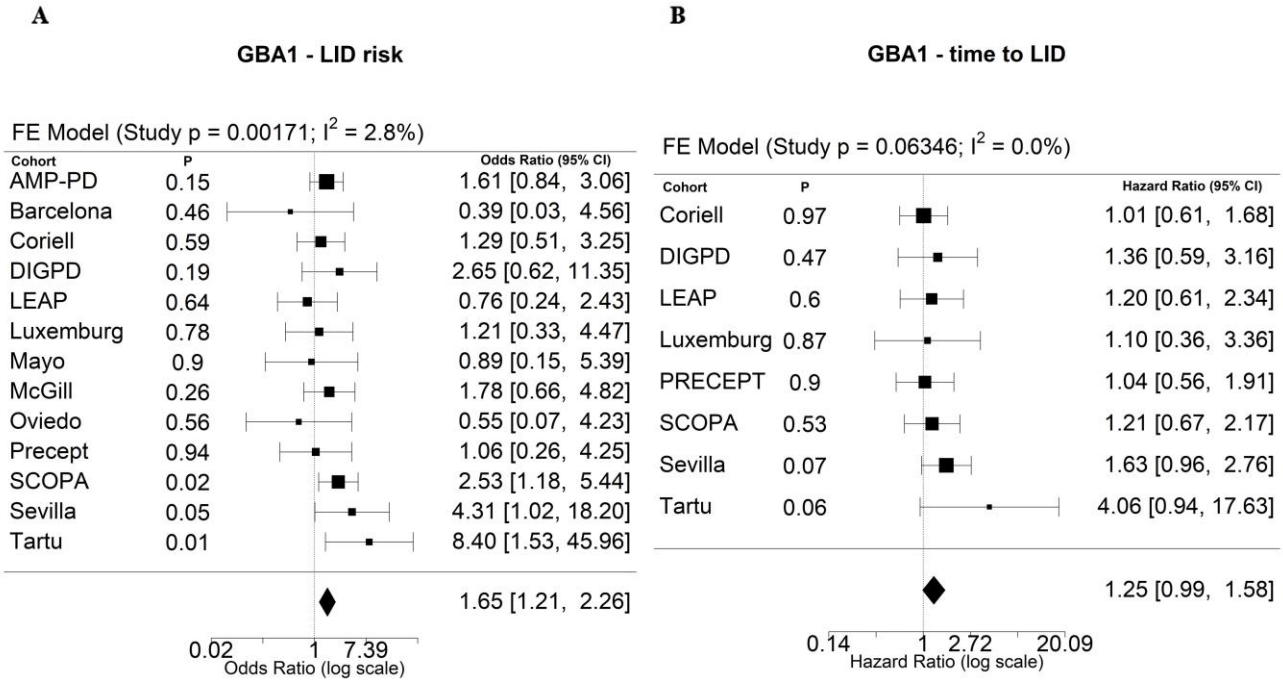

Fig. 1 A-B: Association between *GBA1* variants and LID

The meta-analysis forest plot shows the coefficient (black squares) and 95% confidence interval (bars) of the analyses in each single cohort. The size of the square is proportional to the weight the cohort had on the overall meta-analysis, based on their single standard error. The black diamond at the bottom represents the overall coefficient and confidence interval. **A**. Logistic regression between *GBA1* variants and LID risk; **B**. Cox regression between *GBA1* variants and time to development of LID.

FE: fixed effect model; AMP-PD: Accelerating Medicines Partnership Parkinson's disease, including the New Discovery of Biomarkers (BioFIND), the Harvard Biomarker Study (HBS) and the Parkinson's Disease Biomarkers Program (PDBP) cohorts; Barcelona: Hospital Universitari Mutua de Terrassa, Spain; CORIELL: NINDS Exploratory Trials in PD Long-Term Study 1 (NET-PD LS1), Coriell Institute for Medical Research, USA; DIGPD: Drug Interaction With Genes in Parkinson's Disease, France; LEAP: Levodopa in Early Parkinson's Disease, Netherlands; Luxemburg: Luxembourg Centre for Systems Biomedicine; Mayo: Mayo Clinic, USA; McGill: McGill University, Canada; Oviedo: Central University Hospital of Asturias, Spain; PreCEPT: Parkinson Research Examination of CEP-1347 Trial; SCOPA: SCales for Outcomes in PArkinson's disease; Sevilla: Universidad de Sevilla; Tartu: University of Tartu

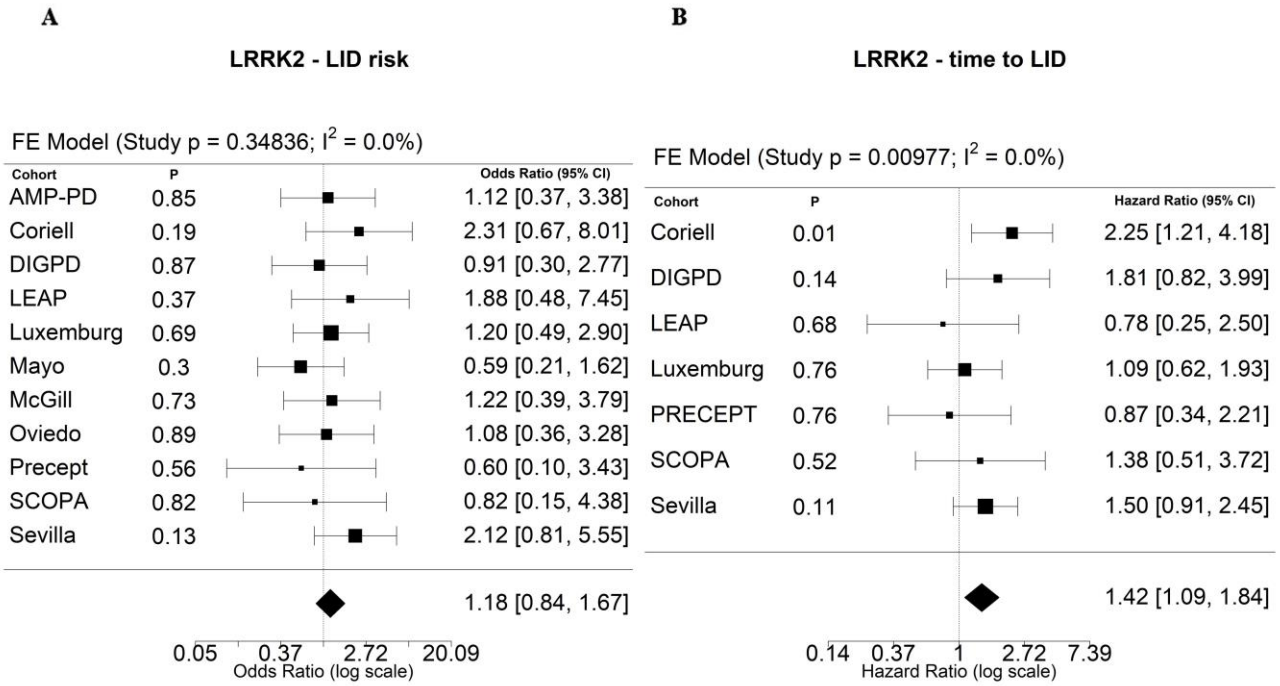

Fig. 2 A-B: Association between *LRRK2* variants and LID

A. Logistic regression between *LRRK2* variants and LID risk; **B**. Cox regression between *LRRK2* variants and time to development of LID.

In the GWAS genomic inflation was evaluated using quantile-quantile plots (Q-Q plots) and the lambda factor, showing no inflation and a slight deflation (lambda logistic regression=0.9709, lambda Cox regression=0.9555, Supplementary Fig. 1-2). GWAS using both logistic and Cox regression showed no significant association with LID risk or time to development of LID, respectively (Supplementary Fig. 3, Supplementary Fig. 4). We further examined whether variants previously associated with LID in the literature[14-18, 21-24] and from the LIDPD website (http://LiDpd.eurac.edu/) showed associations in the current GWAS, but we found no significant results (Supplementary Tables 2-3). A recent GWAS in LID (Martinez et al., 2023, MedRxiv) nominated significant signals in a progression GWAS meta-analysis. However, our study failed to confirm these findings despite the larger sample size. In addition,

the variants nominated by this recent progression GWAS did not reach the nominal significance of 0.05 in our GWAS (Supplementary Table 4).

PD risk variant-based polygenic risk score is associated with increased risk for LID

PRS analyses aggregating PD-associated variants showed that higher values of PRS were associated with a very mild increase in LID risk (OR=1.02, %95 CI=1.002-1.035, p=0.0298, Fig. 3B). When dividing the PRS in quartiles, logistic regression showed a significant association between the fourth quartile and LID, with a greater risk compared to the analyses using PRS as a continuous variable ($OR_{fourth\_quartile}$=1.27, 95% CI=1.03-1.56, p=0.0210, Fig. 3A, Supplementary Table 5). Cox regression did not show any significant associations between PRS and time to development of LID (Supplementary Fig. 5 A-B, Supplementary Table 6).

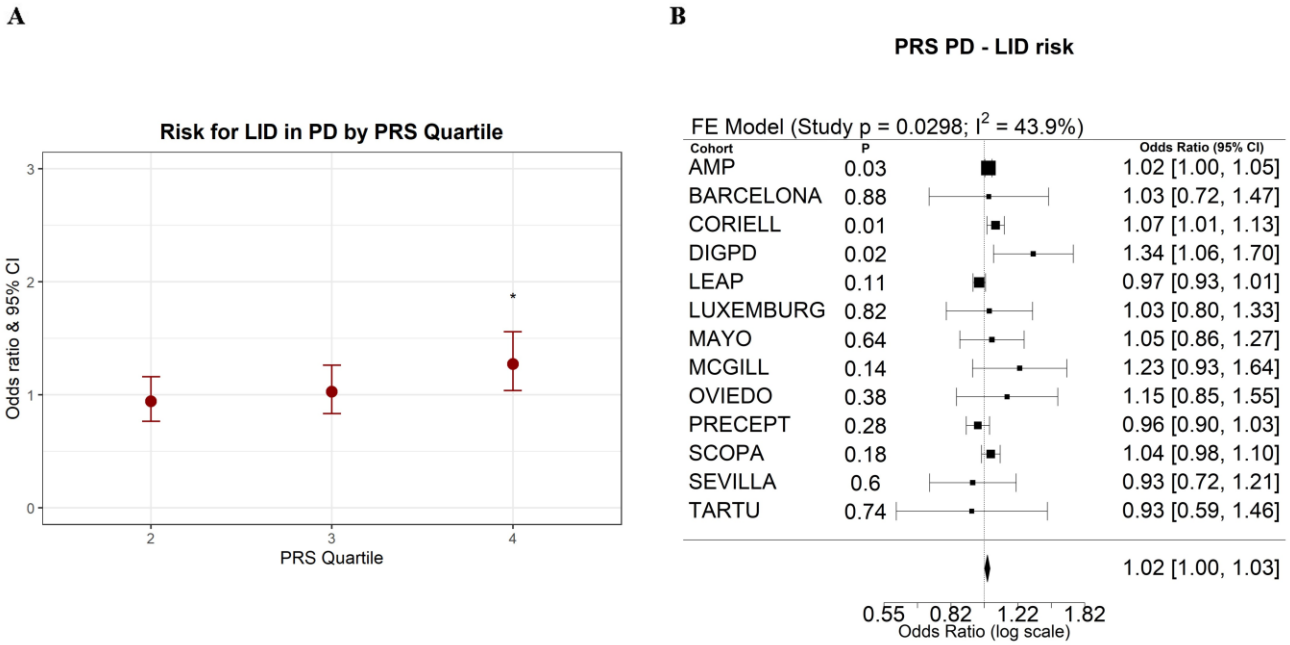

Fig. 3 A-B: Logistic regression between PRS aggregating PD risk variants and LID risk

A. The plot shows the association between each PRS quartile and LID risk compared with the first quartile, meta-analyzing the results across the cohorts. The Y axis represents the PRS quartile, the X axis the odds ratio (red dot) and 95% confidence interval (red bar). The presence

of an asterisk indicates a significant association (p<0.05). **B.** The forest plot shows the association between PRS as a continuous variable and LID risk.

CI: confidence interval.

Dopaminergic transmission pathway polygenic effect score is associated with a reduced time to development of LID

Analyses on the dopaminergic transmission pathway PES showed that higher values of PES were associated with a reduced time to development of LID ((HR=1.10, , 95% CI=1.02-1.18, p=0.0088, Fig. 4B). In addition, the third and fourth PES quartile were also associated with a reduced time to development of LID with a more elevated effect size compared to the analyses on PES as a continuous variable ($HR_{third\_quartile}$=1.38, 95% CI=1.07-1.79, p=0.0128; $HR_{fourth\_quartile}$=1.38, 95% CI=1.06-1.78, p=0.0147, Fig. 4A, Supplementary Table 8). Logistic regression did not show any statistically significant associations between dopaminergic transmission PES and LID risk (Supplementary Fig. 6 A-B, Supplementary Table 7).

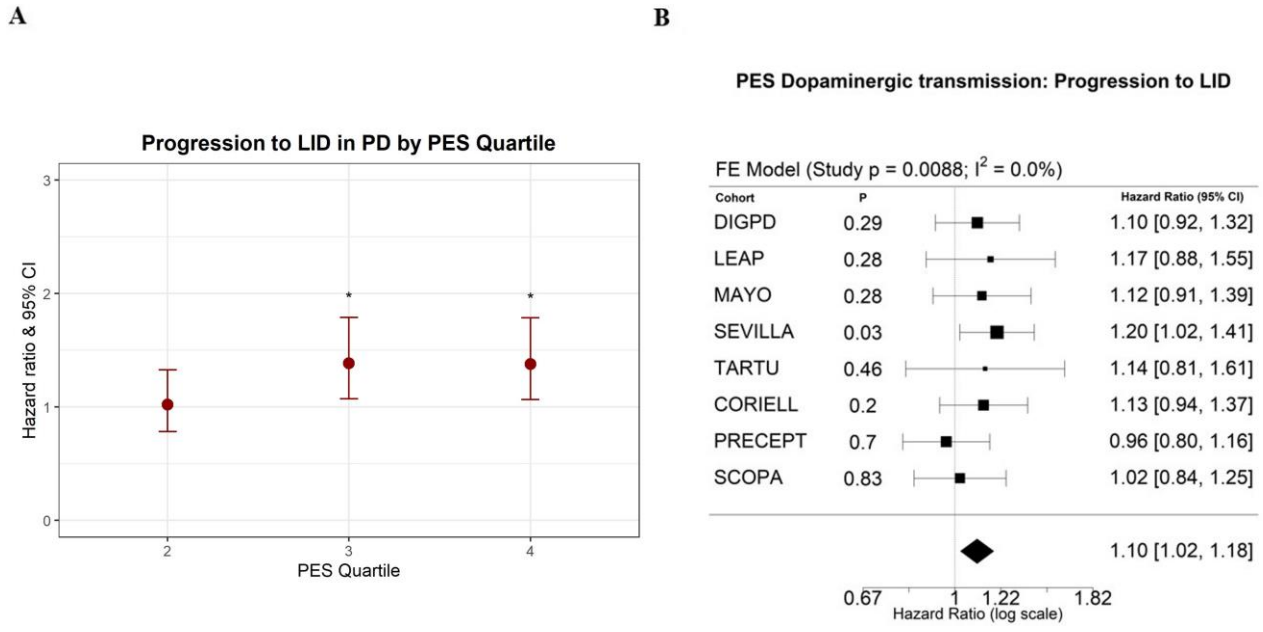

Fig. 4 A-B: Cox regression between the dopaminergic transmission pathway PES and time to development of LID

A. The plot shows the association between each PES quartile and time to development of LID compared with the first quartile, meta-analyzing the results across the cohorts. The Y axis represents the PRS quartile, the X axis the hazard ratio (red dot) and 95% confidence interval (red bar). **B.** The forest plot shows the association between PES as a continuous variable and time to development of LID.

Discussion

In this study, we confirmed that *GBA1* variants were associated with increased risk for LID and demonstrated that *LRRK2* variants were associated with a reduced time to development of LID. Additionally, we found that PD PRS was associated with mildly increased risk for LID and that the dopaminergic transmission pathway PES is associated with a reduced time to development of LID.

Albeit some studies found contradictory results on the association between the *GBA1* and *LRRK2* variants and LID, [39-42] many others have shown that these variants play a role in LID development, [27-32] and in this study we also demonstrated that *LRRK2* variants might also affect the time to development of LID. The absence of significant signals in the risk and progression GWAS and, in general, the difficulty finding congruent results between different genetic studies investigating LID, as also reflected by the divergent results between the recent LID progression GWAS (Martinez et al., 2023, MedRxiv) and our study, may be due to the stronger contribution in LID development of environmental factors, especially pharmacologic- (dosage of dopaminergic drugs, use of amantadine…) and disease-related factors. [9-13]

The significant association between the two PRS analyses suggests that aggregating multiple common variants that might have a scarce effect on LID individually could contribute to uncovering the overall genetic impact on LID. In particular, the association between the PRS including PD risk variants suggests that patients with a stronger genetic risk profile for PD are

also more at risk for LID, a factor to consider for patient counselling and potential clinical trials, although the magnitude of the increased risk was small. We also demonstrated that the dopaminergic synaptic transmission pathway PES was associated with an increased rate of LID development, which is in line with previous pathophysiologic hypotheses[6-8] and studies suggesting an implication of dopamine pathway genes in the development of LID. [14-18, 21-24]

Unravelling the etiologic bases of LID is crucial to implement a tailored therapy for PD patients taking levodopa, adapting the therapeutic choices, dosage and management depending on the individual risk factors of each patient. Over time, it could be beneficial to define a risk profile accounting for the single genetic and environmental factors associated with LID as well as the cumulative genetic risk provided by the PRS. This might lead to a more refined and personalized therapeutic approach for each individual. In addition to the benefits of the current symptomatic therapies, uncovering and confirming genetic factors affecting the risk and time to development of LID could also have important implications for targeted therapies. In particular, *GBA1* and *LRRK2* pathways are already candidate targets for newly developing drugs in clinical trials. [47] A LRRK2 inhibitor, BIIB122/DNL151, reached already experimental phase 3 (https://www.denalitherapeutics.com, 2021). [55] In addition, Ambroxol, a pharmacological chaperone for GCase capable of increasing its enzymatic levels, reached phase 2 and LTI-291, an activator of GCase, reached phase 1B. [56, 57] As these drugs would likely be used in conjunction with symptomatic therapies, knowing that these pathways can be targeted to reduce the risk or delay the time of LID development could considerably improve the compliance and quality of life of PD patients taking dopaminergic treatments.

The current study has several limitations. First, the subjects were all of European ancestry and therefore the results in other population might be different. Despite an overall large sample size, most of the individual cohorts included a limited number of participants, especially those having longitudinal data necessary for Cox regression, this impacted the power

of the study and could have contributed to the lack of association in the GWAS. Some studies suggested that LID is affected more by the disease duration than by the therapy duration, [58] on this line PD AAO would represent a better baseline than levodopa initiation for the time to LID onset. However, this parameter was chosen in accordance with what was previously done with LID GWAS[48] and accounting for the recall bias that PD AAO suffers from, compared to levodopa initiation which represents a report made by the physicians. In addition, understanding the genetic basis of the time to LID from levodopa initiation can be of considerable relevance for patient counselling at the time of treatment administration. Another limitation of this study was that not all the cohorts had the same amount of data available, which limited in part the design of the analytical model.

In conclusion, in the current study we demonstrated that PD risk variants and the dopaminergic transmission PRS are associated with increased risk of LID/time to development of LID. A better understanding of the role of genetics in LID development could reduce the impact of this adverse effect and enhance therapeutic management in PD.

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The authors declare that there are no additional disclosures to report.

Ethical Compliance Statement

IRB Study Number A11-M60-21A (21-11-023) was reviewed and approved by the Research Ethics Offices (REOs). Informed written patient consent was provided in each center before the inclusion of each in the study. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this work is consistent with those guidelines

Data availability

The LID GWAS summary statistics are publicly available on GWAS catalog (https://www.ebi.ac.uk/gwas/). All codes used for the analyses are available at https://github.com/gan-orlab.

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PREFACE TO CHAPTER 5

In the previous chapter, I showed that the PD PRS is associated with LID risk and the dopaminergic transmission pathway with time to LID. I also demonstrated the *GBA1* variants are associated with increased risk for LID and *LRRK2* variants with an increased rate of progression to LID, suggesting a role of these genes as potential targets not only for PD overall but also in the context of dopaminergic symptomatic therapy.

In the following chapter, I further explore the relationship between these two genes in view of the new therapeutics under clinical trials directed toward *GBA1* and *LRRK2* pathways. In particular, I test the association between *LRRK2* variants and GCase activity in peripheral blood, showing an association with increased activity. Biologically speaking, the relationship between LRRK2 kinase and GCase means that therapy targeted toward a pathway might need to test the effects on the other one and their clinical implications.

CHAPTER 5: LRRK2 p.M1646T IS ASSOCIATED WITH GLUCOCEREBROSIDASE ACTIVITY AND WITH PARKINSON'S DISEASE

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Abstract

The LRRK2 p.G2019S Parkinson's disease (PD) variant is associated with elevated glucocerebrosidase (GCase) activity in peripheral blood. We aimed to evaluate the association of other *LRRK2* variants with PD and its association with GCase activity. *LRRK2* and *GBA* were fully sequenced in 1,123 PD patients and 576 controls from the Columbia and PPMI cohorts, in which GCase activity was measured in dried blood spots by liquid chromatography-tandem mass spectrometry. LRRK2 p.M1646T was associated with increased GCase activity in the Columbia University cohort (β=1.58, *p*=0.0003), and increased but not significantly in the PPMI cohort (β=0.29, *p*=0.58). p.M1646T was associated with PD (OR=1.18, 95%CI=1.09-1.28, *p*=7.33E-05) in 56,306 PD patients and proxy-cases, and 1.4 million controls. Our results suggest that the p.M1646T variant is associated with risk of PD with a small effect and with increased GCase activity in peripheral blood.

Introduction

Parkinson's disease (PD) is mostly caused by an interaction between genetic and environmental factors [1]. Variants in *GBA* and *LRRK2* are among the most common genetic risk factors of PD [2, 3]. The frequency of these variants varies in different populations, with *GBA* variants found in 5-20% [2] and *LRRK2* variants reported in 1-40% of PD patients [4].

The activity of the enzyme encoded by *GBA*, β-glucocerebrosidase (GCase), is reduced in carriers of *GBA* variants, but also in a subset of PD patients without *GBA* variants [5, 6]. There are contradicting results regarding the effect of the LRRK2 p.G2019S variant on GCase activity. In peripheral blood, this variant was associated with an increased activity [6], whereas in patient-derived dopaminergic neurons with *LRRK2* variants GCase activity was reduced [7]. GCase protein level, as studied in dopaminergic neurons, was not affected in carriers of *LRRK2* pathogenic variants [7]. A variant in *TMEM175*, p.T393M, has been associated with reduced GCase activity and, together with *GBA* variants and the LRRK2 p.G2019S variant, explain only 23% of the variance in GCase activity in peripheral blood [8]. These observations suggest that other genetic or environmental factors affect GCase activity.

In the current study, we performed full sequencing of *LRRK2* and *GBA* and examined the effect of common *LRRK2* variants on GCase activity in peripheral blood in two cohorts: from Columbia University and from the Parkinson's Progression Markers Initiative (PPMI). We further examined the association of *LRRK2* variants identified through this analysis with risk of PD using data from the International Parkinson's Disease Genomics Consortium (IPDGC), UK biobank and 23andMe, Inc. genome-wide association study (GWAS) meta-analysis [9].

Materials and methods

Study population

To analyze the effects of *LRRK2* variants on GCase activity, two cohorts were included: 1) The Columbia University cohort (n=1,229, PD=797, Controls=432) and 2) The PPMI cohort (n=470, PD=326, Controls=144). Both cohorts have been previously described [6, 10], and their demographic data is detailed in Table 1. The Columbia cohort consisted of patients and controls of mixed ethnicity (mainly of European origin, including 308 individuals of Ashkenazi Jewish descent). Data on the effect of the LRRK2 p.M1646T variant on risk of PD was extracted from the recent PD GWAS, including 37,688 PD patients, 18,618 UK Biobank proxy-cases and 1.4 million control [9]. All PD patients were diagnosed by movement disorder specialists according to the UK brain bank criteria [11] or the MDS clinical diagnostic criteria [12].

Table 1. Demographic data of the cohorts to study *LRRK2* effect on GCase activity.

Cohort	PD	Controls	PD age (mean, SD in years)	Controls age (mean, SD in years)	PD males (N, percentage)	Controls males (N, percentage)
Columbia	797	432	65.80 (11.04)	64.75 (9.94)	512 (64%)	116 (27%)
PPMI	326	144	60.12 (9.67)	61.37 (10.91)	216 (66%)	100 (69%)

PD, Parkinson's disease; SD, standard deviation; Columbia, cohort from Columbia University, NY; PPMI, Parkinson's Progression Markers Initiative cohort.

Standard Protocol Approvals, Registrations, and Patient Consents

The institutional review boards approved the study protocols, and informed consent was obtained from all participants before entering the study. 23andMe participants provided informed consent and participated in the research online, under a protocol approved by the external AAHRPP-accredited IRB, Ethical & Independent Review Services (E&I Review).

Genetic analysis

LRRK2 and GBA Sequencing in the Columbia University cohort

We performed full sequencing of *LRRK2* and *GBA* in the Columbia University cohort using targeted sequencing with Molecular Inversion Probes (MIPs) and Sanger sequencing as previously described [13-15]. The full protocol and the library of MIPs used for sequencing *LRRK2* and *GBA* are available online (https://github.com/gan-orlab/MIP_protocol). A standard quality control protocol was performed as previously described [16], and the code is available at https://github.com/gan-orlab/MIPVar/.

Genetic data from PPMI and IPDGC

Due to the alignment difficulties with *GBA*, data on *GBA* variants in the PPMI cohort were extracted from combined data including whole genome sequencing data, whole exome sequencing data and RNA-seq as previously reported [10]. Data on LRRK2 p.G2019S, p.M1646T, p.N551K-p.R1398H and p.N2081D were extracted from imputed GWAS data (Illumina Immunochip and NeuroX arrays) downloaded from the PPMI project website (https://ida.loni.usc.edu/). To examine the association of *LRRK2* variants with PD, we extracted data from the recent PD GWAS meta-analysis [9].

GCase activity

Dried blood spots (DBS) were obtained as previously described [17, 18]. DBS in the Columbia cohort were prepared from fresh blood [6]. GCase activity was measured in participants from Columbia University at Sanofi laboratories by liquid chromatography-tandem mass spectrometry (LC-MS/MS) from DBS, as a part of multiplex assay with four additional lysosomal enzymes as previously described [6, 19]. PPMI study participants donated blood on the first visit (baseline) and every year, which was frozen and stored in -80C freezer. Samples from

the first three years of the cohort were thawed, and DBS were obtained. Activity was measured as previously described, using the mean GCase activity for each participant across all visits [10].

Statistical Analysis

Linear regression models were used to test for association between common *LRRK2* variants with minor allele frequency (MAF) >1% and GCase activity in the Columbia and PPMI cohorts, adjusting for age, sex, PD status, *GBA* status and ethnicity. In the PPMI cohort additional adjustment for white blood cells count was performed as suggested previously [10]. We then repeated the analysis after excluding LRRK2 p.G2019S, p.M1646T, protective haplotype carriers (tagged by p.R1398H) and *GBA* variants carriers in both Columbia and PPMI cohorts. In addition, to examine whether there are sex-specific effects, we performed additional analyses stratifying the cohorts by sex (code available at https://github.com/gan-orlab/LRRK2_GCase). Bonferroni correction for multiple comparisons was applied as needed. Finally, we evaluated differences in GCase activity between carriers and non-carriers of rare *LRRK2* variants with MAF < 1% in the Columbia cohort. To test the association between GCase activity and *LRRK2* rare variants, t-test was performed. The pathogenicity of such variants was estimated using ClinVar and Varsome annotation [20, 21]. All statistical analyses were performed using R version 3.6.3 or PLINK version 1.9 [22, 23].

Results

In the Columbia University cohort, we identified 26 *LRRK2* common variants with MAF >1% (Supplementary Table 1), including 9 nonsynonymous variants, 12 intronic variants and 5 synonymous variants.

The LRRK2 p.M1646T variant was associated with increased GCase activity compared to non-carriers (12.65 mmol/l/h vs. 11.38 mmol/l/h, respectively, β=1.58, p=0.0003, Table 2, Supplementary Table 1) in the Columbia University cohort.

Table 2. Impact of *LRRK2* variants on GCase activity.

LRRK2 variants	N of carriers	Estimate	SE	p-value*	GCase_mean	Gcase_SD
Columbia cohort						
PD + controls (N=1229)						
p.R1398H	204	0.521	0.233	0.026	11.960	3.804
p.M1646T	58	1.578	0.431	0.0003	12.652	4.529
p.G2019S	61	1.370	0.438	0.0018	12.798	4.340
PD (N=797)						
p.R1398H	123	0.495	0.298	0.097	11.776	3.863
p.M1646T	36	1.736	0.528	0.0011	13.078	4.600
p.G2019S	57	1.440	0.450	0.0014	12.877	4.471
Controls (N=432)						
p.R1398H	81	0.755	0.383	0.050	12.240	3.720
p.M1646T	22	1.367	0.746	0.068	11.956	4.425
p.G2019S	4	-0.030	1.719	0.986	11.658	1.286
PPMI cohort						
PD + controls (N=470)						
p.R1398H	61	-0.724	0.340	0.034	10.936	2.961
p.M1646T	23	0.295	0.543	0.587	12.717	3.510
p.G2019S	6	0.004	1.050	0.997	11.453	2.648
PD (N=326)						
p.R1398H	41	-0.893	0.406	0.028	10.445	3.045
p.M1646T	17	0.073	0.623	0.907	12.385	3.259
p.G2019S	6	-0.078	1.037	0.940	11.453	2.648
Controls (N=144)						
p.R1398H	20	-0.240	0.622	0.701	11.941	2.564
p.M1646T	6	1.169	1.082	0.282	13.657	4.333

SE, standard error; N, number; GCase_mean, mean glucocerebrosidase activity, μmol/l/h; SD, standard deviation; PD, Parkinson's disease; Columbia, cohort from Columbia University, NY; PPMI, Parkinson's Progression Markers Initiative cohort; *, Bonferroni correction significance threshold for Columbia cohort (α=0.05/26=0.0019) and (α=0.05/5=0.01) for PPMI cohort.

The effect of p.M1646T on GCase activity was stronger in PD (GCase=13.08 mmol/l/h, β=1.74, p=0.0011) and did not reach statistical significance in controls (GCase=11.96 mmol/l/h, β=1.37, p=0.068, Table 2, Supplementary Table 2-3). After exclusion of p.G2019S carriers, the association of p.M1646T with increased activity remained strong (GCase=12.64

mmol/l/h, β=1.73, *p*=6.24E-05, Supplementary Table 4-5). The LRRK2 p.G2019S variant was associated with increased GCase activity as previously described [6]. Two variants from the protective haplotype p.N551K-p.R1398H-p.K1423K were nominally associated with GCase activity, but this association was not statistically significant after Bonferroni correction (Table 2). When removing p.M1646T and p.G2019S from the analyses, we observed an increase both in the effect size and in the significance of the association between the protective haplotype and GCase activity (GCase=11.93 mmol/l/h, β=0.66, *p*=0.005, Supplementary Table 8), still not surpassing Bonferroni correction. Using data from the recent PD GWAS meta-analysis [9], including 37,688 PD patients, 18,618 UK Biobank proxy-cases and 1.4 million controls, we then demonstrated that the LRRK2 p.M1646T variant was associated with PD (OR=1.18 95% CI=1.09-1.28, *p*=7.33E-05).

As a replication for GCase activity, we used data from the PPMI cohort, and analyzed the association of p.R1398H (representing the protective haplotype), p.M1646T, p.G2019S and p.N2081D with GCase activity (Table 2, Supplementary Table 9). The p.M1646T variant showed the same direction of effect and similar average GCase activity value as observed in the Columbia cohort, but did not reach statistical significance, possibly due to the small number of carriers (n=23), compared to non-carriers (12.72 mmol/l/h vs. 11.84 mmol/l/h, respectively, β=0.29, *p*=0.59; Table 2). Only six carriers of the LRRK2 p.G2019S variant were included in the PPMI cohort, and the association of this variant with GCase activity, as well as of the protective haplotype, were not statistically significant after Bonferroni correction (Table 2, Supplementary Table 9). Stratified analysis by sex did not identify sex differences in GCase activity in both cohorts (Supplementary Table 6-8).

We have found 32 rare nonsynonymous variants in *LRRK2* gene with MAF < 1% in the Columbia cohort (Supplementary table 10). None of the discovered rare *LRRK2* variants were reported as pathogenic. Rare variant p.E334K, was associated with a decreased GCase activity

(Supplementary Table 10). Among the three carriers of this variant, two were PD patients. This variant has uncertain significance as reported in ClinVar. We further studied association of GCase activity in carriers of all rare variants versus non carriers and did not find any statistically significant difference (Supplementary Table 10).

Discussion

In the current study, we show that the LRRK2 p.M1646T variant is associated with PD and with increased GCase activity in peripheral blood. The association of this variants with PD has been previously demonstrated [3, 24] and we confirmed this association in a larger European cohort. Although the p-value did not reach the GWAS level of statistical significance [9], the association between p.M1646T and PD replicated in different cohorts suggests that this variant plausibly plays a role in PD development. Despite its smaller effect on PD risk compared to the LRRK2 p.G2019S variant, the effect of p.M1646T on GCase activity was larger than the effect of p.G2019S. However, since the results on GCase activity did not fully replicate in the PPMI cohort, additional studies are required to understand the associations between *LRRK2* variants, GCase activity and PD risk.

In a recent study, the *LRRK2* pathogenic variants p.G2019S, p.R1441G, and p.R1441C were associated with reduced GCase activity in patient-derived dopaminergic neurons, and correction of these variants resulted in normalization of GCase activity [7]. Conversely, in the current study, deleterious *LRRK2* variants (p.G2019S and p.M1646T) were associated with increased GCase activity in peripheral blood. There are several potential explanations for these differences in the direction of effects on GCase activity, including: a) different effects of *LRRK2* variants in the central nervous system vs. peripheral blood, b) the possibility that iPSC-derived dopaminergic neurons, which are young cells, are different than patient tissues, due to the natural aging process, and c) the different methods used to measure GCase activity.

Considering the study suggesting that *LRRK2* variants are associated with reduced GCase activity [7], drugs targeting *LRRK2* activity could be repurposed for *GBA*-PD, and drugs that target GCase activity could be used for *LRRK2*-PD. However, this potential association between *LRRK2*, *GBA* and GCase activity should be carefully studied further, since other data suggests that *LRRK2* variants are not associated with reduced GCase activity. Patients with *GBA*-PD (and thus, reduced GCase activity) have a more severe phenotype with faster disease progression and cognitive decline, depression and anxiety, compared to sporadic PD [25-27]. In contrast, *LRRK2* variants carriers have a milder phenotype with slower disease progression and lower frequency of cognitive symptoms compared to sporadic PD [28, 29]. Moreover, two independent studies demonstrated that carriers of both *LRRK2* and *GBA* variants seem to have a benign phenotype, similar to those who carry *LRRK2* variants only [30, 31] . If indeed *LRRK2* variants lead to reduced GCase activity as suggested [7], we would expect that patients with both *LRRK2* and *GBA* variants would have a severe phenotype. Instead, their phenotype is milder [30, 31], which may raise the hypothesis that the increased GCase activity we observed in peripheral blood may have some protective effect on PD phenotype. This hypothesis requires additional studies in human cohorts and disease models.

Our study has several limitations. In our cohorts, difference in sex between PD patients and controls was significant. To address this limitation, we adjusted the regression model with sex as covariate, as well as other covariates. The Columbia cohort differed from the PPMI cohort in terms of ethnicity. The PPMI cohort is predominantly European, while the Columbia cohort included individuals of mixed ethnicity, mainly of European and Ashkenazi Jewish ancestry. This was addressed adjusting the regression models for ethnicity. Due to ethnical differences, the total number of carriers of *LRRK2* variants in PPMI cohort was relatively low comparing to the Columbia cohort. Another limitation is that GCase activity was measured in blood, which does not necessarily reflect GCase activity in the brain. There were also technical

differences in sample preparation: in the Columbia cohort GCase activity was measured in DBS prepared from fresh blood and in the PPMI cohort DBS was prepared from frozen blood.

To conclude, we demonstrated that the LRRK2 p.M1646T variant is associated with increased GCase activity in peripheral blood and with increased risk of PD. The interplay between *LRRK2*, *GBA* and GCase activity should be studied in additional cohorts and relevant disease models.

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CHAPTER 6: DISCUSSION

The main goal of this thesis was to explore the genetics of PD and RBD to show how PD, in its manifest and prodromal stage, is not a single entity. We reported that ~0.3% of iRBD patients carry rare loss of function *PSAP* variants and ~0.2% of them also carry a *GBA1* variant. We also showed that PD with RBD is genetically distinct from PD without RBD. We demonstrated how the differing risk and rates of development of LID are dependent on genetic determinants, including the dopaminergic transmission pathway and genetic variants already associated with PD risk. Finally, we found that PD patients carrying *LRRK2* variants show increased GCase activity compared to non-carriers.

RBD is an ideal stage to implement neuroprotective trials for PD. It represents a window large enough to prevent precipitation of the disease course and experiments conducted in patients with RBD would be free of therapeutic confounders present in clinical PD. More than 90% of iRBD patients will convert into a synucleinopathy and our accuracy in predicting the rate and risk of conversion is progressively increasing.[133, 208] It is, however, pivotal to identify biomarkers that sharpen our ability to differentiate between the trajectories that the disease will take, as the therapeutic approach will be different for individuals who will eventually develop PD, DLB or MSA. Furthermore, albeit most of the iRBD cases will convert into a synucleinopathy, we also need to account for the minority who will not. Multiple biomarkers can serve this purpose. Abnormal alpha-synuclein in different locations has been observed in the majority of iRBD cases, including skin,[140] submandibular and salivary glands,[209, 210] and cerebrospinal fluid (CSF). CSF pathogenic alpha-synuclein, in particular, showed optimal ability to predict phenoconversion in iRBD patients.[211] Imaging can have practical and financial limitations, but can otherwise play an important role in predicting phenoconversion. Changes in glucose metabolism at the 18F-fluorodeoxyglucose (18F-FDG) PET,[212] abnormal DAT at the SPECT[136] and anomalies in the substantia nigra and motor

cortico-subcortical loop at the MRI[213, 214] have all been associated with an increased risk of phenoconversion and can also distinguish different patterns corresponding to the single synucleinopathies.[215] Clinical biomarkers emerging in the prodromal stage of synucleinopathies, including autonomic dysfunction, olfactory loss and neuropsychiatric manifestations, can also contribute to predicting risk and type of phenoconversion. For example, deterioration of cognitive performances has been associated with a higher risk to develop DLB compared to PD.[216] Genetic markers will also play a crucial role to stratify iRBD patients in relation to synucleinopathies. For example, the p.M393T variant in *TMEM175* is associated with both RBD and PD,[150] whereas the 5' *SNCA* rs10005233 variant is associated with both RBD and DLB, but not with PD.[151] *GBA1* variants, on the contrary, are associated with RBD but also with PD, DLB and, plausibly, MSA[215] and it was demonstrated a higher and faster rate of conversion in carriers of *GBA1* variants compared to non-carriers.[217] Therefore, in the future, iRBD patients could also be recruited for neuroprotective clinical trials of therapeutics targeting the *GBA1* pathway. We showed that *PSAP* loss of function mutation could play a role in iRBD. Since two of the carriers also carried a *GBA1* variant and sapC is a coactivator of GCase it is plausible that *PSAP* mutations could act as genetic modifiers of *GBA1* variants, increasing the risk to develop synucleinopathies. Previous studies, in fact, suggested that *PSAP* mutations could be also associated with PD.[94-96] If *PSAP* variants played a role only in RBD and PD they could be used as an additional biomarker to differentiate a pattern of phenoconversion directed towards PD. However, since *PSAP*-driven risk for synucleinopathies is likely intertwined with *GBA1*-driven risk, it is also arguable that *PSAP* variants could have a role in other synucleinopathies, similar to *GBA1* variants. *PSAP* mutations are rare, but as often happens with rare mutations, their impact can be substantial in disease development, especially if carried together with other pathogenic variants like those in *GBA1*. Therefore, they can represent an additional useful biomarker in iRBD that predicts risk for phenoconversion into PD. Future research will need to further explore these possibilities. While clinical,

imaging, biological, genetic and other biomarkers of synucleinopathies alone cannot predict with optimal precision the risk and pattern of phenoconversion of iRBD patients, taken together they can offer an accurate estimation of what will be the destiny of the majority of iRBD individuals and implement the most appropriate clinical trial and, potentially in the future, tailored neuroprotective plans.

When investigating the differences between PD with and without RBD, we found that the discriminating variants were in the *SNCA* and *LRRK2* regions. A previous study identified two PD subtypes based on the patterns of spreading of alpha-synuclein, body-first PD, resembling the typical Braak staging system, and brain-first PD, in which the neuropathology would start in the structures of the CNS, such as the amygdala, substantia nigra and entorhinal cortex.[57] We suggested that *LRRK2* variants could be a signature of brain-first PD, whereas the *SNCA* rs10005233 variant a signature of body-first PD. In the same study, the authors regard *SNCA* as a genetic marker bridging between brain- and body-first PD.[57] This theory also aligns with previous[151] and our own findings on the role played by *SNCA* in PD with and without RBD development. In particular, we demonstrated that the *SNCA* rs10005233 variant is associated with PD with RBD, but other two *SNCA* variants, rs356182 and rs7681154, are associated with PD without RBD. This apparent contradiction can be explained by different processes enacted by different *SNCA* variants, which translate into diverse PD phenotypes. As previously mentioned, the rs10005233 variant was demonstrated to be associated with decreased expression of SNCA-AS1 in multiple cortical regions, spinal cord and cerebellum, which arguably results in increased expression of SNCA.[152] In contrast, a previous study proposed an alternative mechanism underlying the association between PD and the 3' *SNCA* rs356182 variant. Using a model of dopaminergic midbrain neurons, this study showed that the risk allele of this variant was associated with abnormal neuron differentiation. The authors suggested that a reduced proportion of dopaminergic neurons during development might lower the threshold of neuronal loss in the substantia nigra necessary to develop PD motor

symptoms.[218] If this hypothesis were true, the dopaminergic neuron-specific effect of rs356182 would also explain why it is not associated with RBD. Additionally, differently from the rs10005233 variant and what could be expected, the rs356182 risk allele for PD was associated with reduced SNCA expression in dopaminergic midbrain neurons. In line with these findings, another study demonstrated that rs356182 was associated with decreased levels of SNCA expression in the cerebellum[219], an opposite effect to what was shown for the rs10005233 variant.[152] From a clinical standpoint, this variant showed an association with slower motor progression and the tremor-dominant PD subtype,[219] which correlates with less frequent RBD [220], cognitive decline and an overall more benign PD phenotype.[221-223] Further studies will be necessary to elucidate the different mechanisms enacted by different *SNCA* variants. Similar to rs356182, also *LRRK2* variants are associated with the tremor-dominant PD subtype.[109] This subtype shares with *LRRK2* variants the peculiarity of being associated with earlier PD AAO,[219, 224] despite otherwise benign clinical features overall.

The nominal correlation between PD with RBD and attention hyperactivity deficit disorder (ADHD) could be by chance or driven by a partially shared genetic background between the pathogenesis of PD with RBD and ADHD. A previous case report found a patient manifesting ADHD and early onset RBD [225], while an observational study showed that 60% of ADHD also showed RBD.[226] ADHD is strongly associated with greater risk to develop PD.[227-229] Similar to PD, in ADHD altered function of the dopaminergic transmission and function of midbrain dopaminergic neurons is involved. We can also observe in ADHD multiple neuropsychiatric manifestations which, as previously mentioned, are more frequent in PD with RBD than in PD without RBD, with prominent impairment of executive functions, typical of PD cognitive deficits.[227, 230] These pathophysiologic and clinical links suggest that the genetic correlation we reported in our study might not be by chance and future research will need to clarify the relationship between RBD and ADHD.

Although PD with RBD is often in comorbidity with neuropsychiatric disturbances, after correction for multiple comparisons we were not able to find any significant genetic correlation or causative association between PD with RBD and neuropsychiatric disorders. Some limitations of these analyses might explain this result. One limitation is that PD with RBD will arguably share a considerable quota of genetic architecture with PD without RBD and neuropsychiatric manifestations are present also in PD without RBD. This makes a genetic correlation between neuropsychiatric traits and PD with RBD vs PD without RBD more difficult. Another important consideration is the fact that neuropsychiatric symptoms in PD do not equate to neuropsychiatric disorders. For example, PD patients can manifest hallucinations, especially as a response to levodopa therapy or in association with dementia.[231, 232] This single manifestation will arguably have a different genetic background compared to a well-defined and complex disorder such as schizophrenia. Additionally, the genetic architecture of neuropsychiatric manifestations in PD may differ from neuropsychiatric disorders independent of PD. For example, *GBA1* and *SNCA* variants, which are risk factors for both RBD and neuropsychiatric symptoms in PD, are not risk factors for any isolated neuropsychiatric disorders. Similarly, based on the knowledge we have up-to-date, a large number of genes implicated in different neuropsychiatric disorders, such as *PCLO, CCND2, FKBP5, CRHR1* and *DTNBP1*[233-235], do not have a role in the development of neuropsychiatric manifestations in PD based on our knowledge. Another important limitation of these analyses is represented by the fact that summary statistics were downloaded from publicly available databases and thus originate from different studies performed in different centers, posing the problem of data harmonization between the cohorts. While there are multiple studies examining the genetics of cognitive decline in PD, there is a severe lack of similar studies for psychiatric manifestations. Given the burden and frequency of these symptoms in PD, performing GWAS and other large-scale genetic studies to uncover the genetic underpinnings of psychiatric manifestations in PD will be necessary.

Despite the divergence of *GBA1* and *LRRK2* variants for other subtypes of PD, variants in these genes as well as the overall PD polygenic risk score delineate PD subtypes that are more susceptible to LID. It could be argued that *GBA1* variants are not directly associated with LID, but their effect is mediated by the earlier onset and faster disease progression which characterize *GBA1*-PD[236] and represent strong risk factors for LID development.[237] While a mediation effect of these environmental determinants is virtually plausible, we adjusted our analyses by covariates that captured these aspects and, additionally, previous studies also demonstrated that the association between *GBA1* variants and LID are independent of sex, levodopa dose, disease duration and treatment duration.[190, 238] Similarly, although *LRRK2*-PD is overall more benign than *GBA1*-PD, it shows a slightly lower AAO,[109] but, as in the analyses on *GBA1* variants, we adjusted for this and other covariates. Therefore, the association of *GBA1* and *LRRK2* variants with LID is arguably independent of other known environmental risk factors. In addition, a partial mediation by other factors would still make these genes relevant for targeted therapy to manage LID. Targeting *GBA1* and *LRRK2* pathways could, in fact, be beneficial to prevent or delay LID by controlling a direct effect but also by deferring PD onset and/or slowing disease progression.

It has been suggested that the development of LID is not only a consequence of an altered dopaminergic transmission but also a reflection of aberrant plastic changes in the cortico-basal ganglia system.[239] In PD, progressive dopaminergic denervation causes alterations in the dendritic spines of the striatum. Chronic treatment with levodopa has been suggested to stimulate the production of trophic factors which would partially rescue this alteration.[240] However, these plastic changes can also lead to aberrant structural modifications that alter the transmission of dopamine and other neurotransmitters, contributing to LID development.[241] Notably, GCase interacts with BDNF, a neurotrophic factor involved in brain plasticity and also implicated in LID.[178, 242] It is, therefore, possible that *GBA1* variants might be associated with LID through this mechanism and resulting aberrant plastic changes. *LRRK2*

variants have been previously associated with alteration in intracellular trafficking and synaptic transmission,[243] these mechanisms can alter the transmission of dopamine and other potentially implicated neurotransmitters, and thus explain the association between *LRRK2* variants and reduced time to LID. Even though *GBA1* and *LRRK2* genes have been reported in LID in several studies there is still a significant lack of pathophysiologic hypothesis to justify these findings. This thesis provides potential mechanistic explanations for these associations, but future studies will need to further explore these and potential alternative hypotheses with functional studies.

Along with LID, DRT has been associated also with multiple psychiatric manifestations, including impulse control disorder and psychotic symptoms in response to levodopa. In particular, variants in the *DRD3* gene have been implicated in both LID and impulse control disorder.[180, 244] Similarly, *ANKK1* and *SLC6A3* have been implicated in both LID and hallucinations.[179, 182, 245, 246] *ANKK1* encodes PKK2, a kinase that regulates several signal transduction pathways, including gene transcription. Among the genes modulated by *ANKK1,* we find the dopamine receptor *DRD2*.[247] As in LID, it is possible to observe a role of genes involved in the dopaminergic pathway also in the development of psychiatric manifestations, suggesting that this pathway might contribute to defining a specific PD subtype responding more adversely to levodopa. We demonstrated the role in time to LID development of the dopamine transmission pathway, which we selected due to its connection with the dominant pathophysiologic hypothesis in LID. While we used a standard pathway from the MSigDB collection (http://www.gsea-msigdb.org/gsea/msigdb/human/geneset/GOBP_SYNAPTIC_TRANSMISSION_DOPAMINERGIC), one potential limitation is that three genes included in the pathway are also implicated in PD, namely *SNCA, PRKN* and *PINK1.* The latter two have also been previously implicated in LID.[248, 249] It may be argued that the association between LID and the dopaminergic pathway is not different from the association it has with the other PD risk variants, and that the latter

drive the association. However, we were not able to find an association between the PD PRS and time to LID as we did for the dopaminergic pathway PRS. Furthermore, the effect size in the association between the PD PRS and LID risk is extremely small. Finally, the GWAS did not show any statistically significant association between variants in *SNCA, PRKN* and *PINK1* with LID risk or time to LID. So, although we cannot exclude that these variants drive the association more due to a role in PD than in the dopaminergic transmission pathway, this possibility is unlikely. The finding of a role of the dopaminergic pathway in LID is in line with the predominant pathophysiologic hypothesis and could have important implications for the management of DRT, especially given that this relies on the dopaminergic pathway. A different type of therapeutic approach might be implemented in the future in patients who carry dopaminergic pathway-related variants, including the use of different pharmacologic or non-pharmacologic treatments, combined therapy to enable a reduction of levodopa dosage and continuous administration of levodopa to enhance the control of levodopa plasmatic concentration, thus preventing pulsatile stimulation of post-synaptic receptors. Similarly, also PD risk variants associated with LID can contribute to guiding therapeutic management.

An important limitation of this and also previous studies on LID is the lack of information about the subtypes of LID. Similar to PD, LID is an umbrella term encompassing three different entities: peak-dose, off-period and diphasic dyskinesia.[174] These subtypes are associated with polar opposite levodopa concentrations and can be generated by different mechanisms. Genetic risk factors related to the most prevalent subtype, i.e. peak-dose dyskinesia, or those that are in common between all the subgroups could overshadow potential risk factors specific to a certain LID subtype. In addition, different manifestations can occur, with different prevalences, also within these same subgroups, especially peak-dose dyskinesia, including chorea, ballism, dystonia, and myoclonus. Considerable heterogeneity is observable also in the affected parts of the body.[174] Future genetic studies will need to stratify PD patients based on the different clinical manifestations of LID to investigate potentially distinct

etiopathogenesis. Another limitation is represented by the potential differences between the cohorts in the diagnosis of LID, which may vary depending on the operator and the duration of the examination. Potential divergencies between the cohorts were partially addressed by meta-analyzing the results and adjusting the analyses including aforementioned covariates.

Given the relevance that *GBA1* and *LRRK2* might have in future targeted therapy, we analyzed the relationship between *LRRK2* variants and GCase activity. Even though *GBA1* mutations are the main determinants of decreased GCase activity, previous studies already suggested other factors affecting GCase, including variants in *TMEM175* and also in the *LRRK2* gene. In our study, we demonstrated that *LRRK2* variants are associated with increased activity in the peripheral blood in line with a previous study on the same tissue.[250] At the time our study was performed, we discussed potential reasons (i.e., methodological, tissue- or model-related) for the divergent results in a previous study performed in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons, which showed an association between *LRRK2* variants and decreased GCase activity.[251] A more recent work showed opposite direction of effect depending on the tissue for the association between *LRRK2* variants and GCase levels. In brain tissues of p.G2019S knock-in mice and in p.G2109S iPSC-derived neurons GCase levels was decreased. Conversely, in fibroblasts and peripheral blood mononuclear cells, the *LRRK2* p.G2109S variant was associated with increased GCase levels. However, when analyzing GCase activity, the authors reported an association between *LRRK2* variants and increased GCase activity both in peripheral and nervous tissues, including brain tissues of p.G2019S knock-in mice and human iPSC-derived dopaminergic neurons, supporting the findings of our study. [252] Under the assumption that GCase disruption is the main determinant of *GBA1*-PD characteristic malignant phenotype, the association between *LRRK2* variants and increased GCase activity can contribute to explaining the more benign phenotype in *LRRK2*-PD, and feeds into the divergence between these two PD subtypes. Another potential interpretation of this association could be that the more benign *LRRK2*-PD phenotype is

independent of the increased GCase activity, and that this phenomenon might only be a compensatory mechanism. However, GCase activity is decreased also in iPD,[80] suggesting that the increase in GCase activity is a feature associated with *LRRK2*-PD. Furthermore, the association between the *TMEM175* p.M393T variant and decreased GCase activity might contribute to explain the association of this variant also with RBD.[150] The p.M393T variant was shown to affect lysosomal function and alpha-synuclein accumulation and has been suggested as another potential target for future clinical trials.[253]

The association between *LRRK2* variants and increased GCase activity shows the need for caution in the therapy targeting LRRK2 pathway, as it might lead to deficiency of GCase activity, thus potentially aggravating the symptomatology. *LRRK2* knockout macrophages display reduced GCase activity compared to controls, [252] resembling a potentially similar effect to the LRRK2-targeted therapeutics. One option to address this problem might be to combine *LRRK2*- and *GBA1*-targeted therapies in *LRRK2*-PD, however, this possibility will require additional experiments to evaluate the potential beneficial and detrimental effects.

In the previous paragraphs, I often discussed PD dichotomously, e.g., PD with vs without RBD, brain-first vs body-first, PD with or without LID. I traced a connection between these clinical subtypes with genetic subtypes, e.g., *SNCA* rs10005233-PD and body-first PD vs *LRRK2*-PD and brain-first PD. I also did something similar with the iRBD progression, discussing that iRBD can convert into PD, DLB, MSA or not convert at all and illustrating evidence for associations between different biomarkers and the risk/patterns of conversion. These dichotomies remain an average effect that we observe, and thus must be treated as necessary approximations of a much more complex pathophysiologic and clinical picture. Each of them represents a single dimension that we can use to stratify PD in multiple subtypes, these subtypes can overlap and diverge from each other to different degrees. However, to have a full picture of the different PD subtypes it is necessary to gather as many determinants as possible and combine them in a multidimensional approach.

Similarly, the distinctions we make between synucleinopathies like PD and DLB and, in some sense, also from the tauopathies like AD can be useful in the clinical practice but are not free of their own limitations. Sometimes the clinical manifestations can be misleading in classifying synucleinopathies, and it is the reason why the definitive diagnosis of these disorders is autoptic.[9] The current classifications are necessary to account for the clinical manifestations and the effect they produce on the quality of life of the patients. However, a more fine-grained distinction of the disease subtype can be integrated to better predict the disease progression, sharpen selection criteria for clinical trials and decide the most effective and safe therapeutic management. An increasing number of biomarkers, such as those mentioned in the discussion on RBD, are being identified and have the potential to account for the heterogeneity of synucleinopathies. A previous work from Espay and collegues suggested a further step in this understanding, by overturning the clinical phenotype-to-biomarkers into a biomarkers-to-clinical phenotype model. According to this model, the disease classification would not start from the clinical symptoms, as it is currently done in the clinical settings, but rather from an increasing set of confirmed biomarkers.[254] This can be especially useful in the research setting. Starting from genetic mutations and abnormal biological processes, detached from a preconceived clinical classification, it would be possible to study the diseases based on their etiologic determinants and trace a common pathophysiology between subtypes that overlaps across different disorders. New subtypes not previously identified might also emerge more clearly based on their biological homogeneity.[254] In the clinical setting, a multidimensional biomarker signature can be especially useful for the diagnostic classification of those clinical entities that appear clinically borderline but biologically more distinct, to predict disease progression and, as previously discussed, can find a determinant role in neuroprotective trials in RBD.[208] While this approach is, especially at the current time, logistically utopistic and obviously limited if used in isolation, it could be gradually integrated with our currently more accepted phenotype-to-biomarkers model when approaching the

patients therapeutically. In the future, machine learning can be harnessed to gather and make sense of an increasing number of biomarkers associated with synucleinopathies and might represent an effective aid for the work of clinicians. Furthermore, it can be used to enhance inclusion criteria in clinical trials and perform more studies based on biological homogeneity rather than clinical homogeneity, for example finding correlations with clinical and therapeutic outcomes using biologic determinants as independent variables.

The main goal of my thesis was to provide novel instruments to stratify PD based on its underlying genetics. This was done on a prodromal stage, by examining whether rare variants of a gene previously implicated in PD played a role also in patients with iRBD. I then focused on patients with clinically manifest PD, stratifying them based on genetic factors that are associated with a different phenotype, response to symptomatic therapy and potentially different response to future targeted therapy. I discussed how all these genetic factors can be capitalized to identify subtypes that can guide patient counselling, therapeutic management and support the prediction of disease progression, such as iRBD phenoconversion and PD clinical manifestations. I also show how subtyping PD and iRBD can be used to select patients for disease-modifying and neuroprotective clinical trials. Promoting genetic testing, increasing awareness in the general public about the prodromal phase of synucleinopathies, and screening in selected populations will all be necessary building blocks to reach these goals.

CHAPTER 7: CONCLUSION AND SUMMARY

Synucleinopathies are often referred to as a spectrum.[255, 256] In many ways the synucleinopathies can be redefined as a result of multiple spectra, each accounting for a different dimension, and the manifestation of the disease in each individual represents the encounter of these different dimensions, some of which we are aware of, some we are still exploring. In this thesis I aimed to investigate some of these dimensions to further our comprehension of these complex disorders, with a particular focus on PD and RBD.

To evaluate the role of *PSAP* rare variants in iRBD I performed burden test analyses and found a nominal enrichment of lof *PSAP* mutations. I also report the presence of these mutations in 0.3% of the iRBD group and the copresence of *PSAP* lof mutations and *GBA1* variants in 0.2% of iRBD patients.

To investigate the different genetic profile between PD with and without RBD I performed GWAS and demonstrated that a *SNCA* variant was associated with risk for RBD in PD, whereas other three variants, two in the *SNCA* region and one in the *LRRK2* region, were associated with an opposite effect. To test potential relationships between the presence of RBD in PD and neuropsychiatric traits I also assessed their potential genetic correlation and causative association, reporting a nominal correlation with ADHD.

To study the genetic underpinnings of risk and time to develop LID I performed GWAS and analysis focused on genes implicated in LID, PD and tested in clinical trials. I found an association between *GBA1* variants and LID risk and between *LRRK2* variants and reduced time to LID. I also studied the impact on LID of the PD PRS and dopamine transmission pathway, and found an association between PD PRS and LID risk as well as between dopamine transmission pathway and reduced time to LID.

Finally, to examine the association between *LRRK2* variants and GCase activity I performed linear regression between *LRRK2* variants and GCase activity measured in

peripheral blood, finding an association between these variants and increased GCase activity. I also report a potential association between the less characterized p.M1646T variant and a mildly increased risk for PD.

These findings contribute to defining the genetic underpinnings of RBD which can be leveraged for future clinical neuroprotective trials. They also help to stratify PD patients based on their clinical presentation and different responses to symptomatic and targeted therapy, with the ultimate goal to improve patient counselling and therapeutic management. Finally, they contribute to further our understanding of current and future potential targets for genetic therapy, feeding into the growing evolution of precision medicine. A tailored approach to the patient based on their individual genetic, biological and clinical features will translate into enhanced effectiveness of the diagnostic, prognostic and therapeutic approach, resulting in a substantial improvement in their quality of life.

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