Targeted Next-Generation Sequencing Approach to Study the Role of Parkinsonism Genes in REM Sleep Behavior Disorder

Kheireddin Mufti

Master of Science

Department of Human Genetics

McGill University

Montréal, Québec, Canada

August 2020

A thesis submitted to McGill University in partial

fulfillment of the requirements of the degree of

Master of Science

Abstract

Isolated REM sleep behavior disorder (iRBD) is a parasomnia that is characterized by loss of muscle atonia and dream enactment during REM sleep. iRBD is, in most cases, a prodromal synucleinopathy, as more than 80% of patients diagnosed with iRBD will eventually convert to an overt synucleinopathy including Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). Since 40-50% of iRBD patients convert to PD, it is likely that both disorders partially overlap in their genetic background. Although PD genetics have been well-studied over the recent decades, it is only recently that studies have been conducted to understand the genetics of iRBD, and our knowledge on the genetic overlap between iRBD and PD is still limited. Genetic variants in *PRKN*, *PINK1*, *PARK7*, *VPS13C*, *ATP13A2*, *FBXO7*, *PLA2G6*, *LRRK2*, *GCH1* and *VPS35* are known to cause familial forms of parkinsonism (including PD and atypical parkinsonism), while the genes *ACMSD*, *BST1*, *CCDC62*, *DDRGK1*, *DGKQ*, *FGF20*, *GAK*, *GPNMB*, *HIP1R*, *ITGA8*, *LAMP3*, *MAPT*, *MCCC1*, *PM20D1*, *RAB25*, *RAB29*, *RIT2*, *SETD1A*, *SLC41A1*, *STK39*, *SIPA1L2*, *STX1B*, *SYT11*, *TMEM163* and *USP25* have been implicated in PD genome-wide association studies (GWASs). Despite their association with PD, most of these genes have not been studied in iRBD, and it is still unknown whether they play a role in the risk of iRBD.

The aim of the current thesis is to use targeted-sequencing approach to comprehensively explore the association of these parkinsonism-related genes with iRBD risk in 1,039 unrelated iRBD patients and 1,852 unrelated controls of European ancestry. For that, we used multiple approaches to investigate the effect of different types of variants including rare heterozygous and biallelic variants, common variants, and copy number variants. To test whether heterozygous variants in our target genes play role in iRBD, we performed burden and SKAT-O analyses. We examined the effect of copy number variants (CNVs) in *PRKN* on iRBD risk using multiplex ligation-dependent probe amplification and ExomeDepth. Furthermore, we aimed to investigate the effect of three significant nonsynonymous variants in *BST1* found to be associated with iRBD in our analysis on the enzyme function. For that, *in silico* structural mutagenesis of BST1 was performed to assess the potential impact of these variants on the protein stabilization and function. We finally examined the association between common variants in the GWAS PD genes

and iRBD risk using logistic regression adjusted for age and sex. Our results showed novel associations between rare coding variants (p.V85M, p.I101V and p.V272M) in *BST1* and rare non-coding variants (intronic [3:182858302] and 3'UTR rs56682988 [*415T>C]) in *LAMP3* and iRBD risk. The structural analysis of BST1 revealed potential loss-of-function effects of the nonsynonymous variants in *BST1*, suggesting that reduced BST1 activity may decrease the risk of iRBD.

Altogether, these results highlight BST1 and LAMP3 as potential targets for future investigations and functional studies in iRBD and PD. We did not find significant associations between heterozygous, biallelic or common variants in the rest of the genes and risk of iRBD, suggesting that none of the remaining genes seems to play a major role in the development of iRBD and further highlighting the distinct genetic profiles of iRBD and PD.

Résumé

Le trouble du comportement de sommeil REM isolé (iRBD) est une parasomnie qui se caractérise par une perte d'atonie musculaire et des actes de rêve pendant le sommeil REM. iRBD est, dans la plupart des cas, une synucléinopathie prodromique, car plus de 80% des patients diagnostiqués avec iRBD finiront par se convertir pour avoir une synucléinopathie manifeste, y compris la maladie de Parkinson (PD), la démence à corps de Lewy (DLB) et l'atrophie multisystématisée (MSA)). Étant donné que 40 à 50% des patients atteints d'IRBD se convertissent à la MP, il est probable que les deux troubles se chevauchent partiellement dans leur patrimoine génétique. Bien que la génétique PD ait été bien étudiée au cours des dernières décennies, ce n'est que récemment que des études ont été menées pour comprendre la génétique de l'iRBD, et nos connaissances sur le chevauchement génétique entre l'iRBD et la PD sont encore limitées. Les variantes génétiques dans *PRKN, PINK1, PARK7, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* et *VPS35* sont connues pour provoquer des formes familiales de parkinsonisme (y compris la MP et le parkinsonisme atypique), tandis que les gènes *ACMSD, BST1, CCDC62, DDRGK1, DGKQ , FGF20, GAK, GPNMB, HIP1R, ITGA8, LAMP3, MAPT, MCCC1, PM20D1, RAB25, RAB29, RIT2, SETD1A, SLC41A1, STK39, SIPA1L2, STX1B, SYT11, TMEM163* et *USP25* ont été largement associés à des études sur le génome PD (GWAS). Malgré leur association avec la MP, la plupart de ces gènes n'ont pas été étudiés dans l'iRBD, et on ignore encore s'ils jouent un rôle dans le risque d'iRBD.

Le but de la thèse actuelle est d'utiliser une approche de séquençage ciblé pour explorer de manière approfondie l'association de ces gènes liés au parkinsonisme avec le risque d'IRBD chez 1 039 patients iRBD non apparentés et 1 852 témoins non apparentés d'ascendance européenne. Pour cela, nous avons utilisé plusieurs approches pour étudier l'effet de différents types de variantes, y compris des variantes hétérozygotes et biallélіques rares, des variantes communes et des variantes de nombre de copies. Pour tester si les variantes hétérozygotes dans nos gènes cibles jouent un rôle dans l'iRBD, nous avons effectué des analyses de charge et SKAT-O. Nous avons examiné l'effet des variantes du nombre de copies (CNV) dans PRKN sur le risque iRBD en utilisant une amplification de sonde dépendante de la ligature multiplex et ExomeDepth. En outre, nous avons cherché à étudier l'effet de trois variantes non synonymes

significatives dans *BST1* qui se sont révélées être associées à iRBD dans notre analyse sur la fonction enzymatique. Pour cela, une mutagenèse structurale in silico de BST1 a été réalisée pour évaluer l'impact potentiel de ces variants sur la stabilisation et la fonction des protéines. Nous avons finalement examiné l'association entre les variantes communes dans les gènes GWAS PD et le risque iRBD en utilisant une régression logistique ajustée pour l'âge et le sexe. Nos résultats ont montré de nouvelles associations entre des variantes de codage rares (p.V85M, p.I101V et p.V272M) dans BST1 et des variantes rares non codantes (intronic [3: 182858302] et 3'UTR rs56682988 [* 415T> C]) dans Risque *LAMP3* et iRBD. L'analyse structurale de BST1 a révélé des effets potentiels de perte de fonction des variantes non synonymes de *BST1*, suggérant qu'une activité réduite de BST1 pourrait diminuer le risque d'iRBD.

Dans l'ensemble, ces résultats mettent en évidence BST1 et LAMP3 comme cibles potentielles pour de futures investigations et études fonctionnelles dans iRBD et PD. Nous n'avons trouvé aucune association significative entre les variantes hétérozygotes, bialléliques ou communes dans le reste des gènes et le risque d'iRBD, suggérant qu'aucun des gènes restants ne semble jouer un rôle majeur dans le développement d'iRBD et mettant davantage en évidence les profils génétiques distincts de iRBD et PD.

Table of Contents

ABSTRACT 2

RESUMÉ 4

LIST OF ABBREVIATIONS 8

LIST OF FIGURES 12

LIST OF TABLES 13

ACKNOWLEDGNEMTS 14

FORMAT OF THE THESIS 15

CONTRIBUTION OF AUTHORS 16

CHAPTER 1: Introduction 19

1. Background 19
2. Isolated REM sleep behavior disorder pathology 22
3. Genetics of synucleinopathies 23
   - 3.1. Genetics of Parkinson's disease 23
   - 3.2. Genetics of Dementia with Lewy bodies and Multiple system atrophy 25
   - 3.3. Current knowledge on the genetics of REM sleep behavior disorder 25
4. Identifying genetic associations through targeted-sequencing approaches 26
5. Aims and Rational 30

CHAPTER 2: A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder 34

1. Abstract 36
2. Introduction 37
3. Methods 38
4. Results 40
5. Discussion 42
6. References 51

CHAPTER 3: Novel associations of BST1 and LAMP3 with rapid eye movement sleep behavior disorder 55

1. Abstract 57
2. Introduction 58
3. Materials and methods 59
4. Results 61
5. Discussion 64
6. References 77

CHAPTER 4: General Discussion 81

CHAPTER 5: Conclusion & Future Directions 86

CHAPTER 6: Master Bibliography 88

APPENDIX 102

Copyright permissions 102

Ethics certificate 111

List of Abbreviations

AAO	Age at onset
AAS	Age at sampling
ACMSD	Aminocarboxymuconate semialdehyde decarboxylase
AD	Autosomal dominant
APOE	Apolipoprotein E
AR	Autosomal recessive
ATP13A2	ATPase type 13A2
BST1	Bone marrow stromal cell antigen 1
CADD	Combined Annotation Dependent Depletion
CCDC62	Coiled-coil domain containing 62
CCNA	Canadian Consortium on Neurodegeneration in Aging
CDS	Coding sequence
CFREF	Canada First Research Excellence Fund
CI	Confidence interval
CIHR	Canadian Institutes of Health Research
CNS	Central nervous system
CNV	Copy number variant
dbSNP	Single nucleotide polymorphism database
DDRGK1	DRGK domain containing 1
DeNDRoN	Dementias and Neurodegenerative Diseases Research Network
DGKQ	Diacylglycerol kinase theta
DLB	Dementia with Lewy bodies
DOC	Depth of coverage
dNTP	Deoxynucleoside triphosphate
F_A	Frequency in affected
F_C	Frequency in control
FBXO7	F-box protein 7

FGF20	Fibroblast growth factor 20
GAK	Cyclin G associated kinase
GATK	Genome Analysis Toolkit
GBA	Glucosidase, β, acid
GCH1	GTP cyclohydrolase I
GnomAD	Genome Aggregation Database
GPNMB	Glycoprotein (transmembrane) nmb
GTEx	Genotype-tissue expression
GWAS	Genome-wide association study
HBHL	Healthy Brains for Healthy Lives
hg19	Human reference genome version 19
HIP1R	Huntingtin interacting protein 1 related
ICSD	International classification of sleep disorders
iRBD	Isolated RBD
ITGA8	Integrin subunit alpha 8
LAMP3	Lysosomal-associated membrane protein 3
LB	Lewy bodies
LP	Lewy pathology
LRRK2	Leucine-rich repeat kinase 2
LD	Linkage disequilibrium
LOF	Loss-of-function
MAF	Minor allele frequency
MAPT	Microtubule-associated protein tau
MCCC1	Methylcrotonoyl-CoA carboxylase 1 (α)
miRNA	micro RNA
MIPs	Molecular Inversion Probes
MLPA	Multiplex ligation-dependent probe assay
MSA	Multiple system atrophy
NGS	Next-generation sequencing
NIHR	National Institute for Health Research

NS	Nonsynonymous
OR	Odds ratio
PRKN	Parkin RBR E3 ubiquitin protein ligase
PARK7	Parkinson disease protein 7
PCA	Principle component analysis
PD	Parkinson's disease
PINK1	PTEN-induced kinase 1
PLA2G6	85 kDa calcium-independent phospholipase A2
PM20D1	Peptidase M20 domain containing 1
QC	Quality control
QS	Quality score
qPCR	Quantitative polymerase chain reaction
RAB25	RAB25, member RAS oncogene family
RAB29	RAB29, member RAS oncogene family
RBD	REM sleep behavior disorder
REM	Rapid eye movement
RIT2	Ras Like without CAAX 2
SCARB2	Scavenger receptor class B, member 2
SETD1A	SET domain containing 1A, histone lysine methyltransferase
SIPA1L2	Signal induced proliferation associated 1 like 2
SKAT	Sequence kernel association test
SKAT-O	Optimized sequence kernel association test
SLC41A1	Solute carrier family 41 (magnesium transporter), member 1
SNCA	Synuclein, α (non A4 component of amyloid precursor)
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STK39	Serine/threonine kinase 39
STX1B	Syntaxin 1B
SYT11	Synaptotagmin 11
TMEM163	Transmembrane protein 163

USP25	Ubiquitin specific peptidase 25
UTR	Untranslated region
VPS13C	Vacuolar protein sorting-associated protein 13C
VPS35	Vacuolar protein sorting-associated protein 35
vPSG	Video polysomnography

List of Figures

CHAPTER 1

Figure 1. *Normal sleep architecture* 22

Figure 2. *Targeted gene-capture using Molecular Inversion Probes* 31

Figure 3. *Workflow of the current thesis* 34

CHAPTER 3

Figure 1. *Structural analysis of human BST1 variants* 79

List of Tables

CHAPTER 1

Table 1. *Genes of interest targeted in the current study* 33

CHAPTER 2

Table 1. *Summary of all samples with two nonsynonymous variants detected in the present study* ... 49

Table 2. *Summary of results from burden analyses of rare heterozygous variants* 50

CHAPTER 3

Table 1. *Summary of results from burden analyses of rare heterozygous variants* 76

Table 2. *Summary of all samples with two nonsynonymous variants detected in the present study* ... 80

Acknowledgements

First and foremost, my sincere gratitude goes to Almighty God for granting his guidance upon me throughout the whole process. I would also like to express my thankfulness and high appreciation to my supervisor, Dr. Ziv Gan-Or of the Department of Human Genetics at McGill University for his huge support, guidance, feedback and encouragement during this important stage in my career, and without whom this thesis and all of my academic successes during my master's degree would not have been possible. Thank you for being approachable, patient, and kind, and thank you for inspiring me with your solid personality and true leadership.

I would like to wholeheartedly thank all the Gan-Or's lab members for their endless help and guidance. It was great to working with you all. Thank you for answering my inquiries, for sharing ideas on work, food and life with me. A special thank you to Vlad and Eric who taught me a lot in programming when I first joined the lab.

I would also acknowledge my advisory committee members, Dr. Genevieve Bernard and Dr. Thomas Durcan for their time, assistance and wise inspiring discussions with me to improve my work during my graduate journey at McGill University. A huge thank you goes out to all the patients who participated in this study as well as to all of their families. Also, thank you to the collaborators and funding parties who supported this research. Thank you to my friends Rached, Abdulrazzak, Tamer and Ahmad for their continuous social support and tips. I am extremely grateful for your friendship, laughs and stories we have shared together. I will retain these memories forever.

Finally, and most importantly, my deepest and endless gratitude, thankfulness and appreciation goes to my parents (Mrs. Safia Karahamo and Mr. Mohamed Mufti) and all my family members: Fatima, Abdullah, Bana and Bayan, for their unconditional support, sacrifice, and encouraging me throughout my life and this degree.

Format of thesis

This thesis has been prepared following the guidelines provided by the Department of Graduate and Postdoctoral Studies at McGill University, and is presented as a manuscript-based thesis. This thesis is written by Kheireddin Mufti and revised by Dr. Ziv Gan-Or, the supervisor. The study presented herein consists of investigating the potential role of parkinsonism-related genes in the risk of REM sleep behavior disorder.

This thesis includes 6 chapters. Chapter1 provides a general introduction and relevant background to the topic of the thesis, and permissions were obtained for reproducing all the published figures included in this chapter. Chapters 2 and 3 consist of two manuscripts, and they highlight the main findings of this thesis. The first manuscript in Chapter 2 will discuss the role of familial parkinsonism genes in REM sleep behavior disorder risk, it has been submitted to peer-reviewed journal and is currently under review, and a preprint copy of it is available on MedRxiv: https://doi.org/10.1101/2020.03.17.20032664. The second manuscript in Chapter 3 will represent the contribution of genome-wide association studies Parkinson's disease genes to the risk of REM sleep behavior disorder, it has also been submitted to peer-reviewed journal and its preprint can be found on MedRxiv: https://doi.org/10.1101/2020.06.27.20140350. Both chapters (Chapters 2 and 3) are preceded with preface that links the thesis together. I performed most of the analyses described in this thesis according to the lab protocols and under my supervisor directions. Chapter 4 generally discusses of the results presented in this dissertation, and Chapter 5 includes a final conclusion of the work with future directions.

Contribution of Authors

Chapter 2

Manuscript title: A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder

List of authors: Kheireddin Mufti, Uladzislau Rudakou, Eric Yu, Jennifer A. Ruskey, Farnaz Asayesh, Sandra B. Laurent, Dan Spiegelman, Isabelle Arnulf, Michele T.M. Hu, Jacques Y. Montplaisir Jean-François Gagnon, Alex Desautels, Yves Dauvilliers, Gian Luigi Gigli, Mariarosaria Valente, Francesco Janes, Birgit Högl, Ambra Stefani, Evi Holzknecht, Karel Sonka. David Kemlink, Wolfgang Oertel, Annette Janzen, Giuseppe Plazzi, Elena Antelmi, Michela Figorilli, Monica Puligheddu, Brit Mollenhauer, Claudia Trenkwalder, Friederike Sixel-Döring, Valérie Cochen De Cock, Christelle Charley Monaca, Anna Heidbreder, Luigi Ferini-Strambi, Femke Dijkstra, Mineke Viaene, Beatriz Abril, Bradley F. Boeve, Ronald B. Postuma, Guy A. Rouleau, Ziv Gan-Or.

Contributions:

IA, MTMH, JYM, JFG, AD, YD, GLG, MV, FJ, BH, AS, EH, KS, DK, WO, AJ, GP, EA, MF, MP, BM, CT, FSD, VCDC, CCM, AH, LFS, FD, MV, BA, BFB, RBP, GAR: Recruited iRBD patients and controls.

KM performed DNA capture for 50% of the samples, the DNA capture of the remaining samples was done by SBL, JAR, FA.

UR, JAR: Sanger sequencing validations.

KM performed all the post-alignment QC procedures and filtering of variants, UR helped with performing the annotation, DS performed the alignment.

UR: Provided guidance on performing statistical analyses.

EY: Assisted with CNVs analysis.

KM: Performed all the statistical analyses and CNVs analysis.

KM: Wrote the manuscript.

ZGO: Designed the experiments.

ZGO: Supervised the work, provided financial support and contributed to the writing of the manuscript.

Sequencing was performed at the Genome Quebec and McGill University Genome Centre.

All authors revised the manuscript.

Chapter 3

Manuscript title: Novel associations of *BST1* and *LAMP3* with rapid eye movement sleep behavior disorder

List of authors: Kheireddin Mufti, Eric Yu, Uladzislau Rudakou, Lynne Krohn, Jennifer A. Ruskey, Farnaz Asayesh, Sandra B. Laurent, Dan Spiegelman, Isabelle Arnulf, Michele T.M. Hu, Jacques Y. Montplaisir Jean-François Gagnon, Alex Desautels, Yves Dauvilliers, Gian Luigi Gigli, Mariarosaria Valente, Francesco Janes, Andrea Bernardini, Birgit Högl, Ambra Stefani, Evi Holzknecht, Karel Sonka, David Kemlink, Wolfgang Oertel, Annette Janzen, Giuseppe Plazzi, Elena Antelmi, Michela Figorilli, Monica Puligheddu, Brit Mollenhauer, Claudia Trenkwalder, Friederike Sixel-Döring, Valérie Cochen De Cock, Christelle Charley Monaca, Anna Heidbreder, Luigi Ferini-Strambi, Femke Dijkstra, Mineke Viaene, Beatriz Abril, Bradley F. Boeve, Jean-François Trempe, Ronald B. Postuma, Guy A. Rouleau, Ziv Gan-Or.

Contributions:

IA, MTMH, JYM, JFG, AD, YD, GLG, MV, FJ, AB, BH, AS, EH, KS, DK, WO, AJ, GP, EA, MF, MP, BM, CT, FSD, VCDC, CCM, AH, LFS, FD, MV, BA, BFB, RBP, GAR: Recruited iRBD patients and controls.

KM performed DNA capture for 50% of the samples, the DNA capture of the remaining samples was done by SBL, JAR, FA.

UR, JAR: Sanger sequencing validations

LK: Database preparation and processing

KM performed all the post-alignment QC procedures, filtering of variants and annotation, DS performed the alignment.

KM performed statistical analysis for rare heterozygous and biallelic variants, and common variants, EY provided guidance on performing statistical analysis for common variants and helped with designing the scripts.

KM: Wrote the manuscript.

JFT: Performed structural analysis of BST1, contributed to the writing of the manuscript

ZGO: Designed the experiments.

ZGO: Supervised the work, provided financial support and contributed to the writing of the manuscript.

Sequencing was performed at the Genome Quebec and McGill University Genome Centre.

All authors revised the manuscript.

CHAPTER 1: Introduction

1. Background

Sleep is a naturally recurring state of unconsciousness and decreased arousal with reduced interaction with the surrounding, yet it is a very dynamic state in which a normal sleep involves successive cycles of two basic types of sleep: rapid eye movement (REM) sleep and non-REM (NREM) sleep.[1] Together, these two types of sleep make up a single sleep cycle that lasts about 90 minutes and occur multiple times during nocturnal sleep[2] (Figure 1). Disturbance in any of the sleep stages will result in reduced sleep quality. For instance, in normal REM sleep (dreaming stage), the brain is in active state while almost all the body muscles remain inhibited and essentially paralyzed. This muscle inhibition (also known as muscle atonia) is crucial for preventing the physical response to the dream's content and moving around while dreaming. However, loss of the normal muscle paralysis during REM sleep leads to increased muscle activity and dream enactment, causing a parasomnia known as REM sleep behavior disorder (RBD).

RBD is characterized by a history of dream enactment and abnormal motor behaviors occurring during REM sleep, and it has been classified into two types: secondary (secondary RBD) when it occurs as a result of neurological or neurodegenerative disorders,[3] autoimmune diseases[4, 5] or brainstem lessions,[6, 7] as well as when it is initiated by antidepressants consumption,[4] and primary 'or isolated' RBD (iRBD) when RBD onset occurs prior to any other symptoms of neurodegeneration and is not caused by use of antidepressants, autoimmune diseases or brainstem lesions. The prevalence of iRBD is ~1% of individuals at the age of over 60 years,[8, 9] and the confirmed iRBD diagnosis requires detecting the presence of abnormal motor behaviors during REM sleep by video polysomnography (vPSG), that includes a synchronized video coupled with: electroencephalography (EEG) to measure the brain activity and identify REM sleep stage, electrooculography (EOG) to measure eye movements and confirm REM sleep, and electromyography (EMG) to detect the excess tonic and phasic motor activity.[1] In fact, iRBD has a highly variable clinical presentation; the abnormal behaviors that could occur during REM sleep range from unnoticed sleep disruption and non-violent behaviors including laughing, crying or singing to severe self-injurious and/or injuries of bed partners

through violent behaviors like punching, biting or kicking, depending on the dream content. Although most motor events during dream enactment are simple elementary movements, sleep-related injuries to the self and/or the bedpartner are common in patients with iRBD. However, the most important implication of iRBD is its association with neurological diseases. More than 80% of iRBD patients eventually develop a neurodegenerative disorder associated with α-synuclein pathology,[10] including Parkinson's disease (PD), Dementia with Lewy bodies (DLB), and multiple system atrophy (MSA).[11] Therefore, iRBD is now considered not only as a parasomnia, but also as a prodromal manifestation of these neurodegenerative disorders, collectively termed as synucleinopathies. The high rate of conversion from iRBD to overt synucleinopathies suggests that the genetic background of iRBD may overlap, or at least partially overlap, with the genetic background of these disorders. Thus, studying and exploring this potential genetic overlap between iRBD and synucleinopathies would be of great importance for several reasons: a) identifying genes and variants specifically involved in iRBD, b) better understanding of the molecular mechanisms underlying iRBD and its conversion c) identifying genetic markers for patient stratification, and d) identifying potential targets for drug development.

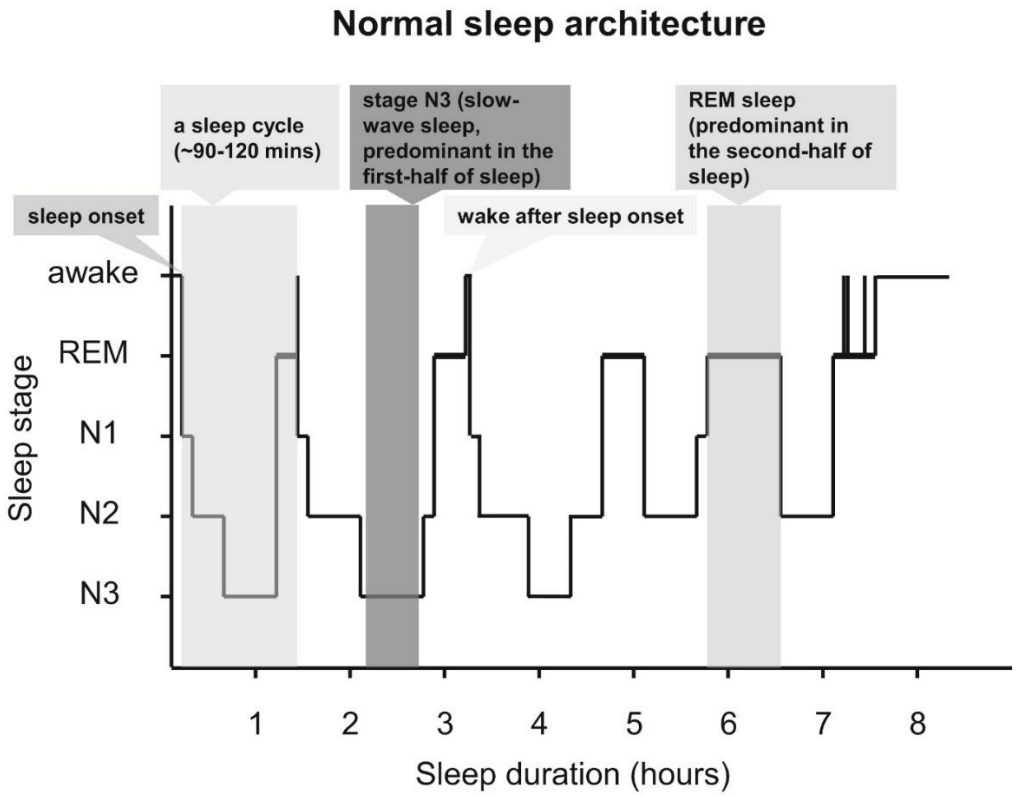


Figure 1. Normal sleep architecture. Sleep is divided into four main stages (3 NREM stages and 1 REM stage). Each sleep stage is characterized with certain brain activity and brain waves type. After sleep onset, the brain activity decreases from awake to N1, N2, N3 stages and then increases again to reach REM sleep stage which is the light sleep stage. Each sleep cycle consists of the 3 NREM and 1 REM sleep stages and lasts to about 90 minutes. The nocturnal sleep usually consists of 4–6 sleep cycles. This figure is adapted with permission from Tan, X *et al* 2019.[12]

2. Isolated REM sleep behavior disorder pathology

Multiple studies have been carried out in order to identify the neuronal system responsible for the REM sleep genesis. These studies have suggested multiple neurons and brain regions to be involved in RBD, such as the coeruleus/subcoeruleus and laterodorsal/sublaterodorsal tegmental nucleus comples.[13] . However, animal studies coupled with functional neuroimaging and post-mortem studies demonstrated that the GABAergic and glycinergic ventral medullary neurons in the brainstem are likely to generate the muscle atonia during REM sleep,[1] and thus, RBD is likely to be caused by specific neurodegeneration of these neurons being associated with Lewy bodies (LB). The association between the neurodegeneration and LB has not yet been explained. LB are cellular inclusions that are made by the accumulation of misfolded proteins. One of these proteins is α-synuclein, which is considered a major component of LB. α-synuclein is normally a water-soluble protein. However, under certain conditions, this protein can start forming insoluble dimers, oligomers, fibrils, and aggregates which are suspected to be neurotoxic.[14] Although the exact function of α-synuclein is still unclear, it is suggested to play an important role in vesicular transportation and recycling in the nigrostriatal presynaptic terminals, as well as in dopamine storage and compartmentalization.[15]

Since iRBD is a prodromal symptom of synucleinopathies, it suggests that α-synuclein pathology (or Lewy pathology, LP) can be present in the nervous system long before the emergence of other neurological symptoms of these disorders. This disease progression has been described by Braak et al.[16] According to their hypothesis, LP propagate from the periphery to the medulla oblongata and the olfactory system via the vagus nerve or olfactory nerves, leading to autonomic and olfactory disturbances. This propagation then continues further to reach the brainstem where the prodromal sleep disturbances and RBD occur with other initial motor symptoms. Then, the LP finally progresses into the other brain regions that are responsible for the onset of the associated synucleinopathies, such as the limbic system and neocortical regions that when degenerated cause cognitive and behavioral impairments associated with late stages of PD.

3. Genetics of synucleinopathies

Synucleinopathies are a group of neurological disorders that include mainly PD, DLB, and MSA, as well as other rarer neurological disorders. The common neuropathological hallmark of these disorders is the accumulation of α-synuclein in neurons or in glial cells of the nervous system in the form of LBs or Lewy neurites.[17] The current advancements in sequencing technologies developed over the recent decades and the emergence of population-scale cohorts have enabled conducting large studies in order to analyze phenotype and genotype data on neurological disorders. Coupled with the rapid development of various genetic methods, these technologies have helped obtaining a wealth of information about the genetic component of synucleinopathies, revealing genetic loci and genes that are implicated in the risk of synucleinopathies:

3.1. Genetics of Parkinson's disease

More than 40% of iRBD patients convert to PD, which is one of the most common progressive neurodegenerative disorders. PD is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta and α-synuclein protein aggregates.[18] Unlike DLB and MSA, the genetic component of PD has been thoroughly explored through familial and genome-wide association studies (GWASs). The advancement of the genetic methods over the recent decades has helped us identify several genes and variants that either cause or affect PD risk. Genes that are known to be involved in PD can be divided into three main groups: 1) genes or loci with common genetic variants that have a small effect on the disease risk, 2) genes with less common variants with incomplete penetrance that have moderate effect on the disease risk, and 3) rare genetic variants with a complete or near complete penetrance that are responsible for about 1-5% of the total PD cases.[19]

To date, GWASs have identified more than 90 known independent risk factors in 80 genetic loci to be associated with the risk of PD.[20-23] In addition, several genes have been shown to contribute to monogenic (or Mendelian) forms of PD, either through autosomal dominant (single copy of the mutant allele is enough to cause the disease) or autosomal recessive (both alleles are needed to be mutated to cause the disease) inheritance patterns. For example, rare genetic variants in *PRKN*, *PINK1* and *PARK7 (DJ1)* may cause autosomal recessive early-onset forms of

PD[17] with biallelic (homozygous and compound heterozygous) variants in *PRKN* being the most common, accounting for ~8.6% of all early-onset PD cases.[24] It is demonstrated that biallelic variants in *PRKN* are linked with atypical neuropathology of PD as they are associated with pure neurodegeneration in the substania nigra with the absence of LP,[25, 26] suggesting that patients carrying biallelic *PRKN* variants represent either a distinct subgroup of PD, or possibly a distinct disease that has similar clinical features.[26] *PRKN* is known to be located in a genomic region that is prone to rearrangements.[27, 28] However, while the role of biallelic variants in *PRKN* has been clearly established, the role of heterozygous variants, including single nucleotide variants (SNVs) and copy number variants (CNVs), remains unclear although they are frequent in *PRKN*. Variants in *PINK1* and *PARK7* are less common, accounting for up to 1-8%, and 1-2% of early-onset sporadic cases, respectively.[29-31] Although rare in frequency, variants in these genes have been established as PD-causative and account for most recessive PD cases.[32]

Furthermore, genetic variants in *SNCA, LRRK2, GCH1* and *VPS35* are linked with autosomal dominant inheritance of PD.[17, 33, 34] *SNCA* encodes α-synuclein, and its role in PD has been clearly established from the early familial reports[35] to the most recent GWASs findings.[22, 23] Different types of variations can be found in *SNCA*, including SNVs, duplications and triplications, and they are all reported to be associated with autosomal dominant forms of parkinsonism (with incomplete penetrance found in a few cases).[36] Another autosomal dominant cause of PD, although often with incomplete penetrance, is *LRRK2*, which encodes the large multidomain protein leucine-rich repeat kinase (or dardarin) that has been suggested to play important roles in many cellular processes including vesicular trafficking, microtubule binding, autophagy and mitophagy.[37] The most common variant found in *LRRK2* is the pathogenic p.G2019S variant, which is known to be present in approximately 1% of all PD cases,[38] and up to 40% in some populations.[39, 40] While 65% of p.G2019S carriers show neurodegeneration associated with LB, 70% of PD patients carrying other *LRRK2* pathogenic variants do not have LP.[41] Furthermore, although being strong risk factors of PD, some *LRRK2* variants, such as p,G2019S, have age-dependent penetrance; up to 30% after the age of 50, and 75% above the age of 70.[42, 43]

Finally, there are other genes such as *ATP13A2*, *FBXO7*, *VPS13C* and *PLA2G6* that are have been implicated in atypical forms of parkinsonism,[17, 44] with LP reported to be associated with

ATP13A2,[45] *FBXO7*,[46] and *PLA2G6*.[47] Variants in *GBA*, which encodes for the enzyme glucocerebrosidase, are common in PD, found in 5-20% of patients depending on their ethnicity, with a penetrance of 10-30%.[48] Since *GBA* has already been thoroughly studied in iRBD,[49] we will not study it further in the current thesis. Similarly, *SNCA* has also been thoroughly studied in iRBD[50] and will not be studied here.

3.2. Genetics of Dementia with Lewy bodies and Multiple system atrophy

DLB is the second most common dementia after Alzheimer's disease.[51] Despite this fact, the genetic background that underlies DLB is still not well-studied. To date, the only large-scale genetic study of DLB was a GWAS conducted on 1,743 DLB patients and 4,454 controls, showing several genome-wide significant loci associated with DLB including *APOE*, *SNCA* and *GBA*.[52] Other genes such as *SCARB2* and *MAPT* were also implicated in DLB, but without enough replications.[53, 54]

MSA is a rare, adult-onset neurodegenerative disorder with clinical features that include parkinsonism, pyramidal signs and dysautonomia.[55] Unlike DLB and PD, MSA-associated α-synuclein pathology is found to be present in oligodendroglia cells instead of neurons.[56] Only a minority of iRBD patients (~5%) convert to MSA, and only little is known about the genetics underlying MSA compared to PD and DLB. Since multiplex familial cases of MSA are rarely reported, it was previously thought that MSA is a non-genetic disease.[57] However, MSA has been reported to involve higher incident of parkinsonism in first-degree relatives compared with controls, suggesting that there might be a genetic component responsible for the development of MSA symptoms.[58] There is one GWAS on 918 MSA patients and 3,864 controls which failed to find loci significantly associated with MSA risk.[59] Additional genes such as *COQ2*,[60] *GBA*[61] and others have also been implicated in MSA, but with contradictory results which require additional replications.

3.3. Current knowledge on the genetics of REM sleep behavior disorder

Although the association between iRBD and synucleinopathies was initially reported more than two decades age, the first studies focusing on iRBD genetic component have emerged only recently. The previous genetic studies have demonstrated that there is no full overlap in the genetic background between iRBD and that of related synucleinopathies. For example, variants

in *GBA* are known to be common genetic risk factors for PD and DLB,[62] and were also found to be associated with increased iRBD risk.[11] Furthermore, the p.N551K-p.R1398H-p.K1423K protective haplotype of *LRRK2* in PD has been also suggested to be associated with reduced risk of iRBD, although this study was performed on a small population and requires replication.[63] Other genes have been reported to have independent risk variants for PD and iRBD. In the *TMEM175* locus, while there are two independent PD risk variants, only one of these variants, p.M393T, has been associated with iRBD.[64] Although RBD and DLB variants are found to be in Linkage disequilibrium (LD), a distinct pattern of association with PD and iRBD was found in the *SNCA* locus. Specific variants at the 5' untranslated region (UTR) (tagged by rs10005233) in *SNCA* are associated with increased risk of PD, iRBD, and DLB. Other independent variants (tagged by rs356182) in the 3' UTR of the *SNCA* locus are associated with increased risk of PD but found to have an opposite direction of effect in iRBD.[50] On the other hand, genetic studies found no association between other key PD or DLB pathogenic variants in *LRRK2*,[65] *MAPT* haplotypes,[66] *APOE* ε4[67] and *SMPD1*[68] risk variants and haplotypes.

4. Identifying genetic associations through targeted-next generation sequencing

Since next-generation sequencing (NGS) technology is based on the ability to sequence millions of DNA fragments in massively parallel manner, it provides a dramatic increase in the speed and content of sequencing at a lower fraction of cost compared to previous technologies such as Sanger sequencing, microarray technology and quantitative PCR (qPCR).[69] The rapid and inexpensive NGS methods offer broadly accessible technologies for high-throughput sequencing analysis of large genomic regions in a large number of samples with a single experiment. Variant detection has been shown to have about 95% sensitivity and 100% specificity for a variety of alterations such as single nucleotide polymorphisms (SNPs), small insertions and deletions, and splicing variants. Therefore, with some NGS technologies such as whole genome sequencing (WGS), genomes can be interrogated without bias as WGS can be unselective,[70] and does not require prior information about the gene structure and sequence location. However, this also means that the position of each sequence read on the genome is unclear. Thus, the short reads must be mapped back to the human reference genome. To map these individual reads to the human reference genome and process the massive raw data that NGS generates, bioinformatic

pipelines and tools are required. Other NGS disadvantages include limited detection of copy number variations (CNVs), bias against sequences of GC-rich DNA regions[71] and difficulties in detecting structural variants[72] and sequencing genetic areas where pseudogenes occur.[73] One example of that is the difficulty of detecting *GBA* variants due to the presence of the *GBA* pseudogene (*GBAP1*) which shares 96% homology with *GBA*.[74]

The application of NGS technologies include the use of WGS in which the DNA sequence of the whole genome is determined, whole-exome sequencing (WES) by which most of the protein-coding sequences can be captured, and targeted sequencing.[75] More recently, long-read sequencing has also been developed, which allows for sequencing of "difficult" regions and identification of large structural variants.[76] Compared to the higher cost of WGS, WES and long-read sequencing, targeted NGS provides more cost-effective method that can be used for genetic studies of large cohorts. In targeted sequencing, only the selected and captured genetic regions will be sequenced and analyzed. Since the captured regions of the genome are usually the most relevant to the disease, targeted NGS provides a faster approach with simpler data analysis requirements compared to WGS and WES.

In this thesis, the targeted capture method used is called molecular inversion probes (MIPs), designed by O'Roak., et al[77] (Figure 2). MIPs are single-stranded oligonucleotides containing two annealing arms (each of 18-25 bases) complimentary to the 5' and 3' ends of the target of interest in the genome. The two arms are linked with an internal common linking sequence of 35-50 bases containing universal PCR primer binding sites.[78] MIPs belong to the class of capture-by-circularization techniques;[79] when the designed probe hybridize and captures the genomic target, the arms undergo an inversion in configuration and forms circular structure with the intended DNA target captured between the flanking arms (Figure 2). MIPs are used because of many advantages they provide: due to the use of target probe design, MIPs provide high overall capture specificity (>99% of target overlap), consistent capture reproducibility (~98% of captured target), with a multiplex scalability (thousands of capture targets) for high-throughput sequencing.[80, 81] The circular design allows for removal of all linear DNA by exonucleases and retaining only the target sequences. Furthermore, adding unique barcode sequences to each DNA sample facilitates the quantification of individual capture reads, allowing for highly sensitive variant-calling.[82] Another advantage of MIPs is the relative low

sample DNA input requirement (less than 100 ng in some settings) compared to other target capture methods, as they can be directly applied to the genomic DNA and no shotgun library preparation is required.[83] Therefore, combining MIPs with NGS represents a simple and efficient approach that can selectively enrich thousands of genomic DNA targets and determine their sequence in parallel on large scale for the detection of common, rare and *de-novo* genetic variants in large disease-cohorts with relatively low cost per sample.[81, 84,85]

Targeted gene-capture using Molecular Inversion Probes

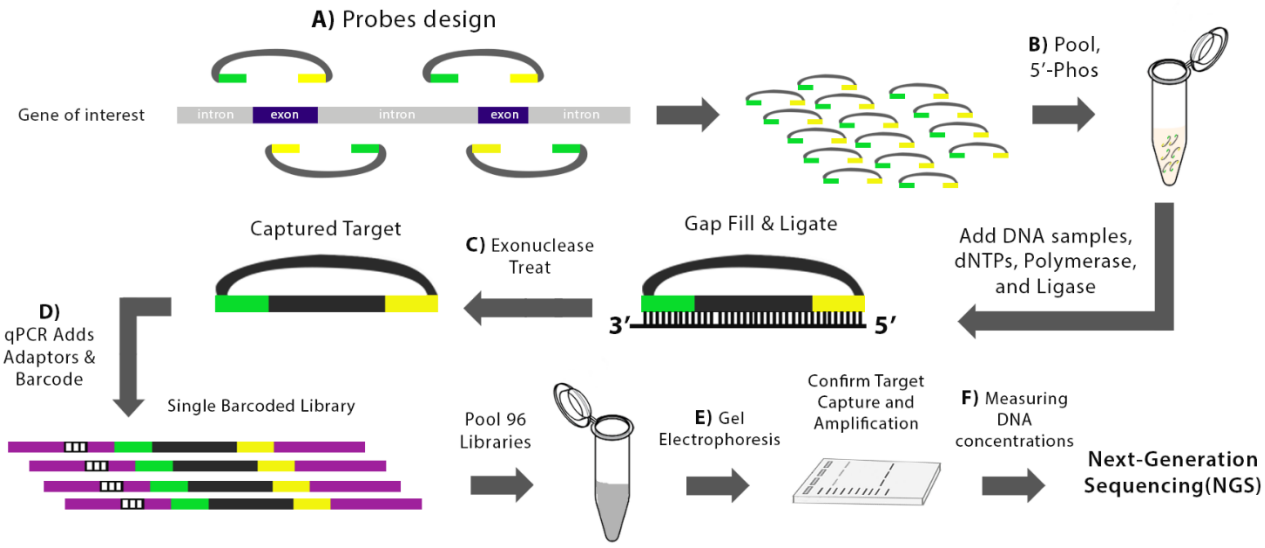


Figure 2. This figure illustrates targeted gene-capture using molecular inversion probes (MIPs). (A) The design and generation of probes: probes and their annealing arms are designed to capture specific DNA sequences of the target genetic region (B) Phosphorylation and MIP capture: probes are added to the genomic DNA samples with dNTPs, polymerase and ligase. The polymerase facilitates the extension between the targeting arms and the intervening sequence, and the addition of 5'-phosphate helps to form phosphodiester bonds between the targeted DNA fragment with the two arms of the probe to fill the gap and complete the covalently closed circular molecule. (C) Exonuclease treatment: adding exonuclease to degrade linear DNA (non-reacted probes and non-treated DNA) and keep target sequences only. (D) Amplification of target reads with PCR using universal primers complementary to the MIP backbone followed by adding specific barcode sequences to each sample to recognize it. (E) Pooling all samples together followed by gel-electrophoresis to confirm target capture and amplification. (F) Measuring the DNA concentrations and sequencing of the targeted DNA samples. This figure was produced using *Adobe Photoshop CC 2015* based on figure of O'Roak., *et al*. 2012 after obtaining permission.[77]

5. Aims and Rational

General aim

Since the conversion from iRBD to PD has been well-studied, we aimed to take advantage of the abundant data pertaining to PD genetics to investigate the role of PD-related genes in the risk of iRBD and further explore the genetic overlap between these disorders. Previous studies on iRBD genetics have demonstrated that while iRBD and PD have some common genetic risk factors, there is no full overlap in their genetic background. For instance, it was shown that while some genes such as *GBA*,[11] *TMEM175*[64] and *SNCA*[50] are associated with both PD and iRBD, other genes such as *MAPT*,[66] *SMPD1*[68] and pathogenic variants in *LRRK2*[63] were reported to be associated only with PD and not with iRBD. However, most of PD- and Parkinsonism-associated genes have not been investigated in iRBD yet. Hence, we aimed to further explore the potential overlap between PD and iRBD by examining the role of PD-related genes in iRBD risk. In this thesis, we hypothesized that rare and common variants in PD and Parkinsonism-related familial and GWAS genes could contribute to the risk of iRBD. To test our hypothesis, we selected a total of 35 genes: 10 familial PD or atypical parkinsonism genes, and 25 GWAS PD genes to examine their association with iRBD risk (Table 1 and Figure 2).

Specific aims and rationale

Aim 1 (chapter 2): To study the role of familial PD and atypical Parkinsonism genes in iRBD.

Rationale: The role of familial PD and atypical Parkinsonism genes in iRBD is unknown. We hypothesize that these genes will have no role or only minor role in RBD, as disease-causing variants in these genes are rare, and in some of these disorders (e.g. PRKN-associated PD) there is no α-synuclein pathology in most cases.

Aim 2 (chapter 3): To study the role of PD GWAS genes in iRBD.

Rationale: Since about half of iRBD patients will convert to PD, it is likely that some genes involved in PD may also be involved in iRBD. We hypothesize that rare variants in some of these genes will be associated with iRBD.

Genes of interest targeted in the current study

Familial genes				
PRKN	*PARK7*	*PINK1*	*VPS13C*	*ATP13A2*
LRRK2	*GCH1*	*VPS35*	*FBXO7*	*PLA2G6*
GWAS genes				
ACMSD	*BST1*	*CCDC62*	*DDRGK1*	*DGKQ*
FGF20	*GAK*	*GPNMB*	*HIP1R*	*ITGA8*
LAMP3	*MAPT*	*MCCC1*	*PM20D1*	*RAB25*
RAB29	*RIT2*	*SETD1A*	*SLC41A1*	*STK39*
SIPA1L2	*STX1B*	*SYT11*	*TMEM163*	*USP25*

Table 1. This table details the target genes that have been analyzed in the current project. Detailed explanation on how these genes were selected can be found in the following chapters. In blue: autosomal recessive PD genes; green: autosomal dominant PD genes; red: genes implicated with atypical parkinsonism.

Workflow of the Current Thesis

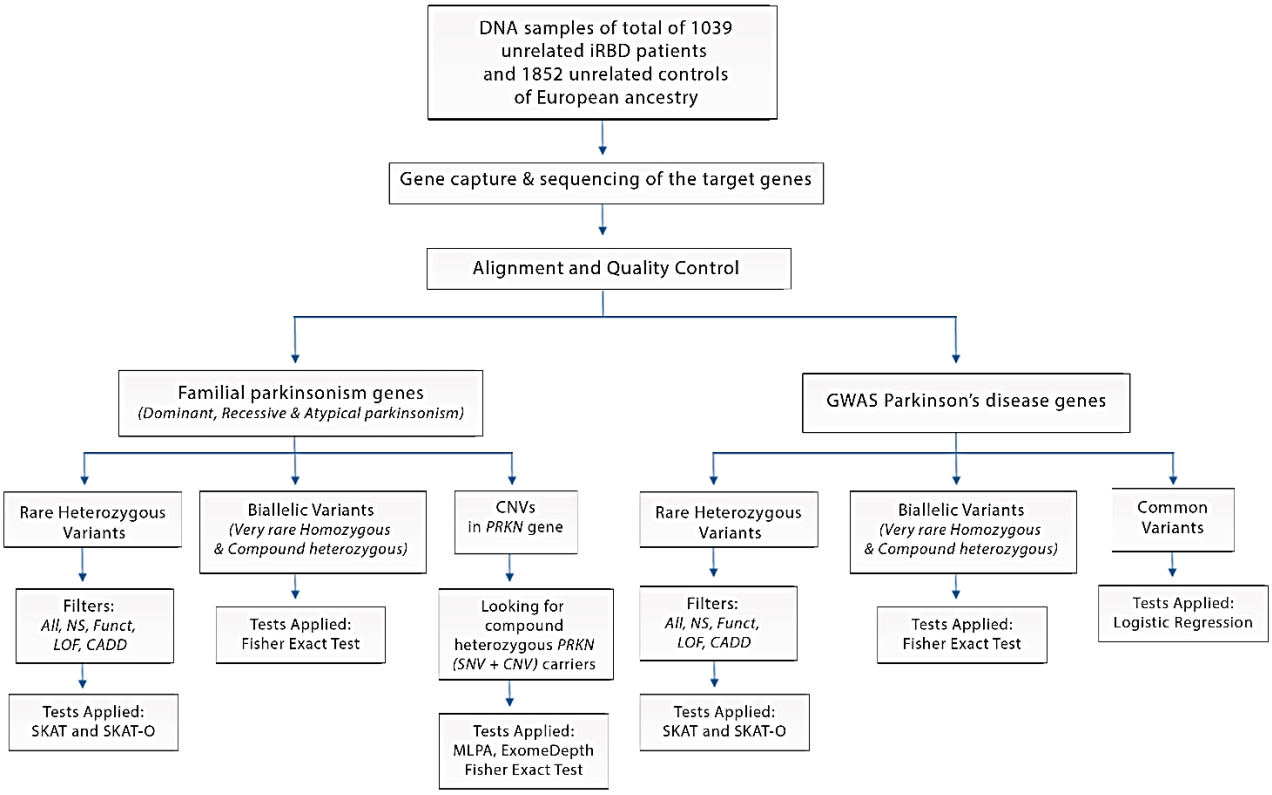


Figure 3. This figure shows the general flow of the analyses discussed in the current dissertation. Abbreviations: All = All rare variants; CADD = Combined annotation dependent depletion; CNVs = Copy number variants; Funct = Potentially functional variants; LOF = Loss-of-function; NS = Nonsynonymous; SKAT-O = Optimized sequence kernel association test; SKAT = Sequence Kernel association test; SNVs = Single nucleotide variants.

Preface to Chapter 2

Genetic variants in *PRKN, PARK7, PINK1, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* have been implicated in familial forms (autosomal dominant and autosomal recessive) of PD and atypical parkinsonism.[17, 24, 33, 34, 44] Previous reports have demonstrated that some genetic risk factors for PD are also involved in iRBD (e.g. *GBA, SNCA*), whereas other PD genes (e,g, *PINK1, LRRK2, MAPT*) are not. Using a much larger cohort, we aimed to examine whether these 10 familial genes associated with PD or atypical parkinsonism are also important in iRBD. The work detailed in Chapter 2 is focused on using targeted next-generation sequencing to study the association of rare heterozygous and bi-allelic variants – and copy number variants in *PRKN* - in these genes and iRBD in a large European ancestry cohort of 1,039 iRBD patients and 1,852 controls.

CHAPTER 2: A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder

Kheireddin Mufti[1,2], Uladzislau Rudakou[1,2], Eric Yu[1,2], Jennifer A. Ruskey[2,3], Farnaz Asayesh[2,3], Sandra B. Laurent[2,3], Dan Spiegelman[2,3], Isabelle Arnulf[4], Michele T.M. Hu[5,6], Jacques Y. Montplaisir[7,8], Jean-François Gagnon[7,9], Alex Desautels[7,10], Yves Dauvilliers,[11], Gian Luigi Gigli[12,13], Mariarosaria Valente[12,14], Francesco Janes[12], Birgit Högl[15], Ambra Stefani[15], Evi Holzknecht[15], Karel Sonka[16], David Kemlink[16], Wolfgang Oertel[17], Annette Janzen[17], Giuseppe Plazzi[18,19], Elena Antelmi[18,20], Michela Figorilli[21], Monica Puligheddu[21], Brit Mollenhauer[22,23], Claudia Trenkwalder[22.23], Friederike Sixel-Döring[17,22], Valérie Cochen De Cock[24,25] Christelle Charley Monaca[26], Anna Heidbreder[27], Luigi Ferini-Strambi[28], Femke Dijkstra[29,30,31], Mineke Viaene[29,30], Beatriz Abril[32], Bradley F. Boeve[33], Ronald B. Postuma[2,3,7], Guy A. Rouleau[1,2,3], Ziv Gan-Or[1,2,3].

Affiliations: 1. Department of Human Genetics, McGill University, Montréal, QC, Canada. 2. Montreal Neurological Institute, McGill University, Montréal, QC, Canada. 3. Department of Neurology and Neurosurgery, McGill University, Montréal, QC, Canada. 4. Sleep Disorders Unit, Pitié Salpêtrière Hospital, Centre de Recherche de l'Institut du Cerveau et de la Moelle Epinière and Sorbonne University, Paris, France. 5. Oxford Parkinson's Disease Centre (OPDC), University of Oxford, Oxford, United Kingdom. 6. Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom. 7. Centre d'Études Avancées en Médecine du Sommeil, Hôpital du Sacré-Cœur de Montréal, Montréal, QC, Canada. 8. Department of Psychiatry, Université de Montréal, Montréal, QC, Canada. 9. Department of Psychology, Université du Québec à Montréal, Montréal, QC, Canada. 10. Department of Neurosciences, Université de Montréal, Montréal, QC, H3T 1J4, Canada. 11. National Reference Center for Narcolepsy, Sleep Unit, Department of Neurology, Gui-de-Chauliac Hospital, CHU Montpellier, University of Montpellier, Inserm U1061, Montpellier, France. 12. Clinical Neurology Unit, Department of Neurosciences, University Hospital of Udine, Udine, Italy. 13.

DMIF, University of Udine, Udine, Italy. 14. Department of Medicine (DAME), University of Udine, Udine, Italy. 15. Sleep Disorders Clinic, Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria. 16. Department of Neurology and Centre of Clinical Neuroscience, Charles University, First Faculty of Medicine and General University Hospital, Prague, Czech Republic. 17. Department of Neurology, Philipps University, Marburg, Germany. 18. Department of Biomedical and Neuromotor Sciences (DIBINEM), Alma Mater Studiorum, University of Bologna, Bologna, Italy. 19. IRCCS, Institute of Neurological Sciences of Bologna, Bologna, Italy. 20. Neurology Unit, Movement Disorders Division, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy. 21. Department of Medical Sciences and Public Health, Sleep Disorder Research Center, University of Cagliari, Cagliari, Italy. 22. Paracelsus-Elena-Klinik, Kassel, Germany. 23. Department of Neurology, University Medical Centre Göttingen, Göttingen, Germany. 24. Sleep and Neurology Unit, Beau Soleil Clinic, Montpellier, France. 25. EuroMov, University of Montpellier, Montpellier, France. 26. University Lille North of France, Department of Clinical Neurophysiology and Sleep Center, CHU Lille, Lille, France. 27. Department of Sleep Medicine and Neuromuscular Disorders, University of Müenster, Müenster, Germany. 28. Department of Neurological Sciences, Università Vita-Salute San Raffaele, Milan, Italy. 29. Laboratory for Sleep Disorders, St. Dimpna Regional Hospital, Geel, Belgium. 30. Department of Neurology, St. Dimpna Regional Hospital, Geel, Belgium. 31. Department of Neurology, University Hospital Antwerp, Edegem, Antwerp, Belgium. 32. Sleep disorder Unit, Carémeau Hospital, University Hospital of Nîmes, France. 33. Department of Neurology, Mayo Clinic, Rochester, MN, USA.

This manuscript was submitted to *Neurology*. March 2020, and it was published in MedRxiv: https://doi.org/10.1101/2020.03.17.20032664

1. Abstract

Objective: To examine the role of autosomal dominant (AD) and recessive (AR) Parkinsonism genes in the risk of isolated rapid-eye-movement (REM) sleep behavior disorder (iRBD).

Methods: Ten genes implicated in AD and AR Parkinsonism were fully sequenced using targeted next-generation sequencing in 1,039 iRBD patients and 1,852 controls of European ancestry. These include the AR genes *PRKN, DJ-1 (PARK7), PINK1, VPS13C, ATP13A2, FBXO7* and *PLA2G6,* and the AD genes *LRRK2, GCH1* and *VPS35*. To examine the role of rare heterozygous variants in these genes, burden test and SKAT-O analyses were performed. The contribution of homozygous and compound heterozygous variants was further examined in the AR genes. Copy number variants (CNVs) in *PRKN* were tested in a subset of samples (n=374) using multiplex ligation-dependent probe amplification followed by analysis of all samples using ExomeDepth.

Results: We found no association between rare heterozygous variants in the tested genes and risk for iRBD. Several homozygous and compound heterozygous carriers were identified with variants of unknown significance, yet there was no overrepresentation in iRBD patients versus controls.

Conclusion: Our results do not support a major role for variants in *PRKN, PARK7, PINK1, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* in the risk of iRBD.

2. Introduction

Isolated rapid eye movement (REM)-sleep behavior disorder (iRBD) is a prodromal neurodegenerative disease. More than 80% of iRBD patients diagnosed with video-polysomnography (vPSG) will eventually convert to an overt α-synucleinopathy.[1] These include mostly Parkinson's disease (PD) and dementia with Lewy bodies (DLB), and a small minority will convert to multiple system atrophy (MSA).[2]

While not much is known about the genetic background of DLB and MSA, accumulating data from the last two decades have unraveled the role of common and rare genetic variants in PD. Currently, 90 independent risk factors of PD in 78 genetic loci are known, discovered through genome-wide association studies (GWAS).[3] Other, less common genetic variants, have been implicated in familial forms of PD, including autosomal dominant (AD) inherited variants in genes such as *SNCA, LRRK2, GCH1* and *VPS35,*[4-6] and autosomal recessive (AR) inherited variants in *PRKN, PINK1* and *PARK7.*[7] Bi-allelic mutations in other genes, including *ATP13A2, VPS13C, FBXO7* and *PLA2G6* may cause AR atypical syndromes with Parkinsonism,[4,8] in some of which α-synucleinopathy has also been reported.[9-11]

The genetic background of iRBD has only been studied in recent years, with studies showing that there is no full genetic overlap between the genetic background of iRBD and that of PD or DLB. For example, *GBA* mutations are associated with risk of iRBD, PD and DLB,[2,12] but pathogenic *LRRK2* mutations seem to be involved only in PD and not in iRBD and DLB.[8,13,14] *MAPT* and *APOE* variants are important risk factors of PD and DLB, respectively,[15,16] but both genes are not associated with iRBD.[15,17] In the *SNCA* locus, there are independent risk variants of PD, DLB and iRBD; specific 3' variants are associated with PD, and other, independent variants at the 5' of *SNCA* are associated with iRBD and DLB.[18] Within the *TMEM175* locus, there are two independent risk factors of PD, but only one of them, the coding polymorphism p.M393T, has also been associated with iRBD.[19]

Thus far, the role of most of the familial PD genes or genes involved in rare forms of atypical parkinsonism has not been studied in iRBD. Here, since *GBA* and *SNCA* have been studied previously,[2,18] we aimed to thoroughly examine the roles of *PRKN, PINK1, PARK7* (*DJ-1*), *VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* in iRBD.

3. Methods

3.1. Population

A total of 1,039 unrelated iRBD patients and 1,852 unrelated controls were included in this study, all of European ancestry (confirmed by principal component analysis of GWAS data). Approximately 81% of the patients were male, the mean reported age at onset (AAO) was 60.1 ± 10.5 years and the average age at diagnosis was 65.3 ± 8.7 years. Data on sex and age were available for 1,032 and 1,004 patients, respectively. Among the controls, about 51% were male, and the mean age at sampling was 52.3 ± 14.3 years, age was not available for nine controls. RBD diagnosis was done with video polysomnography according to the ICSD-2/3 criteria (International Classification of Sleep Disorders, version 2 or 3).[20]

3.2. 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.

3.3. Genetic analysis

The coding sequences and 5' and 3' untranslated regions (UTRs) of *PRKN, PINK1, DJ-1, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* were captured using molecular inversion probes (MIPs) designed as previously described,[21] and the full protocol is available upon request. Details of the MIPs used in the current study are listed in Supplementary Table 1. The library was sequenced on illumina HiSeq 2500\4000 platform at the McGill University and Génome Québec Innovation Centre. Sequencing reads were mapped to the human reference genome (hg19) using the Burrows-Wheeler Aligner.[22] Post-alignment quality control and variant calling were done using the Genome Analysis Toolkit (GATK, v3.8),[23] and annotation with ANNOVAR.[24] The Frequency of each variant was extracted from the Genome Aggregation Database (GnomAD).[25] We used ClinVar and specific searches on PubMed to examine whether variants that were found in these genes are known or suspected to be pathogenic in PD or atypical parkinsonism.

3.4. Quality control

To perform quality control (QC), we used the PLINK software. We excluded variants with: genotyping rate lower than 90%, deviation from Hardy-Weinberg equilibrium set at p=0.001 threshold and when the variant was identified in <25% of the reads for a specific variant. To be included in the analysis, the minimum quality score (QS) was set to 30. Threshold for rate of missingness difference between cases and controls was set at p=0.05, and variants below this threshold were removed. Genotyping rate cut-off for individuals was 90%, and individuals with a lower genotyping rate were excluded. After the QC steps, 1,039 patients and 1,852 controls were included in the analysis. Since we aimed to examine the role of variants that cause monogenic PD, only rare variants (minor allele frequency [MAF]<0.01) were included in the analysis. To ensure that we capture high quality variants, we performed analyses for variants with coverage depth of >30X and variants with >50X.

3.5. Data and statistical analysis

We used different approaches to examine the effect of multiple variants on iRBD risk. To examine whether there is a burden of rare (MAF<0.01) heterozygous variants in each of our targeted genes, we used optimized sequence Kernel association test (SKAT-O, R package)[26] and burden tests for different types of variants: all rare variants, potentially functional rare variants (nonsynonymous, frame-shift, stop-gain and splicing), rare loss-of-function variants (frame-shift, stop-gain and splicing), and rare nonsynonymous variants only. We then examined the association between variants predicted to be pathogenic based on Combined Annotation Dependent Depletion (CADD) score of ≥12.37 (representing the top 2% of potentially deleterious variants) and iRBD. For this analysis, we used burden test (R package SKAT) since the direction of the association was presumed as pathogenic prior to the test. In addition, since copy number variants (CNVs) are frequent in the *PRKN* gene,[27] we included CNVs when we analyzed the association of *PRKN* variants with iRBD. To call CNVs, we first performed multiplex ligation probe amplification (MLPA, the gold standard for CNV detection in *PRKN*) analysis of 374 samples using the SALSA MLPA Probemix P051 Parkinson mix 1 according to the manufacturer's instructions (MRC Holland). Then, using the ExomeDepth tool,[28] we determined the ideal parameters for CNV calls using the MIPs data, with sensitivity of 100% and specificity of 97% when compared to the MLPA results. These parameters were subsequently

applied to call CNVs from the MIPs data across all iRBD patients and controls. The contribution of homozygous and compound heterozygous variants in all the genes was also examined by comparing the frequencies of the very rare (MAF<0.001) nonsynonymous, splice-site, frame-shift and stop-gain variants between patients and controls. Bonferroni correction for multiple comparisons was applied in all analyses.

3.6. Availability of data and materials

Data used for the analysis is available in the supplementary tables. Anonymized raw data can be shared upon request from any qualified investigator.

4. Results

4.1. Quality of coverage

The average coverage of the 10 genes analyzed in this study was >144X for all genes, and the coverage of 8 of the genes was >900X. The per-gene coverage for all 10 genes, although not perfect, is better than the coverage of these specific genes in gnomAD. Supplementary Table 2 details the average coverage and the percentage of nucleotides covered at 20X and 50X for each gene. There were no differences in the coverage across the samples (patients and controls).

4.2. Rare homozygous and compound heterozygous variants are not enriched in iRBD patients

To examine whether homozygous or compound heterozygous variants in our genes of interest may cause iRBD, we compared the carrier frequencies of very rare (MAF <0.001) bi-allelic variants between iRBD patients and controls. Three carriers (one patient and two controls) were identified with homozygous variants across all genes. All three carried homozygous non-coding and synonymous variants that are not likely to cause a disease: one male patient with AAS of 76 years who carried the *PINK1* variant rs181532922, c.*717T>C at the 3’ UTR of the gene, one female control recruited at age 72 who carried the *DJ-1* rs7534132, an intronic variant, and one control recruited at the age of 26 who carried the *LRRK2* rs72546315 synonymous (p.H275H) variant.

For the analysis of compound heterozygous carriers, since phasing could not be performed, we considered carriers of two rare variants as compound heterozygous carriers, with two

exceptions: 1) when variants were physically close and we could determine their phase based on the sequence reads and 2) if the same combination of very rare variants appeared more than once, we assumed that the variants are likely on the same allele. We found a total of 9 patients and controls, presumably compound heterozygous carriers in the studied genes (Table 1). Three affected and three unaffected carriers of compound heterozygous variants in *VPS13C* were identified, with no overrepresentation in iRBD patients (Fisher test, p=1).

4.3. Rare heterozygous variants are not enriched in any of the studied genes

In order to further study the role of rare (MAF<0.01) heterozygous variants, we performed SKAT-O and burden tests, repeated twice for variants detected at coverage depth of >30X and variants detected at >50X (see methods). All rare heterozygous variants identified in each gene are detailed in supplementary table 3. We performed SKAT-O and burden tests at 5 different levels: all rare variants, all potentially functional variants (nonsynonymous, splice site, frame-shift and stop-gain), loss-of-function variants (frame-shift, stop-gain and splicing), nonsynonymous variants only, and variants with CADD score ≥12.37 (Table 2). The Bonferroni corrected p value for statistical significance was set on p<0.001. We found no statistically significant association between iRBD and any of the variant types in any of the genes, suggesting that these genes either have no role in iRBD or have a minor role that we could not detect with this sample size. The nominal association between *PARK7* and iRBD in the SKAT-O analysis of rare functional variants is driven by the nonsynonymous variant rs71653622 (p.A179T) which was ~10 times more frequent in iRBD patients (0.003074) compared to controls (0.000277), but not statistically significant (p=0.09, see supplementary table 3). We did not identify any iRBD patient with known biallelic pathogenic variants in *PARK7, PINK1, VPS13C* and *ATP13A2,* or heterozygous pathogenic variants in *LRRK2, GCH1* and *VPS35*. Two controls were found with the pathogenic *LRRK2* p.G2019S variant. We identified 9 (0.86%) iRBD patients and 13 (0.70%, p=0.65) controls who were heterozygous carriers of the potentially pathogenic variant p.R275W in *PRKN*, and two additional controls with the *PRKN* p.T240M pathogenic variant. One patient and one control with the pathogenic variant p.R299C in *FBXO7* were also found.

4.4. Analysis of copy number variants in *PRKN*

We further examined the association between deletions and duplications in *PRKN* and risk for iRBD. Using ExomeDepth, 7 patients (0.7 %) and 17 controls (0.9%, p=0.53) were found to

carry CNVs in *PRKN*, and none of the patients found to have an additional nonsynonymous variant. Therefore, there were no homozygous or compound heterozygous carriers of rare *PRKN* variants among the iRBD patients. Supplementary table 4 lists all the CNVs found in our cohort.

5. Discussion

The present study provides the first large-scale, full sequencing analysis to examine the possible role of the dominant and recessive parkinsonism genes *PRKN, PARK7, PINK1, VPS13C, ATP13A2, FBXO7, PLA2G6, LRRK2, GCH1* and *VPS35* in iRBD. We did not find evidence for association of any of these genes with iRBD. In the recessive genes, there was no over-representation of carriers of homozygous or compound heterozygous variants in iRBD patients, and no single patient with bi-allelic pathogenic variants. In the dominant genes, we did not find any known pathogenic variants in these genes, and SKAT-O and burden analyses did not identify burden of rare heterozygous variants in any of these 10 genes.

Whether heterozygous carriage of mutations in recessive PD or atypical parkinsonism related genes is a risk factor for PD is still controversial.[29] *PRKN*-associated PD is characterized by pure nigral degeneration without α-synuclein accumulation,[30] and reports on synucleinopathy and Lewy bodies in *PINK1*-associated PD are inconclusive, as some studies identified Lewy bodies while others did not.[31,32] Since iRBD is a prodromal synucleinopathy, it is not surprising that we did not identify bi-allelic mutations or burden of heterozygous variants in any of these genes. Of note, 380 (36.5%) of the iRBD cohort had a self-reported AAO <50 years. In the case of iRBD, reported AAO may be especially unreliable, as patients may have had RBD symptoms long before they were noticed by themselves or their bed partners. Therefore, the true percentage of iRBD patients with AAO <50 is likely higher, yet none of the known genes involved in early onset PD seems to be involved in early onset iRBD.

Recently, we have shown that the *SNCA* locus is important in RBD, yet with different and distinct variants that are associated with risk of PD.[18] In the same study, *SNCA* was fully sequenced and no known PD-causing variants were found in iRBD patients. We and others have previously reported that pathogenic *LRRK2* variants were not identified in smaller cohorts of iRBD,[14] which was further confirmed in the current study. In addition, several studies of PD patients with and without RBD have shown reduced prevalence of RBD [33-36] or reduced scores in RBD questionnaires among *LRRK2* mutation carriers. *VPS35* mutations have not been identified

in iRBD in the current study, although pathogenic *VPS35* mutations are generally rare.[37,38] Altogether, these results provide no evidence that known, well-validated familial gene mutations involved in PD (including *SNCA*, *LRRK2*, *VPS35*, *PRKN*, *PINK1* and *PARK7*) are also involved in iRBD. *GBA* is the only gene in which strong risk variants associated with PD are also associated with iRBD.[2]

Our study has some limitations. While being the largest genetic study of iRBD to date, it may still be underpowered to detect rare variants in familial PD-related genes. Therefore, our study does not completely rule out the possibility that variants in these genes may lead to iRBD in very rare cases. Another potential limitation of the study design is the earlier age and the different sex distribution in the control population, and the fact that they have not been tested for iRBD. However, since iRBD is not common, found in about 1% of the population,[1] age would have a minimal or no effect on the results. The differences in sex ratios are less likely to have an effect, since in AD and AR Mendelian diseases, the risk is typically similar for men and women.

To conclude, the lack of association between different PD and Parkinsonism genes may suggest that either iRBD is an entity more affected by environmental factors, or that there are other, yet undetected genes that may be involved in iRBD. To examine these possibilities, larger studies that include carefully collected epidemiological data and more extensive genetic data such as whole-exome or whole-genome sequencing will be required. Our study also suggests that screening for variants in the tested genes will have a very low yield.

Table 1. Summary of all samples carrying two nonsynonymous variants detected in the present study

Gene	Sample	Sex	AAS	dbSNP	Allele*	Substitution	F_A	F_C	gnomAD ALL	gnomAD NFE
PRKN	C	M	46	rs137853054	G/A	p.T212M	0	0.0005504	0.0004	0.0003
				rs9456735	G/T	p.M192L	0	0.001101	0.0043	0.0003
PINK1	C	M	57	rs370906995	C/T	p.T257I	0	0.0002756	7.02E-05	0.0001
				rs372280083	C/G	p.L268V	0	0.0002756	9.34E-05	0.0001
VPS13C	A	M	75	15:62165489	C/A	p.D3469Y	0.0005092	0	-	-
				15:62204039	A/C	p.E2862D	0.0005139	0	-	-
VPS13C	C	F	60	rs746819519	C/T	p.G3172D	0	0.001096	1.76E-05	0.00003753
				rs202056315	A/C	p.V2235G	0	0.0002744	4.06E-05	0.00001793
VPS13C	C	M	30	rs780081183	G/C	p.A2368P	0	0.0002738	1.24E-05	0.00002724
				15:62302740	C/G	p.E271D	0	0.0002738	-	-
VPS13C	C	M	52	rs767080349	A/G	p.M2344T	0	0.0002738	1.87E-05	0.0000187
				rs370832130	C/T	p.M1416V	0	0.0002738	0.0001	0.0001
VPS13C	A	M	64	rs760460320	G/C	p.D1496H	0.0005081	0	1.75E-05	0.00002803
				rs765303583	G/C	p.Q660E	0.0005081	0	0	0
VPS13C	A	M	59	rs141515062	T/A	p.S522T	0.001016	0	0.0002	0.0004
				rs376219715	C/T	p.Y365C	0.001016	0	1.63E-05	0.00003598
LRRK2	C	M	63	rs886344692	A/T	p.R1282S	0	0.000275	1.63E-05	2.69E-05
				rs202179802	A/G	p.T2310A	0	0.000275	4.47E-05	7.17E-05

Abbreviations: A = Affected; C = Control; M = Male; F = Female; AAS Age at sampling; dbSNP = Single nucleotide polymorphism database; *Allele = Reference allele/mutant allele; F_A = Frequency in affected; F_C = Frequency in controls; gnomAD ALL= Exome allele frequency in all populations; gnomAD NFE = Exome allele frequency in non-Finnish European.

Table 2. Summary of results from burden analyses of rare heterozygous variants

DOC	Gene	All rare (*p* value)		Rare functional (*p* value)		Rare LOF (*p* value)		Rare NS (*p* value)		Rare CADD (*p* value)	
		SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden
30x	Recessive genes										
	PRKN	0.4316	0.484	0.388	0.240	NV	NV	0.508	0.331	1	0.889
	PARK7	0.104	0.254	0.008	0.369	0.175	0.174	0.005	0.005	NV	NV
	PINK1	0.703	0.505	0.117	0.605	NV	NV	0.117	0.605	0.124	0.494
	Recessive (atypical) genes										
	ATP13A2	0.543	0.383	0.379	0.227	NV	NV	0.379	0.227	0.201	0.121
	FBXO7	0.525	0.562	0.266	0.140	0.163	0.252	0.327	0.160	0.228	0.279
	PLA2G6	0.325	0.859	0.222	0.663	0.260	0.193	0.243	0.948	0.196	0.688
	VPS13C	0.018	0.047	0.334	0.206	0.237	0.137	0.343	0.207	0.468	0.834
	Dominant genes										
	GCH1	0.361	0.217	0.730	0.804	0.730	0.804	NV	NV	NV	NV
	LRRK2	0.601	0.827	0.578	0.888	0.134	0.199	0.590	0.966	0.610	0.871

	VPS35	0.159	0.111	0.161	0.247	0.382	0.522	0.161	0.247	0.434	0.807
50x	Recessive genes										
	PRKN	0.085	0.084	0.452	0.609	NV	NV	0.452	0.609	0.771	0.564
	PARK7	0.180	0.288	0.017	0.436	NV	NV	0.010	0.010	NV	NV
	PINK1	0.572	0.546	0.050	0.133	NV	NV	0.050	0.133	0.050	0.133
	Recessive (atypical) genes										
	ATP13A2	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	FBXO7	0.618	0.624	0.209	0.125	0.331	0.613	0.256	0.148	0.540	0.309
	PLA2G6	0.528	0.853	0.360	0.680	0.680	0.452	0.360	0.680	0.680	0.452
	VPS13C	0.101	0.055	0.073	0.038	0.777	0.971	0.149	0.082	0.332	0.227
	Dominant genes										
	GCH1	0.901	0.817	0.734	0.760	0.734	0.760	NV	NV	NV	NV
	LRRK2	0.030	0.019	0.279	0.173	0.062	0.088	0.525	0.377	0.527	0.365
	VPS35	0.453	0.549	NV	NV	NV	NV	NV	NV	NV	NV

Abbreviations: DOC = Depth of coverage; CADD = Combined annotation dependent depletion; NS = Nonsynonymous; LOF = Loss of function; SKAT-O = Optimized sequence kernel association test; SKAT = Kernel association test; NV = No variants were found for this filter

Supplementary tables 1&3 can be accessed online through medRxiv:

https://doi.org/10.1101/2020.03.17.20032664

Supplementary Table 2. Average coverage of the genes analyzed in the current study

Gene	Average coverage	%20x	%50x
PARK2	1742.91	0.973	0.973
PARK7	2138.28	0.935	0.887
PINK1	1383.01	0.905	0.905
VPS13C	1460.67	0.926	0.893
ATP13A2	144.02	0.856	0.621
FBXO7	1583.55	0.970	0.920
PLA2G6	344.74	0.894	0.788
GCH1	939.84	0.982	0.936
LRRK2	993.70	0.946	0.915
VPS35	1096.60	0.953	0.953

Supplementary Table 4. All copy number variants identified in *PRKN*

start.p	end.p	type	nexons	start	end	chr	ID	BF	Reads expected	Reads observed	Reads ratio	correlation	numRef	S_Number	Affected?
896	898	deletion	3	162683534	162683853	6	chr6:162683534-162683853	43.1	3642	1967	0.54	0.991419	6	S02762	C
866	868	deletion	3	161770165	161770456	6	chr6:161770165-161770456	34	6152	4274	0.695	0.995788	13	S04524	C
866	868	deletion	3	161770165	161770456	6	chr6:161770165-161770456	14.6	2285	1592	0.697	0.993801	16	S04784	C
899	901	deletion	3	162864271	162864599	6	chr6:162864271-162864599	42.7	1515	803	0.53	0.991709	8	S05512	C
866	868	deletion	3	161770165	161770456	6	chr6:161770165-161770456	14.1	1117	727	0.651	0.98786	5	S17295	C
881	891	duplication	11	161807807	162394535	6	chr6:161807807-162394535	68	12959	19120	1.48	0.989475	10	S21514	C
858	860	deletion	3	161769350	161769677	6	chr6:161769350-161769677	65.7	4776	1930	0.404	0.990457	14	S21533	C
899	901	duplication	3	162864271	162864599	6	chr6:162864271-162864599	21.2	5612	7465	1.33	0.992047	5	S21652	C
898	898	deletion	1	162683743	162683853	6	chr6:162683743-162683853	10.7	1091	583	0.534	0.988085	3	S32119	C
894	898	deletion	5	162622143	162683853	6	chr6:162622143-162683853	53.1	5795	3227	0.557	0.993717	20	S32268	C
868	868	deletion	1	161770346	161770456	6	chr6:161770346-161770456	10.2	610	335	0.549	0.994275	20	S32275	C
883	885	deletion	3	161969861	161970142	6	chr6:161969861-161970142	21.3	6543	4274	0.653	0.993908	16	S32612	C
868	868	deletion	1	161770346	161770456	6	chr6:161770346-161770456	9.26	413	240	0.581	0.995402	20	S32897	C
896	898	deletion	3	162683534	162683853	6	chr6:162683534-162683853	35	2023	1037	0.513	0.990884	10	S32915	C
899	901	deletion	3	162864271	162864599	6	chr6:162864271-162864599	49.6	1385	629	0.454	0.994694	18	S32929	C
894	898	deletion	5	162622143	162683853	6	chr6:162622143-162683853	66.3	3337	1723	0.516	0.993689	11	S32937	C
899	901	deletion	3	162864271	162864599	6	chr6:162864271-162864599	64.6	3126	1413	0.452	0.995172	10	S33018	C
894	898	deletion	5	162622143	162683853	6	chr6:162622143-162683853	30.8	8666	4346	0.502	0.978159	3	S28069	A
899	901	duplication	3	162864271	162864599	6	chr6:162864271-162864599	9.08	546	877	1.61	0.978138	4	S33133	A
866	868	deletion	3	161770165	161770456	6	chr6:161770165-161770456	10.8	4004	2789	0.697	0.991844	7	S33200	A
898	901	deletion	4	162683743	162864599	6	chr6:162683743-162864599	20.7	4106	2046	0.498	0.979679	15	S34321	A
896	901	duplication	6	162683534	162864599	6	chr6:162683534-162864599	9.6	9969	12604	1.26	0.989351	6	S34393	A
896	901	duplication	6	162683534	162864599	6	chr6:162683534-162864599	21.1	11755	18068	1.54	0.981061	10	S34412	A
896	901	duplication	6	162683534	162864599	6	chr6:162683534-162864599	12	7416	9974	1.34	0.985364	8	S34418	A

Acknowledgment

This work was supported by the Michael J. Fox Foundation, the Canadian Consortium on Neurodegeneration in Aging (CCNA), Parkinson Canada and the Canada First Research Excellence Fund (CFREF), with funds awarded to McGill University for the Healthy Brains for Healthy Lives (HBHL) program. The Montreal cohort was funded by the Canadian Institutes of Health Research (CIHR) and the W. Garfield Weston Foundation. The Oxford Discovery study is funded by the Monument Trust Discovery Award from Parkinson's UK and supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, the NIHR Clinical Research Network and the Dementias and Neurodegenerative Diseases Research Network (DeNDRoN). JFG holds a Canada Research Chair in Cognitive Decline in Pathological Aging. WO is Hertie Senior Research Professor, supported by the Hertie Foundation. EAF holds a Canada Research Chair (Tier 1) in Parkinson disease. GAR holds a Canada Research Chair in Genetics of the Nervous System and the Wilder Pen field Chair in Neurosciences. ZGO is supported by the Fonds de recherche du Québec–Santé Chercheur-Boursier award and is a Parkinson Canada New Investigator awardee. We thank D. Rochefort, H. Catoire, and V. Zaharieva for their assistance.

References

1. Postuma RB, Iranzo A, Hu M, et al. Risk and predictors of dementia and parkinsonism in idiopathic REM sleep behaviour disorder: a multicentre study. Brain 2019;142:744-759.
2. Gan-Or Z, Mirelman A, Postuma RB, et al. GBA mutations are associated with Rapid Eye Movement Sleep Behavior Disorder. Annals of clinical and translational neurology 2015;2:941-945.
3. Nalls M, Blauwendraat C, Vallerga C, et al. Expanding Parkinson's disease genetics: novel risk loci, genomic context, causal insights and heritable risk. bioRxiv. Epub, 2019.
4. Gan-Or Z, Alcalay RN, Rouleau GA, Postuma RB. Sleep disorders and Parkinson disease; lessons from genetics. Sleep Medicine Reviews 2018;41:101-112.
5. Chenbhanich J, Sringean J, Bhidayasiri R. Beyond the Classic Segawa Disease, GCH1-Associated Neurodegenerative Parkinsonism: Practical Considerations for Physicians. JMD 2017;10:102-104.
6. Guella I, Sherman HE, Appel-Cresswell S, Rajput A, Rajput AH, Farrer MJ. Parkinsonism in GTP cyclohydrolase 1 mutation carriers. Brain 2014;138:e349-e349.
7. Kilarski LL, Pearson JP, Newsway V, et al. Systematic review and UK-based study of PARK2 (parkin), PINK1, PARK7 (DJ-1) and LRRK2 in early-onset Parkinson's disease. Movement Disorders 2012;27:1522-1529.
8. Blauwendraat C, Nalls MA, Singleton ABJTLN. The genetic architecture of Parkinson's disease. 2019.
9. Usenovic M, Tresse E, Mazzulli JR, Taylor JP, Krainc D. Deficiency of ATP13A2 Leads to Lysosomal Dysfunction, α-Synuclein Accumulation, and Neurotoxicity. 2012;32:4240-4246.
10. Conedera S, Apaydin H, Li Y, et al. FBXO7 mutations in Parkinson's disease and multiple system atrophy. 2016;40:192. e191-192. e195.
11. Guo Y-P, Tang B-S, Guo J-F. PLA2G6-Associated Neurodegeneration (PLAN): Review of Clinical Phenotypes and Genotypes. Front Neurol 2018;9:1100-1100.
12. Gan-Or Z, Rouleau GA. Genetics of REM Sleep Behavior Disorder. In: Schenck CH, Högl B, Videnovic A, eds. Rapid-Eye-Movement Sleep Behavior Disorder. Cham: Springer International Publishing, 2019: 589-609.
13. Heckman MG, Soto-Ortolaza AI, Sanchez Contreras MY, et al. LRRK2 variation and dementia with Lewy bodies. Parkinsonism & Related Disorders 2016;31:98-103.

14. Bencheikh BOA, Ruskey JA, Arnulf I, et al. LRRK2 protective haplotype and full sequencing study in REM sleep behavior disorder. 2018;52:98-101.
15. Li J, Ruskey JA, Arnulf I, et al. Full sequencing and haplotype analysis of MAPT in Parkinson's disease and rapid eye movement sleep behavior disorder. 2018;33:1016-1020.
16. Dickson DW, Heckman MG, Murray ME, et al. APOE ε4 is associated with severity of Lewy body pathology independent of Alzheimer pathology. 2018;91:e1182-e1195.
17. Gan-Or Z, Montplaisir JY, Ross JP, et al. The dementia-associated APOE ε4 allele is not associated with rapid eye movement sleep behavior disorder. 2017;49:218. e213-218. e215.
18. Krohn L, Wu RYJ, Heilbron K, et al. Fine-Mapping of SNCA in Rapid Eye Movement Sleep Behavior Disorder and Overt Synucleinopathies. 2020;87(4):584-598.
19. Krohn L, Öztürk TN, Vanderperre B, et al. Genetic, Structural, and Functional Evidence Link TMEM175 to Synucleinopathies. 2020;87:139-153.
20. Thorpy MJJN. Classification of sleep disorders. 2012;9:687-701.
21. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619-1622.
22. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. bioinformatics 2009;25:1754-1760.
23. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research 2010;20:1297-1303.
24. Li X, Patel JC, Wang J, et al. Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. J Neurosci 2010;30:1788-1797.
25. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016;536:285-291.
26. Lee S, Emond MJ, Bamshad MJ, et al. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. 2012;91:224-237.
27. Lücking CB, Dürr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. New England Journal of Medicine 2000;342:1560-1567.

28. Plagnol V, Curtis J, Epstein M, et al. A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. 2012;28:2747-2754.
29. Reed X, Bandrés-Ciga S, Blauwendraat C, Cookson MR. The role of monogenic genes in idiopathic Parkinson's disease. Neurobiology of Disease 2019;124:230-239.
30. Schneider SA, Alcalay RN. Neuropathology of genetic synucleinopathies with parkinsonism: review of the literature. Movement Disorders 2017;32:1504-1523.
31. Samaranch L, Lorenzo-Betancor O, Arbelo JM, et al. PINK1-linked parkinsonism is associated with Lewy body pathology. Brain 2010;133:1128-1142.
32. Takanashi M, Li Y, Hattori N. Absence of Lewy pathology associated with PINK1 homozygous mutation. Neurology 2016;86:2212-2213.
33. Fernández-Santiago R, Iranzo A, Gaig C, et al. Absence of LRRK2 mutations in a cohort of patients with idiopathic REM sleep behavior disorder. 2016;86:1072-1073.
34. Zhang J, Xu C-Y, Liu J. Meta-analysis on the prevalence of REM sleep behavior disorder symptoms in Parkinson's disease. BMC Neurol 2017;17:23-23.
35. Zhang X, Sun X, Wang J, Tang L, Xie A. Prevalence of rapid eye movement sleep behavior disorder (RBD) in Parkinson's disease: a meta and meta-regression analysis. Neurological Sciences 2017;38:163-170.
36. Jiang H, Huang J, Shen Y, et al. RBD and Neurodegenerative Diseases. Molecular Neurobiology 2017;54:2997-3006.
37. Lesage S, Condroyer C, Klebe S, et al. Identification of VPS35 mutations replicated in French families with Parkinson disease. 2012;78:1449-1450.
38. Kumar KR, Weissbach A, Heldmann M, et al. Frequency of the D620N Mutation in VPS35 in Parkinson Disease. Archives of Neurology 2012;69:1360-1364.

Preface to Chapter 3

While Chapter 2 discussed 10 genes that are known to cause familial forms of PD and atypical parkinsonism, the next logical step is to study genes within GWAS loci associated with PD. The work described in Chapter 3 is focused on 25 genes identified within PD GWAS loci.[21, 86] Although recent GWASs identified more than 90 genes from 80 loci,[20-23] this study was designed earlier, with the target genes selected from previous PD GWASs.[21, 86] We selected 25 genes based on several criteria including expression profile and biological plausibility. As we hypothesized that some of these genes might be associated with iRBD, we aimed to test this hypothesis by thoroughly examining whether common or rare variants (heterozygous and bi-allelic) in these 25 genes could be associated with iRBD.

CHAPTER 3: Novel associations of *BST1* and *LAMP3* with rapid eye movement sleep behavior disorder

Kheireddin Mufti[1,2], Eric Yu[1,2] Uladzislau Rudakou[1,2], Lynne Krohn[1,2], Jennifer A. Ruskey[2,3], Farnaz Asayesh[2,3], Sandra B. Laurent[2,3], Dan Spiegelman[2,3], Isabelle Arnulf[4], Michele T.M. Hu[5,6], Jacques Y. Montplaisir[7,8], Jean-François Gagnon[7,9], Alex Desautels[7,10], Yves Dauvilliers[11], Gian Luigi Gigli[12,13], Mariarosaria Valente[12,14], Francesco Janes[12], Andrea Bernardini[12], Birgit Högl[15], Ambra Stefani[15], Evi Holzknecht[15], Karel Sonka[16], David Kemlink[16], Wolfgang Oertel[17], Annette Janzen[17], Giuseppe Plazzi[18,19], Elena Antelmi[20], Michela Figorilli[21], Monica Puligheddu[21], Brit Mollenhauer[22,23], Claudia Trenkwalder[22.23], Friederike Sixel-Döring[17,22], Valérie Cochen De Cock[24,25] Christelle Charley Monaca[26], Anna Heidbreder[27], Luigi Ferini-Strambi[28], Femke Dijkstra[29,30,31], Mineke Viaene[29,30], Beatriz Abril[32], Bradley F. Boeve[33], Jean-François Trempe[34], Guy A. Rouleau[1,2,3], Ronald B. Postuma[2,3,7], Ziv Gan-Or[1,2,3].

Affiliations: 1. Department of Human Genetics, McGill University, Montréal, QC, Canada. 2. Montreal Neurological Institute, McGill University, Montréal, QC, Canada. 3. Department of Neurology and Neurosurgery, McGill University, Montréal, QC, Canada. 4. Sleep Disorders Unit, Pitié Salpêtrière Hospital, Centre de Recherche de l'Institut du Cerveau et de la Moelle Epinière and Sorbonne University, Paris, France. 5. Oxford Parkinson's Disease Centre (OPDC), University of Oxford, Oxford, United Kingdom. 6. Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom. 7. Centre d'Études Avancées en Médecine du Sommeil, Hôpital du Sacré-Cœur de Montréal, Montréal, QC, Canada. 8. Department of Psychiatry, Université de Montréal, Montréal, QC, Canada. 9. Department of Psychology, Université du Québec à Montréal, Montréal, QC, Canada. 10. Department of Neurosciences, Université de Montréal, Montréal, QC, H3T 1J4, Canada. 11. National Reference Center for Narcolepsy, Sleep Unit, Department of Neurology, Gui-de-Chauliac Hospital, CHU Montpellier, University of Montpellier, Inserm U1061, Montpellier, France. 12. Clinical Neurology Unit, Department of Neurosciences, University Hospital of Udine, Udine, Italy. 13. DMIF, University of Udine, Udine, Italy. 14. Department of Medicine (DAME), University of

Udine, Udine, Italy. 15. Sleep Disorders Clinic, Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria. 16. Department of Neurology and Centre of Clinical Neuroscience, Charles University, First Faculty of Medicine and General University Hospital, Prague, Czech Republic. 17. Department of Neurology, Philipps University, Marburg, Germany. 18. Department of Biomedical and Neuromotor Sciences (DIBINEM), Alma Mater Studiorum, University of Bologna, Bologna, Italy. 19. IRCCS, Institute of Neurological Sciences of Bologna, Bologna, Italy. 20. Neurology Unit, Movement Disorders Division, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy. 21. Department of Medical Sciences and Public Health, Sleep Disorder Research Center, University of Cagliari, Cagliari, Italy. 22. Paracelsus-Elena-Klinik, Kassel, Germany. 23. Department of Neurology, University Medical Centre Göttingen, Göttingen, Germany. 24. Sleep and Neurology Unit, Beau Soleil Clinic, Montpellier, France. 25. EuroMov, University of Montpellier, Montpellier, France. 26. University Lille North of France, Department of Clinical Neurophysiology and Sleep Center, CHU Lille, Lille, France. 27. Department of Sleep Medicine and Neuromuscular Disorders, University of Müenster, Müenster, Germany. 28. Department of Neurological Sciences, Università Vita-Salute San Raffaele, Milan, Italy. 29. Laboratory for Sleep Disorders, St. Dimpna Regional Hospital, Geel, Belgium. 30. Department of Neurology, St. Dimpna Regional Hospital, Geel, Belgium. 31. Department of Neurology, University Hospital Antwerp, Edegem, Antwerp, Belgium. 32. Sleep disorder Unit, Carémeau Hospital, University Hospital of Nîmes, France. 33. Department of Neurology, Mayo Clinic, Rochester, MN, USA. 34. Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, Canada.

This manuscript was submitted to *Brain*. June 2020, and it was published in MedRxiv: https://doi.org/10.1101/2020.06.27.20140350

1. Abstract

Isolated rapid-eye-movement (REM) sleep behavior disorder (iRBD) is a parasomnia, characterized by loss of muscle atonia and dream enactment occurring during REM sleep phase. Since a large subgroup of iRBD patients will convert to Parkinson's disease, and since previous genetic studies have suggested common genes, it is likely that there is at least a partial overlap between iRBD and Parkinson's disease genetics. To further examine this potential overlap and to identify genes specifically involved in iRBD, we fully sequenced 25 genes previously identified in genome-wide association studies of Parkinson's disease. The genes were captured and sequenced using targeted next-generation sequencing in a total of 1,039 iRBD patients and 1,852 controls of European ancestry. The role of rare heterozygous variants in these gene was examined using burden tests and optimized sequence Kernel association tests (SKAT-O), adjusted for age and sex. The contribution of biallelic (homozygous and compound heterozygous) variants was further tested in all genes. To examine the association of common variants in the target genes, we used logistic regression adjusted for age and sex. We found a significant association between rare heterozygous nonsynonymous variants in *BST1* and iRBD (p=0.0003 at coverage >50X and 0.0004 at >30X), mainly driven by three nonsynonymous variants (p.V85M, p.I101V and p.V272M) found in a total of 22 (1.2%) controls vs. two (0.2%) patients. Rare non-coding heterozygous variants in *LAMP3* were also found to be associated with reduced iRBD risk (p=0.0006 at >30X). We found no statistically significant association between rare heterozygous variants in the rest of genes and risk of iRBD. Several carriers of biallelic variants were identified, yet there was no overrepresentation in iRBD patients vs. controls. To examine the potential impact of the rare nonsynonymous *BST1* variants on the protein structure, we performed *in silico* structural analysis. All three variants seem to be loss-of-function variants significantly affecting the protein structure and stability. Our results suggest that rare coding variants in *BST1* and rare non-coding variants in *LAMP3* are associated with iRBD, and additional studies are required to replicate these results and examine whether loss-of-function of *BST1* could be a therapeutic target.

2. Introduction

Isolated rapid-eye-movement sleep behavior disorder (iRBD) is a prodromal synucleinopathy, as more than 80% of iRBD patients will eventually convert to an overt neurodegenerative syndrome associated with α-synuclein pathology. Typically, iRBD patients will convert to Parkinson's disease (about 40-50% of patients), dementia with Lewy bodies or unspecified dementia (40-50%), or, in much fewer cases, to multiple system atrophy (5-10%) [1, 2]. While our understanding of the genetic background of dementia with Lewy bodies or multiple system atrophy is limited, the rapid development of various genetic methods during the recent decades has led to wealth of data on the role of common and rare genetic variants in Parkinson's disease. To date, there are 80 genetic loci found to be associated with Parkinson's disease risk discovered through genome-wide association studies (GWASs) [3, 4], and several genes have been implicated in familial Parkinson's disease [5-7].

In order to study the genetic background of iRBD and its conversion to α-synucleinopathies, recent studies have examined whether Parkinson's disease- or dementia with Lewy bodies-related genes are also associated with iRBD. These studies have suggested that while there is some overlap between the genetic backgrounds of iRBD and Parkinson's disease or dementia with Lewy bodies, this overlap is only partial. For example, it was demonstrated that *GBA* variants are associated with iRBD risk, Parkinson's disease and dementia with Lewy bodies [5, 8], but pathogenic *LRRK2* variants are found to only be associated with Parkinson's disease, and not with iRBD and dementia with Lewy bodies [7, 9, 10]. We have recently reported that the familial Parkinson's disease and atypical parkinsonism genes *PRKN*, *PARK7*, *GCH1*, *VPS35*, *ATP13A2*, *VPS13C*, *FBXO7* and *PLA2G6* are not likely to be involved in iRBD [11]. Heterozygous variants in *SMPD1* have been reported to be associated with Parkinson's disease risk [12, 13], yet no association was found with iRBD [14]. Whereas variants in *MAPT* are associated with Parkinson's disease and *APOE* haplotypes are important risk factors of dementia with Lewy bodies, [15, 16], neither are linked to iRBD [15, 17]. Furthermore, there are independent risk variants of Parkinson's disease, dementia with Lewy bodies and iRBD within *SNCA* locus; specific variants in the 3' untranslated region (UTR) are associated with Parkinson's disease but not with iRBD, and other, independent variants at 5' UTR are associated with Parkinson's disease, iRBD and dementia with Lewy bodies [18]. In the *TMEM175* locus, there are two independent Parkinson's disease risk variants, but only one of them, p.M393T, has also been associated with iRBD risk [19].

Thus far, the role of most Parkinson's disease GWAS genes has not been thoroughly studied in iRBD. In the current study, we aimed to examine whether rare and common variants in 25 Parkinson's disease-related GWAS genes are associated with iRBD. The entire coding regions with the exon-intron boundaries as well as the regulatory 3' and 5' UTRs were captured and sequenced. We then performed different genetic analyses to investigate the association of rare and common variants in these genes with iRBD.

3. Materials and methods

3.1. Study population

This study included a total of 2,891 subjects, composed of 1,039 unrelated individuals diagnosed with iRBD (according to the International Classification of Sleep Disorders criteria, version 2 or 3) and 1,852 controls. Details on age and sex of patients and controls have been previously described [11] and can be found in Supplementary Table 1. Differences in age and sex were taken into account as needed in the statistical analysis. All patients and controls were of European ancestry (confirmed by principal component analysis [PCA] of GWAS data compared to data from HapMap v.3 and hg19/GRCh37).

3.2. Standard protocol approvals, registrations, and patient consents

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

3.3. Selection of genes and genetic analysis

The current study was designed and performed before the publication of the recent Parkinson's disease GWAS [3], therefore, the genes for analysis were selected from previous GWASs [20, 21]. A total of 25 genes were selected for analysis, including: *ACMSD*, *BST1*, *CCDC62*, *DDRGK1*, *DGKQ*, *FGF20*, *GAK*, *GPNMB*, *HIP1R*, *ITGA8*, *LAMP3*, *MAPT*, *MCCC1*, *PM20D1*, *RAB25*, *RAB29*, *RIT2*, *SETD1A*, *SLC41A1*, *STK39*, *SIPA1L2*, *STX1B*, *SYT11*, *TMEM163* and *USP25*. These genes were selected based on the presence of one or more of the following: quantitative trait loci, expression in brain, potential interaction with known Parkinson's disease-associated genes and involvement in pathways implicated in Parkinson's disease, such the autophagy-lysosomal pathway, mitochondria quality control and endolysosomal recycling. The 25 genes were fully

captured (coding sequence and 3'- and 5'- untranslated regions) using molecular inversion probes (MIPs) designed as previously described [22]. The full protocol is available upon request. Supplementary Table 2 details the probes used in the current study for the MIPs capture. Targeted next-generation sequencing (NGS) was performed post-capture using illumina HiSeq 2500\4000 platform at the McGill University and Génome Québec Innovation Centre. Sequencing reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler Aligner [23]. Genome Analysis Toolkit (GATK, v3.8) was used for post-alignment quality control and variant calling [24], and ANNOVAR for annotation [25]. The Frequency of each variant was extracted from the Genome Aggregation Database (GnomAD) [26].

3.4. Quality control

Quality control (QC) was performed using PLINK v1.9 [27]. We excluded variants that deviated from Hardy-Weinberg equilibrium in controls with a threshold set at p=0.001, and those identified in <25% of the reads for a specific variant. We also filtered out variants with genotyping rate lower than 90%. The same genotyping rate cut-off was used for exclusion of individual samples. Threshold for rate of missingness difference between patients and controls was set at p=0.05, and variants below this threshold were excluded from the analysis. To be included in the analysis, the minimum genotype quality score was set to 30. We used two coverage thresholds for rare variants (minor allele frequency [MAF] <0.01), >30X and >50X, and all analyses were repeated using these thresholds. For the analysis of common variants, coverage of >15X was used.

3.5. Statistical analysis

To test whether rare heterozygous variants (defined by MAF<0.01) in each of our target genes are associated with iRBD, we performed sequence kernel association test (SKAT, R package) [28] and optimized sequence kernel association test (SKAT-O) on different groups of variants: all rare variants, potentially functional rare variants (including nonsynonymous, frame-shift, stop-gain and splicing), rare loss-of-function variants (frame-shift, stop-gain and splicing), and rare nonsynonymous variants only. In addition, we further tested whether rare variants that are predicted to be pathogenic based on Combined Annotation Dependent Depletion (CADD) score of ≥12.37 (representing the top 2% of potentially deleterious variants) are enriched in iRBD patients. To test the association between biallelic variants and iRBD risk, we compared the

frequencies of carriers of two vary rare (MAF<0.001) nonsynonymous, splice-site, frame-shift and stop-gain variants between patients and controls using Fisher's exact test. Bonferroni correction for multiple comparisons was applied as necessary. We tested the association between common variants (MAF>0.01) in the target genes and iRBD risk using logistic regression adjusted for age and sex using PLINK v1.9. Linkage disequilibrium between the discovered variants and the respective GWAS top hits was examined using the non-Finnish European reference cohort on LDlink (https://ldlink.nci.nih.gov/) [29]. Effects of common variants on expression was viewed using the genotype-tissue expression database (GTEx - https://www.gtexportal.org). We further performed in silico structural analysis of *BST1* to test whether the rare coding variants that were found to be associated with iRBD in our analysis could potentially affect the enzyme structure and activity. The atomic coordinates of human *BST1* bound to ATP-γ-S were downloaded from the Protein Data Bank (ID 1isg). The steric clashes induced by each variant were evaluated using the "mutagenesis" toolbox in PyMol v. 2.2.0.

3.6. Data availability

Data used for the analysis is available in the supplementary tables. Anonymized raw data can be shared upon request.

4. Results

4.1. Coverage and identified variants

The average coverage of the 25 genes analyzed in this study was >647X (range 73-1162, median 790). An average of 95% of the target regions were covered with >15X, 93% with >30X and 90% with >50X. The average coverage of each gene and the percentage of the nucleotides covered at 15X, 30X and 50X are detailed in Supplementary Table 3. Finally, there were no differences in the coverage between patients and controls. A total of 1,189 rare variants were found with coverage of > 30X, and 570 rare variants with > 50X (Supplementary Table 4). We identified 125 common variants across all genes (Supplementary Table 5) with a coverage of >15X.

4.2. Rare heterozygous variants in *BST1* and *LAMP3* are associated with iRBD

To examine whether rare heterozygous variants in our genes of interest may be associated with iRBD risk, we performed SKAT and SKAT-O tests, repeated twice for variants detected at depths

of coverage of >30X and >50X (see methods). Supplementary Table 4 details all rare heterozygous variants identified in each gene and included in the analysis. We applied both SKAT and SKAT-O on five different groups of variants: all rare variants, all potentially functional variants (nonsynonymous, splice site, frame-shift and stop-gain), loss-of-function variants (frame-shift, stop-gain and splicing), nonsynonymous variants only, and variants with CADD score ≥12.37 (Table 1). The Bonferroni-corrected *p*-value threshold for statistical significance was set at $p<0.001$ after correcting for the number of genes and depths of coverage.

We found a statistically significant association of rare heterozygous functional variants in *BST1* (SKAT $p=0.0004$ at >30X and $p=0.0003$ at >50X for rare functional variants), found more in controls than in iRBD patients. This association is mainly driven by the nonsynonymous variants p.V85M (rs377310254, found in five controls and none in patients), p.I101V (rs6840615, found in seven controls and none in patients), and p.V272M (rs144197373, found in 10 controls and two patients). Overall, these variants were found in 22 (1.2%) controls vs. 2 (0.2%) patients. Another statistically significant association was found between rare variants in *LAMP3* gene and reduced iRBD risk in SKAT-O analysis. This association is driven by two non-coding variants (one intronic [location - chr3:182858302] and one at the 3' UTR of *LAMP3* [rs56682988, c.*415T>C]) found only in controls (15 and nine controls, respectively). In order to further examine whether these variants indeed drive the association in both *BST1* and *LAMP3*, we excluded them and repeated the analysis (SKAT and SKAT-O), which resulted in loss of statistical significance for both genes (Supplementary table 6). There were no additional statistically significant associations of the remaining genes with iRBD after correcting for multiple comparisons ($p<0.001$).

4.3. Structural analysis of *BST1* variants suggests that loss-of-function may be protective in iRBD

To investigate the potential impact of the three *BST1* nonsynonymous variants (p.V85M, p.I101V and p.V272M) on the structure and activity of the enzyme, we performed *in silico* mutagenesis and evaluated potential clashes with surrounding residues. Figure 1 depicts the structure of *BST1* with the respective locations of the three nonsynonymous variants that drive the *BST1* association detected in our analysis. The structure of human *BST1* was solved by X-ray crystallography in complex with five substrate analogues [30]. All structures revealed a homodimeric assembly, with the catalytic clefts facing the cavity at the interface of the two chains (Figure 1A).

The sidechain of p.V85M points towards the hydrophobic core of the protein, behind a helix facing the nucleotide binding site. The amino-acid change from valine to the bulkier sidechain of methionine results in clashes with other residues in the core, for all rotamers (Figure 1B). This variant would therefore likely destabilize the enzyme active site and potentially unfold the protein. The sidechain of the variant p.I101V is located underneath the active site towards the hydrophobic core. Although the amino-acid change from isoleucine to the smaller sidechain of valine does not create clash (Figure 1C), it reduces the packing in the core, which could also destabilize the enzyme. Finally, the p.V272M variant is located in a helix at the C-terminus of the protein that forms symmetrical contacts with the same helix in the other chain of the dimer. The p.V272M variant would create clashes with sidechain and main-chain atoms located in the other chain of the dimer (Figure 1D). As p.V272M resides at the dimer interface of the enzyme and probably helps maintaining the two subunits together, this variant would most likely lead to the disruption of the dimer. Overall, all the disease-associated nonsynonymous variants in *BST1* (p.V85M, p.I101V, and p.V272M) appear to be "loss-of-function", suggesting that reduced BST1 activity may be protective in iRBD. This is supported by the top Parkinson's disease GWAS hit in the *BST1* locus, the rs4698412 G allele, which is associated with reduced risk of Parkinson's disease [3]. This allele is also associated with reduced expression of BST1 in blood in GTEx (normalized effect size =-0.07, p=1.5e-6), suggesting that reduced expression might be protective.

4.4. Very rare bi-allelic variants are not enriched in iRBD patients

In order to examine whether bi-allelic variants in our genes of interest are enriched in iRBD, we compared the carrier frequencies of very rare (MAF<0.001) homozygous and compound heterozygous variants between iRBD patients and controls. To analyze compound heterozygous variants, since phasing could not be performed, we considered carriers of two very rare variants as compound heterozygous carriers, with the following exceptions: 1) when variants were physically close (less than 112 base pairs [bp]; probes' target length) and we could determine their phase based on the sequence reads, and 2) if the same combination of very rare variants appeared more than once across samples, we assumed that the variants are most likely to be on the same allele. We found five (0.5%) iRBD patients and seven (0.4%) controls presumably carriers of bi-allelic variants in the studied genes (Table 2, p=0.731, Fisher test).

4.5. Association of common variants in the target genes with iRBD

To test whether common variants in our target genes are associated with iRBD, we performed logistic regression (using PLINK v1.9 software) adjusted for age and sex for common variants (MAF>0.01) detected at coverage depth of >15X. A nominal association was observed in 12 variants across all genes (Supplementary table 5), but no association remained statistically significant after Bonferroni correction for multiple comparisons (set at $p<0.0005$).

Of the variants with nominal associations, one variant in the *ITGA8* 3' UTR (rs896435, OR=1.15, 95% CI = 1.01-1.32, $p=0.04$) is the top hit from the most recent Parkinson's disease GWAS [3], and two other *ITGA8* 3' UTR variants are almost in perfect LD (D'=1.0, $R^2>0.99$, $p<0.0001$) with rs896435. Four variants in the 3' UTR of *RAB29* were almost in perfect LD (Supplementary table 5) and are associated with expression of RAB29 in multiple tissues in GTEx, including the brain. Three *MAPT* variants were in partial LD with Parkinson's disease GWAS hits in the *MAPT* locus and were associated with expression of multiple genes in multiple tissues in GTEx, demonstrating the complexity of this genomic region.

5. Discussion

In the current study, we studied a large cohort of iRBD patients by fully-sequencing and analyzing 25 Parkinson's disease-related GWAS genes and their association with iRBD. Our results identify *BST1* and *LAMP3* as novel genes potentially associated with iRBD. Based on *in silico* models, the three nonsynonymous *BST1* variants that drive the association with iRBD may be loss-of-function variants, suggesting that reduced *BST1* activity may reduce the risk of developing iRBD. The variants driving the association of *LAMP3* are in noncoding regions and could be regulatory. These hypotheses will require confirmation in functional studies in relevant models. While some common variants were nominally associated with iRBD, none of them remained statistically significant after correction for multiple comparisons.

BST1, also called CD157, is a glycosyl phosphatidylinositol (GPI) anchored membrane protein initially found in bone marrow stromal cells and is essential for B-lymphocyte growth and development. It has an extracellular enzymatic domain that produces cyclic ADP-ribose (cADPR). This metabolite acts as a second messenger that can trigger Ca^{2+} release from intracellular stores [31], a process that plays a role in cellular function and death. Specific features of calcium

homeostasis have been suggested to be responsible for the specific vulnerability of dopaminergic neurons in Parkinson's disease [32], yet whether BST1 is involved in calcium homeostasis in human neurons is still unclear, as most work was done in non-human models. Another mechanism by which BST1 may be involved in Parkinson's disease is immune response and neuroinflammation, which are likely important in the pathogenesis of the disease.[33] BST1 serves as a receptor which regulates leukocyte adhesion and migration, and plays a role in inflammation [34]. However, its potential role in microglia activation and neuroinflammation is yet to be determined. Our *in-silico* analysis suggested that the *BST1* variants found mostly in controls are loss-of-function variants. We can therefore hypothesize that these variants may reduce immune response and lead to a reduced risk of iRBD, and that inhibition of *BST1* could be a therapeutic target for iRBD and Parkinson's disease treatment or prevention.

LAMP3 encodes the lysosomal-associated membrane protein 3, which plays a role in the unfolded protein response (UPR) that contributes to protein degradation and cell survival during proteasomal dysfunction [35]. Furthermore, LAMP3 knockdown impairs the ability of the cells to complete the autophagic process, and high LAMP3 expression is associated with increased basal autophagy levels [36]. Numerous Parkinson's disease-related genes have been implicated in the autophagy-lysosomal pathway,[37] and genes associated with iRBD such as *GBA,*[38] *TMEM175*[19] and *SNCA*[39] are all involved in this pathway.[37] Our current findings further strengthen the potential association between the autophagy-lysosomal pathway and iRBD.

Our study has several limitations. First, despite being the largest genetic study of iRBD to date, it may be still underpowered to detect rare variants in GWAS Parkinson's disease-related genes, as well as common variants with a small effect size. Therefore, we cannot completely rule out the possibility that rare and common variants in these genes may contribute to iRBD risk. A second limitation is the younger age and the differences in sex distribution between iRBD patients and controls, for which we adjusted in the statistical analysis as needed. Another potential limitation is the possibility that there were undiagnosed iRBD patients among the control population. However, since iRBD is found in only ~1% of the population [1], the effect of having undiagnosed iRBD patients in the controls would be minimal, given the large sample size.

To conclude, our results suggest two novel genetic associations with iRBD; an association with rare functional variants in *BST1,* and with rare non-coding variants in *LAMP3*. All the

association-driving coding variants found in *BST1*, mainly in controls, appear to potentially cause loss-of-function, suggesting that reduced BST1 activity may reduce the risk of iRBD. Further studies would be required to confirm our results and to examine the biological mechanism underlying the effect of disease-associated variants in both *LAMP3* and *BST1*. The absence of evidence of association between rare and common variants in the remaining genes and iRBD risk suggests that these genes either have no effect in iRBD or have a minor effect that we could not detect with this sample size. Environmental factors and environment-gene interactions are likely to play a major role on iRBD, and larger studies that include carefully collected epidemiological data and more extensive genetic data such as whole-exome or whole-genome sequencing will be required to clarify these issues.

Table 1. Summary of results from burden analyses of rare heterozygous variants

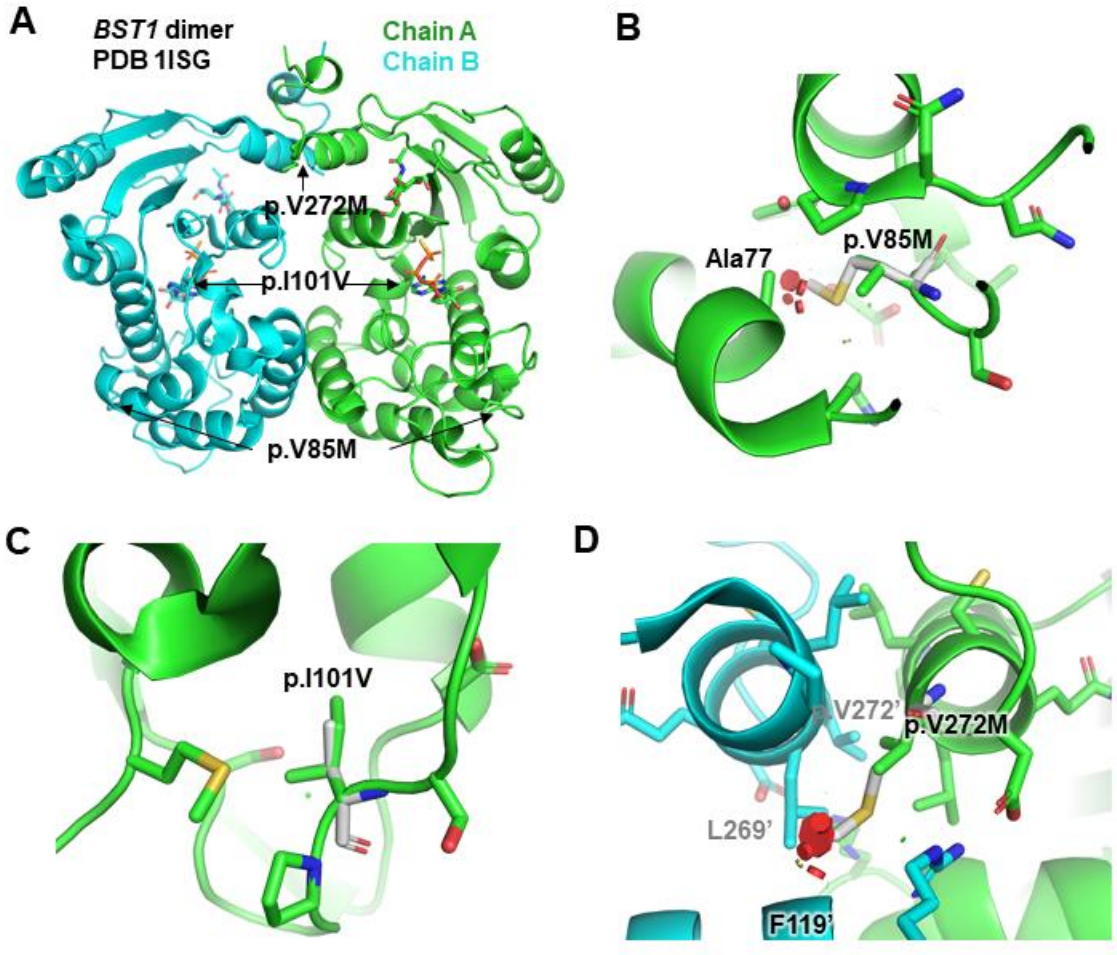
DOC	Gene	All rare (*p*-value)		Rare functional (*p*-value)		Rare LOF (*p*-value)		Rare NS (*p*-value)		Rare CADD (*p*-value)	
		SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden
30x	***ACMSD***	0.124	0.788	0.101	0.475	NV	NV	0.101	0.475	0.098	0.299
	BSTI	0.040	0.020	**0.0009**	**0.0004**	0.348	0.653	0.001	**0.0007**	0.011	0.005
	CCDC62	0.006	0.357	0.048	0.975	0.080	0.596	0.282	0.203	0.134	0.074
	DDRGK1	0.152	0.112	0.745	0.527	0.765	0.587	0.746	0.742	0.919	0.677
	DGKQ	0.067	0.062	0.067	0.062	NV	NV	0.183	0.188	NV	NV
	FGF20	0.059	0.020	0.142	0.552	0.162	0.149	0.162	0.149	0.052	0.041
	GAK	0.195	0.836	0.386	0.565	0.885	0.642	0.781	0.613	0.701	0.684
	GPNMB	0.189	0.798	0.311	0.205	0.547	0.665	0.447	0.304	0.530	0.348
	HIP1R	0.166	0.940	0.219	0.609	NV	NV	0.072	0.993	0.056	0.671
	ITGA8	0.282	0.726	0.379	0.945	0.346	0.648	0.382	0.997	0.288	0.873
	LAMP3	**0.0006**	0.478	0.189	0.322	0.787	0.300	0.318	0.724	0.524	0.601
	MAPT	0.063	0.132	0.003	0.001	1	1	0.063	0.037	0.165	0.101
	MCCC1	0.426	0.413	0.303	0.743	0.347	0.649	1	0.866	0.886	0.690
	PM20D1	0.333	0.463	0.844	0.643	0.674	0.464	0.645	0.479	0.569	0.416
	RAB25	0.561	0.395	0.807	0.802	0.777	0.968	0.565	0.637	NV	NV
	RAB29	0.252	0.222	0.660	0.425	NV	NV	0.777	0.967	0.777	0.967

	RIT2	0.395	0.242	0.023	0.266	NV	NV	0.576	0.495	0.576	0.495
	SETD1A	0.667	0.500	0.241	0.452	0.171	0.166	0.300	0.940	0.310	0.399
	SLC41A1	0.073	0.167	0.055	0.031	0.060	0.065	0.754	0.433	0.257	0.213
	STK39	0.098	0.789	0.055	0.094	0.174	0.171	0.160	0.247	0.160	0.247
	SIPA1L2	0.024	0.095	0.028	0.176	0.886	0.806	0.032	0.379	0.044	0.084
	STX1B	0.504	0.682	NV	NV	NV	NV	NV	NV	NV	NV
	SYT11	0.365	0.887	0.737	0.582	1	1	0.673	0.465	0.673	0.465
	TMEM163	0.598	0.411	0.170	0.165	NV	NV	0.170	0.165	0.170	0.165
	USP25	0.012	0.325	0.102	0.150	NV	NV	0.048	0.067	0.052	0.096
50x	***ACMSD***	0.127	0.538	0.102	0.188	NV	NV	0.102	0.188	0.087	0.093
	BSTI	0.018	0.009	**0.0007**	**0.0003**	0.671	0.470	0.001	**0.0009**	0.010	0.006
	CCDC62	0.011	0.012	0.031	0.018	0.056	0.047	0.027	0.014	0.005	0.004
	DDRGK1	0.368	0.314	0.899	0.811	NV	NV	0.899	0.811	0.899	0.811
	DGKQ	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	FGF20	0.015	0.030	0.164	0.153	0.164	0.153	NV	NV	0.164	0.153
	GAK	0.195	0.836	0.386	0.565	0.885	0.642	0.781	0.613	0.701	0.684
	GPNMB	0.046	0.995	0.177	0.127	0.108	0.413	0.343	0.215	0.283	0.189
	HIP1R	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	ITGA8	0.184	0.315	0.402	0.540	0.340	0.634	0.428	0.584	0.345	0.942
	LAMP3	0.004	0.930	0.047	0.458	0.785	0.304	0.020	0.016	0.458	0.513
	MAPT	0.605	0.503	0.619	0.659	NV	NV	0.619	0.659	0.619	0.659

	MCCC1	0.26	0.151	0.972	0.752	0.334	0.618	0.951	0.745	0.958	0.800
	PM20D1	0.903	0.934	0.873	0.758	0.662	0.487	1	0.758	0.816	0.666
	RAB25	0.173	0.169	NV	NV	NV	NV	NV	NV	NV	NV
	RAB29	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	RIT2	0.953	0.874	0.667	0.478	NV	NV	0.667	0.478	0.667	0.478
	SETD1A	0.212	0.147	0.671	0.469	NV	NV	0.671	0.469	0.671	0.469
	SLC41A1	0.132	0.189	NV	NV	NV	NV	NV	NV	NV	NV
	STK39	0.073	0.808	0.051	0.087	0.160	0.146	0.160	0.146	0.160	0.146
	SIPA1L2	0.163	0.098	0.598	0.861	NV	NV	0.777	0.963	0.777	0.963
	STX1B	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
	SYT11	0.0896	0.671	0.672	0.467	NV	NV	NV	NV	NV	NV
	TMEM163	0.308	0.237	0.169	0.163	NV	NV	0.169	0.163	0.169	0.163
	USP25	0.127	0.418	0.027	0.019	NV	NV	0.027	0.019	0.069	0.037

The table shows *p*-values of the SKAT and SKAT-O analysis in the specified subgroups; in bold - result significant after Bonferroni correction for the number of genes and depths; DOC: Depth of coverage; LOF: Loss of function; NS: Nonsynonymous; CADD: Combined annotation dependent depletion; SKAT-O: Optimized sequence kernel association test; SKAT: Kernel association test; NV: No variants were found for this filter.

Figure 1. Structural analysis of human *BST1* variants



This figure was produced using the software PyMOL v.2.2.0, and represents: (A) Structure of the BST1 dimer bound to ATP-γS (pdb 1ISG). The position of each variant sites is indicated. The ATP-γS molecule in the active site is shown as sticks. (B) Close-up view of the p.V85M variant site. The mutated residue is shown in white. The variant would create clashes (red disks) with nearby Ala77 in the core. (C) Close-up view of the p.I101V variant site. The residue is located in the core of the protein, but the variant to a smaller residue results in no clash. (D) Close-up view of the p.V272M variant site. Primed (‘) residues correspond to chain B. This residue is located at the dimer interface and the variant would create clashes with the other chain, resulting in a destabilization of the dimer.

Table 2. Summary of all samples carrying two nonsynonymous variants detected in the present study

Gene	Sample	Sex	AAS/ AAO	dbSNP	Allele*	Substitution	F_A	F_C	gnomAD ALL	gnomAD NFE
GPNMB	A	M	75/50	7:23300122	T/C	p.S250P	0.000528	0	-	-
				7:23313795	G/T	p.Q545H	0.0005285	0	-	-
MAPT	A	M	85/66	17:44051771	G/T	p.D81Y	0.0005656	0	-	-
				rs63750612	G/A	p.A120T	0.0005423	0	0.0035	7.17E-05
SIPA1L2	A	F	64/48	rs184013125	G/A	p.S1482L	0.002665	0.008451	0.0057	0.0092
				rs200917620	G/A	p.R1089W	0.000547	0.000293	9.44E-05	0.0002
SIPA1L2	A	F	66/-	rs184013125	G/A	p.S1482L	0.002665	0.008451	0.0057	0.0092
				rs200293380	C/T	p.D1088N	0.006031	0.004695	0.0028	0.0041
USP25	A	M	68/-	rs377694221	C/T	p.A56V	0.000533	0	5.35E-05	7.23E-05
				rs200059109	G/T	p.D815Y	0.000533	0	0.0002	1.90E-05
MCCC1	C	M	35	rs149017703	C/T	p.G648S	0.001063	0.001683	0.0005	0.0009
				3:182810207	T/G	p.H88P	0	0.0002804	8.12E-06	8.95E-06
PM20D1	C	M	50	rs145195839	G/A	p.A332V	0.001059	0.000844	0.0003	0.0004
				rs14160575	G/T	p.P281Q	0.005325	0.005936	0.0057	0.0058
SIPA1L2	C	M	48	1:232615440		p.Y673F	0	0.0002817	-	-
				rs761063595		p.S266N	0	0.0002817	4.07E-06	8.99E-06
SIPA1L2	C	M	-	rs61729754	T/G	p.M1427L	0.009062	0.005634	0.0058	0.0088
				rs200293380	C/T	p.D1088N	0.006031	0.004695	0.0028	0.0041
SIPA1L2	C	M	28	rs184013125	G/A	p.S1482L	0.002665	0.008451	0.0057	0.0092
				1:232581435	T/C	p.T1065A	0	0.000282	-	-
SIPA1L2	C	M	50	rs184013125	G/A	p.S1482L	0.002665	0.008451	0.0057	0.0092
				rs966761148	C/T	p.V822M	0	0.000282	2.03E-05	2.69E-05

The table represents all the carriers of biallelic nonsynonymous variants in the target genes; A: Affected; C: Control; M: Male; F: Female; AAS: Age at sampling; AAO: Age at onset of iRBD; dbSNP: Single

nucleotide polymorphism database; *Allele: Reference allele/mutant allele; F_A: Frequency in affected; F_C: Frequency in controls; gnomAD ALL: Exome allele frequency in all populations; gnomAD NFE: Exome allele frequency in non-Finnish European.

Acknowledgments

We thank the participants for contributing to this study. JYM holds a Canada Research Chair in Sleep Medicine. JFG holds a Canada Research Chair in Cognitive Decline in Pathological Aging. WO is Hertie Senior Research Professor, supported by the Hertie Foundation. EAF holds a Canada Research Chair (Tier 1) in Parkinson disease. GAR holds a Canada Research Chair in Genetics of the Nervous System and the Wilder Pen field Chair in Neurosciences. JFT holds a Canada Research Chair (Tier 2) in Structural Pharmacology. ZGO is supported by the Fonds de recherche du Québec–Santé Chercheur-Boursier award and is a Parkinson Canada New Investigator awardee. We thank D. Rochefort, H. Catoire, and V. Zaharieva for their assistance.

Funding

This work was financially supported by the Michael J. Fox Foundation, the Canadian Consortium on Neurodegeneration in Aging (CCNA), Parkinson Canada the Canada First Research Excellence Fund (CFREF), awarded to McGill University for the Healthy Brains for Healthy Lives (HBHL) program. The Montreal cohort was funded by the Canadian Institutes of Health Research (CIHR) and the W. Garfield Weston Foundation. The Oxford Discovery study is funded by the Monument Trust Discovery Award from Parkinson's UK and supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, the NIHR Clinical Research Network and the Dementias and Neurodegenerative Diseases Research Network (DeNDRoN).

Competing interests

JYM received fees from Takeda, Eisai and Paladin Pharma for consultancies in unrelated fields. ZGO received consultancy fees from Lysosomal Therapeutics Inc. (LTI), Idorsia, Prevail Therapeutics, Inceptions Sciences (now Ventus), Ono Therapeutics, Denali, Deerfield, Neuron23

and Handl Therapeutics. None of these companies were involved in any parts of preparing, drafting and publishing this review.

Supplementary material

Supplementary Table 1. Details on age and sex of samples

Supplementary Table 2. MIPs used for each gene

Supplementary Table 3. Average coverage of genes

Supplementary Table 4. All variants included in SKAT-O and SKAT analyses

Supplementary Table 5. All common variants detected at 15x

Supplementary Table 6. Burden and SKAT-O results without *BST1* and *LAMP3* association-driving variants

Supplementary tables 2,4 and 5 can be accessed online through medRxiv:
https://doi.org/10.1101/2020.06.27.20140350

Supplementary Table 1. Details on age and sex of samples

	Number of Samples	Sex		Age at sampling (AAS)		Age at onset (AAO)		Age at diagnosis (AAD)		Pheno-conversion to overt neurodegenerative disease	
		Data available	Percentage of males	Data available	Mean	Data available	Mean	Data Available	Mean	Data available	Converted
iRBD Patients	1039	1032	81%	1005	67.9 ± 9.1 years	601	60.1 ± 10.5 years	608	65.3 ± 8.7 years	540	190
Controls	1852	1852	51%	1843	52.5 ± 14.3 years	NA	NA	NA	NA	NA	NA

iRBD: isolated REM sleep behavior disorder; AAS: age at sampling; AAO: age at onset; AAD: age at diagnosis; NA: non-applicable

Supplementary Table 3: Average coverage details for target genes

Gene	Average coverage	Percent_15x	Percent_30x	Percent_50x
SYT11	988	100	100	100
RAB25	383	100	100	100
RAB7L1	901	97	97	97
SLC41A1	809	93	93	93
PM20D1	911	100	100	100
SIPA1L2	1064	100	98	98
TMEM163	891	88	88	88
ACMSD	790	100	100	100
STK39	883	92	92	92
MCCC1	691	100	100	100
LAMP3	1162	100	100	97
GAK	184	98	98	89
DGKQ	73	79	68	54
BST1	883	100	100	96
GPNMB	1044	100	100	100
FGF20	291	100	100	100
ITGA8	682	98	98	95
CCDC62	911	97	97	97
HIP1R	86	93	82	65
SETD1A	175	89	83	71
STX1B	107	88	79	72
MAPT	377	96	95	90
RIT2	886	100	100	100
DDRGK1	379	86	86	86
USP25	627	91	88	83

Supplementary Table 6. Burden and SKAT-O tests results after excluding the association-driving variants in *BST1* and *LAMP3*

DOC	Gene	All rare (*p* value)		Rare functional (*p* value)		Rare LOF (*p* value)		Rare NS (*p* value)		Rare CADD (*p* value)	
		SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden	SKAT-O	SKAT Burden
30x	*BST1*	0.382	0.233	0.048	0.025	0.348	0.653	0.076	0.052	0.012	0.011
	LAMP3	0.046	0.256	0.688	0.896	0.787	0.300	0.318	0.724	0.524	0.601
50x	*BST1*	0.225	0.142	0.032	0.016	0.671	0.470	0.080	0.057	0.017	0.012
	LAMP3	0.003	0.058	0.468	0.431	0.785	0.304	0.020	0.016	0.458	0.513

DOC: Depth of coverage; LOF: Loss of function; NS: Nonsynonymous; CADD: Combined annotation dependent depletion; SKAT-O: Optimized sequence kernel association test; SKAT: Kernel association test; NV: No variants were found for this filter.

References

1. Postuma RB, Iranzo A, Hu M, et al. Risk and predictors of dementia and parkinsonism in idiopathic REM sleep behaviour disorder: a multicentre study. Brain 2019;142:744-759.
2. Iranzo A, Santamaria J, Tolosa E. Idiopathic rapid eye movement sleep behaviour disorder: diagnosis, management, and the need for neuroprotective interventions. The Lancet Neurology 2016;15:405-419.
3. Nalls MA, Blauwendraat C, Vallerga CL, et al. Expanding Parkinson's disease genetics: novel risk loci, genomic context, causal insights and heritable risk. BioRxiv 2019:388165.
4. Foo JN, Chew EGY, Chung SJ, et al. Identification of Risk Loci for Parkinson Disease in Asians and Comparison of Risk Between Asians and Europeans: A Genome-Wide Association Study. JAMA neurology 2020.
5. Gan-Or Z, Rouleau GA. Genetics of REM Sleep Behavior Disorder. Rapid-Eye-Movement Sleep Behavior Disorder: Springer, 2019: 589-609.
6. Gan-Or Z, Alcalay RN, Rouleau GA, Postuma RB. Sleep disorders and Parkinson disease; lessons from genetics. Sleep medicine reviews 2018;41:101-112.
7. Blauwendraat C, Nalls MA, Singleton AB. The genetic architecture of Parkinson's disease. The Lancet Neurology 2020;19:170-178.
8. Gan-Or Z, Mirelman A, Postuma RB, et al. GBA mutations are associated with rapid eye movement sleep behavior disorder. Ann Clin Transl Neurol 2015;2:941-945.
9. Heckman MG, Soto-Ortolaza AI, Contreras MYS, et al. LRRK2 variation and dementia with Lewy bodies. Parkinsonism & related disorders 2016;31:98-103.
10. Bencheikh BOA, Ruskey JA, Arnulf I, et al. LRRK2 protective haplotype and full sequencing study in REM sleep behavior disorder. Parkinsonism & related disorders 2018;52:98-101.
11. Mufti K, Rudakou U, Yu E, et al. A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder. medRxiv 2020.
12. Alcalay RN, Mallett V, Vanderperre B, et al. SMPD1 mutations, activity, and α-synuclein accumulation in Parkinson's disease. Movement Disorders 2019;34:526-535.
13. Gan-Or Z, Ozelius LJ, Bar-Shira A, et al. The p. L302P mutation in the lysosomal enzyme gene SMPD1 is a risk factor for Parkinson disease. Neurology 2013;80:1606-1610.

14. Rudakou U, Futhey NC, Krohn L, et al. SMPD1 variants do not have a major role in REM sleep behavior disorder. Neurobiology of Aging 2020.
15. Li J, Ruskey JA, Arnulf I, et al. Full sequencing and haplotype analysis of MAPT in Parkinson's disease and rapid eye movement sleep behavior disorder. Movement Disorders 2018;33:1016-1020.
16. Dickson DW, Heckman MG, Murray ME, et al. APOE ε4 is associated with severity of Lewy body pathology independent of Alzheimer pathology. Neurology 2018;91:e1182-e1195.
17. Gan-Or Z, Montplaisir JY, Ross JP, et al. The dementia-associated APOE ε4 allele is not associated with rapid eye movement sleep behavior disorder. Neurobiology of aging 2017;49:218. e213-218. e215.
18. Krohn L, Wu RY, Heilbron K, et al. Fine-Mapping of SNCA in Rapid Eye Movement Sleep Behavior Disorder and Overt Synucleinopathies. Annals of Neurology 2020;87:584-598.
19. Krohn L, Öztürk TN, Vanderperre B, et al. Genetic, structural, and functional evidence link TMEM175 to synucleinopathies. Annals of Neurology 2020;87:139-153.
20. Nalls MA, Pankratz N, Lill CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nature genetics 2014;46:989.
21. Chang D, Nalls MA, Hallgrímsdóttir IB, et al. A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci. Nature genetics 2017;49:1511.
22. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619-1622.
23. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. bioinformatics 2009;25:1754-1760.
24. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research 2010;20:1297-1303.
25. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic acids research 2010;38:e164-e164.
26. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016;536:285-291.

27. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. The American journal of human genetics 2007;81:559-575.
28. Lee S, Emond MJ, Bamshad MJ, et al. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. The American Journal of Human Genetics 2012;91:224-237.
29. Machiela MJ, Chanock SJ. LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. Bioinformatics 2015;31:3555-3557.
30. Yamamoto-Katayama S, Ariyoshi M, Ishihara K, Hirano T, Jingami H, Morikawa K. Crystallographic studies on human BST-1/CD157 with ADP-ribosyl cyclase and NAD glycohydrolase activities. Journal of molecular biology 2002;316:711-723.
31. Ishihara K, Hirano T. BST-1/CD157 regulates the humoral immune responses in vivo. Chemical immunology 2000;75:235-255.
32. Chan CS, Gertler TS, Surmeier DJ. Calcium homeostasis, selective vulnerability and Parkinson's disease. Trends in neurosciences 2009;32:249-256.
33. Kannarkat GT, Boss JM, Tansey MG. The Role of Innate and Adaptive Immunity in Parkinson's Disease. Journal of Parkinson's Disease 2013;3:493-514.
34. Malavasi F, Deaglio S, Ferrero E, et al. CD38 and CD157 as receptors of the immune system: a bridge between innate and adaptive immunity. Molecular medicine; 2006: BioMed Central: 334-341.
35. Dominguez-Bautista JA, Klinkenberg M, Brehm N, et al. Loss of lysosome-associated membrane protein 3 (LAMP3) enhances cellular vulnerability against proteasomal inhibition. European journal of cell biology 2015;94:148-161.
36. Nagelkerke A, Sieuwerts AM, Bussink J, et al. LAMP3 is involved in tamoxifen resistance in breast cancer cells through the modulation of autophagy. Endocr Relat Cancer 2014;21:101-112.
37. Senkevich K, Gan-Or Z. Autophagy lysosomal pathway dysfunction in Parkinson's disease; evidence from human genetics. Parkinsonism & Related Disorders 2019.
38. Krohn L, Ruskey JA, Rudakou U, et al. GBA variants in REM sleep behavior disorder: a multicenter study. Neurology 2020.

39. Krohn L, Wu R, Ruskey J, et al. Fine-mapping of SNCA variants in REM sleep behavior disorder identifies distinct associations. 2019.

CHAPTER 4: General Discussion

The purpose of this MSc dissertation was to study the role of familial and GWAS parkinsonism genes in iRBD using targeted next-generation sequencing. In the current thesis, we performed the most comprehensive genetic study of iRBD to date by fully sequencing a total of 35 genes known to be implicated in PD and parkinsonism in the largest iRBD genetic cohort available, including 1,039 unrelated iRBD patients and 1,852 unrelated controls. Patients and controls were recruited consecutively in clinics and hospitals in Europe and Canada, and collected data included information on sex, age at sampling, age at onset for iRBD patients, and pheno-conversion from iRBD to synucleinopathies. Combining the two studies described in Chapters 2 and 3, our results provided no evidence that variants in familial PD and atypical parkinsonism genes are involved in iRBD risk, which further highlights the distinct genetic background of PD and its prodromal manifestation, iRBD. On the other hand, two PD GWAS genes were highlighted by our data as associated with iRBD. Both genes - *BST1* and *LAMP3* - are involved in the peripheral immune response. In fact, although PD is mainly considered a brain disorder, evidence from several studies has implicated the role of peripheral inflammation and adaptive immune response in PD pathogenesis,[87, 88] suggesting that activation of the peripheral immune system may later activate central inflammatory response and synergistically drive neurodegeneration.[89] Evidence of inflammation and immune response involvement in PD pathogenesis includes increased serum levels of proinflammatory cytokines such as tumor necrosis factor (TNFα), interleukin 6 (IL-6) and interferon γ (IFNγ) in PD patients compared to controls.[90-92] These peripheral inflammatory factors appear to accompany the preclinical nonmotor symptoms of PD in the early stages described by Braak et al[16] (see Chapter 1).

Another remarkable difference between PD patients and controls has been reported in the composition of the immune cells, suggesting a potential role of these immune cells in the prodromal stage of PD.[93,94] Interestingly, there is an obvious interplay between the peripheral inflammatory reactions and the central system response which, in turn, may cause neurodegeneration. In normal conditions, the central nervous system (CNS) is protected by the blood brain barrier (BBB) that prevents the pass of pathogens and immune cells into the brain.[95] However, BBB dysfunction increases the vascular permeability and allows for immune cells and

inflammatory factors to cross into the brain parenchyma,[96] which appears to play an important role in the pathology of numerous neurodegenerative diseases including PD.[97] The activation of the peripheral immune response also increases the blood levels of cytotoxic T-cell lymphocytes, which were found to cross the BBB and infiltrate the substantia nigra in PD patients, possibly causing an autoimmune response against α-synuclein by recognizing it as a foreign antigen.[98,99]

In the brain, BBB dysfunction and associated central neuroinflammation in PD have been shown to occur as a result of microglial cells activation.[100] These innate immune cells play defensive neuroprotection functions in the brain by constituting the first-line immune defense of the CNS, as well as having essential roles in the physiology and survival of neurons. Microglia-associated central neuroinflammation has been reported as a pathological hallmark of PD since the first post-mortem study over 30 years ago, which reported presence of activated microglia in PD patients,[101] to more recent clinical data.[102] Although these central inflammatory processes may occur as a secondary effect of neurodegeneration, under certain conditions, inflammation and excess immune-response could also be a primary event that leads to neuronal cell death. The activation of microglial cells can cause a neurotoxic phenotype that generates an inflammatory response releasing cytokines and interleukins including TNFα, IL-1, IL-6, and IFNγ.[97] These inflammatory factors mediate the neurotoxic effects of the activated microglia and drive excess inflammatory events that eventually cause damage and death to the nigral cells associated with PD.[89] Although our knowledge on the involvement of inflammation and immune response in the progression of iRBD is still lacking, the high prevalence of autoimmune disorders among iRBD patients such as narcolepsy and multiple sclerosis may suggest a potential link between immune dysregulation and iRBD.[4,103]

Hence, our results further highlight this pathway as potentially important in iRBD pathogenesis. *BST1* is known to encode the Bone marrow stromal antigen 1 (or CD157), a protein that is highly expressed in the blood and important for B-lymphocytes growth and development, which may favor the involvement of *BST1* in PD through immune-response activation. However, whether *BST1* is expressed in microglia and important in their activation is still unclear and requires additional studies. The association of *BST1* with iRBD was mainly driven by three coding variants that were enriched in controls (six-fold higher frequency in controls). Structural *in-silico* analysis of BST1 suggested that all three variants may destabilize

the enzyme structure. Therefore, these variants are most likely loss-of-function variants, which requires additional confirmation by future functional studies. However, our findings may suggest that loss-of-function of BST1 may be protective against iRBD. If this is true, these are encouraging results, since designing pharmaceutical inhibitors is usually much easier than creating activators, making BST1 an interesting target for therapy. In addition, we have identified an association of *LAMP3*, encoding lysosomal associated membrane protein 3, with iRBD. Unlike the variants found in *BST1*, the main variants driving the association in *LAMP3* are non-coding (intronic and 3' UTR), making it more challenging to design functional studies to test their effects. These variants might have a regulatory function and can probably affect LAMP3 expression. In humans, the LAMP3 protein is enriched in the immune system, mainly in dendritic cells (DCs),[104] suggesting that LAMP3 plays an important role for DCs maturation and function and is involved in adaptive immunity.[105]

LAMP3 has also been reported to have an important role in the autophagy-lysosomal pathway (ALP),[106] as its knockdown impacts the ability of the cells to complete the autophagic process, and its high expression is associated with increased basal autophagy levels.[107] ALP is known to be a very important pathway in PD, and lysosomal dysfunction leads to accumulation of α-synuclein which may eventually trigger neuronal cell death.[108-110] Lysosomes are cellular organelles that contain hydrolytic enzymes capable of degrading intracellular components through several degradation pathways, including endocytosis, phagocytosis, and autophagy.[106, 110] These organelles are known to be responsible for the clearance of proteins, such as α-synuclein, or other defective organelles, such as depolarized mitochondria.[110] Although there is another main pathway for protein degradation in the cells, the ubiquitin proteasomal systems (UPS),it was shown that only the ALP is essential for α-synuclein degradation in neuronal cells (its inhibition leads to the accumulation of α-synuclein).[111, 112] Indeed, both mitochondrial dysfunction and α-synuclein aggregation are known as major pathogenic events that occur in PD,[113, 114] and numerous genes involved in PD are found to have essential functions within the ALP pathway including genes such as *GBA* and *LRRK2* which are known to harbor some of the most common mutations associated with PD.[64, 86, 115]

Furthermore, previous genetic studies have revealed lysosomal PD genes such as *GBA*, *TMEM175* and *SNCA* to also be associated iRBD risk. Genetic variants in *GBA* have been found

to be associated with increased risk of iRBD.[116] *GBA* encodes for glucocerebrosidase (GCase), and different *GBA* variants have been found to reduce GCase activity.[110] Several mechanisms have been suggested for the association of *GBA* variants in the development of neurodegeneration, including alterations in the composition of lysosomal membrane that may affect autophagy and mitophagy,[106, 117] and accumulation of the misfolded GCase and endoplasmic reticulum stress.[118] Severe and mild *GBA* variants appear to have different effects on the risk, age at onset, and the conversion to overt synucleinopathies in iRBD. Carriers of severe *GBA* variants were found to convert to PD with higher frequency and earlier onset compared to those who carry mild variants or non-carriers of *GBA* mutations.[116] In addition to *GBA*, genetic variants in other genes have also been reported to reduce GCase activity, including a coding variant in *TMEM175*, which encodes a transmembrane endolysosomal potassium channel responsible for regulation of lysosomal function. *TMEM175* is the 4th strongest risk locus in GWAS of PD and has been found to be involved in iRBD.[64] In that study, the *TMEM175*p.M393T variant was associated with reduced GCase activity, which in other studies has been shown to cause α-synuclein accumulation.[119] α-synuclein is encoded by the *SNCA* gene, in which 5'UTR region variants (tagged by rs10005233) were also reported to be associated with increased iRBD risk.[50] Together with *LAMP3* association with iRBD discussed in the current thesis, ALP appear to be a potentially important pathway in iRBD risk and conversion.

Finally, PD is not a single entity, it is rather a multisystem disease with many heterogeneous subgroups of disorders. For example, some PD patients progress faster than others, some have α-synuclein pathology while pure degeneration occurs in others, and some have RBD whereas others do not. As described in Chapter 1, sequencing-based genetic studies have helped to reveal multiple genes and loci associated with PD, but since GWASs do not separate between these different subtypes of PD, looking at each specific gene and studying it in iRBD further helps to identify which genetic factors are involved in this specific subtype, which - in turn - could help us understand the underlying mechanisms of its progression and conversion, identify targets for future basic and clinical studies, and improve clinical trials by stratifying the cohorts by the genetic profiles of the participants. The work described in this thesis provides evidence that targeted sequencing methods are a viable and scalable approach that may help identifying specific genes or variants responsible for the onset of an important prodromal neurodegenerative disorder such as iRBD, which is crucial for our understanding of

the disease mechanism and progression, better identifying individuals at high risk of conversion and identifying novel targets for drug development.

CHAPTER 5: Conclusion and Future Directions

iRBD is a powerful prodromal symptom of synucleinopathies. Patients diagnosed with iRBD in sleep centres have a high risk of converting to neurodegenerative diseases associated with α-synuclein pathology over time. This conversion, which is a gradual process, may occur very rapidly or within years or even decades. Presently, it is still not possible to predict in patients with iRBD whether they will eventually convert into an overt synucleinopathy and how fast. Understanding the genetic components that lead iRBD patients to develop synucleinopathies would make it possible to identify patients with specific genetic markers for prognosis. PD can be viewed as a large umbrella for numerous heterogenous neurological disorders with overall similar clinical presentation. Thus, determining the genetic link between iRBD and PD would help to decipher the puzzle of this complex condition through applying these genetic findings for disease-classification and subtyping.

As preliminary studies have confirmed the partial genetic overlap between iRBD and PD, we aimed in this research to further explore this overlap by studying genes known to be involved in PD and atypical parkinsonism and their role in the risk of iRBD. In this dissertation, we have investigated for the first time the role of rare variants in 10 familial parkinsonism genes in iRBD risk and examined the association of rare and common variants in 25 PD GWAS genes with iRBD. In brief, this body of work has contributed towards expanding our knowledge of the distinct genetics between PD and iRBD while further confirming their partial genetic overlap. Our data showed that *BST1* and *LAMP3* could potentially play a role in the risk of iRBD, which makes them appealing targets for further investigation. Interestingly, loss-of-function of BST1 appears to be protective in iRBD. Since BST1 in expressed in the peripheral blood and may be involved in immune predisposition, BST1 could be a potential target for systemic therapy in the future. As a follow-up, functional studies could use knock-out *BST1* and *LAMP3* or knock-in models with the specific variants we have identified, or use human-derived cell models from individuals who carry these variant and their isogenic controls. Using these models will allow for assessing how rare variants in these two genes can affect specific mechanisms, such as inflammatory response and lysosomal function, and better understand how the excess or reduced

activity of their respective proteins could contribute to the pathogenesis of iRBD and α-synuclein pathology.

In addition, since the design of the current work was done before the recent GWASs, another way to follow-up on our research is to expand the targeted analysis to test all the genes found in loci associated with PD in the more recent GWASs. In the current research, we performed targeted analysis of specific genes, and by that, we cannot rule out the potential role of other genes. Performing WGS on our iRBD cohort will potentially facilitate future identification of other genes involved in iRBD, although we will be limited by the sample size. Nevertheless, as our cohort keeps growing, performing WGS will be a good approach, also allowing for combining data with other synucleinopathy cohorts. It will also be crucial to study iRBD patients of other ethnicities, such as African, South American, Asian and others, since there could be population-specific variants or genes that are involved in iRBD. In addition to genetic variants, epigenetic changes have also been suggested to play a role in the progression of PD. Changes in various epigenetic mechanisms that regulate gene expression such as DNA methylation, histone modifications or micro RNAs (miRNAs) have all been suggested to be linked to PD.[120] More recently, epigenetic changes in *SNCA* have also been implicated in iRBD.[121] Thus, expression analysis would also be useful to further investigate the effect of expression changes in these genes on iRBD development.

Finally, one of the advantages of association studies is that they can serve as a corner stone for future designed studies. The type of work discussed in this thesis would ultimately help to reveal genetic markers that will advance our ability to more accurately prognose disease-development and to tailor personalized treatments for each individual patient in the future. The main goal of such treatments in iRBD patients, would be to delay, or preferably halt, the progression of LP and conversion to an overt synucleinopathy. This would reduce the huge burden incurred on patients, their caregivers and the entire society, healthcare expenses and time consumed for treatments, improve efficacy therapies and decrease errors and side effects. While all these goals could still be far out of our reach, the current work provides an additional step towards this direction.

CHAPTER 6: Master Bibliography

1. Dauvilliers Y, Schenck CH, Postuma RB, et al. REM sleep behaviour disorder. Nature Reviews Disease Primers 2018;4:1-16.
2. McCarley RW. Neurobiology of REM and NREM sleep. Sleep medicine 2007;8:302-330.
3. Iranzo A, Santamaria J, Tolosa E. Idiopathic rapid eye movement sleep behaviour disorder: diagnosis, management, and the need for neuroprotective interventions. The Lancet Neurology 2016;15:405-419.
4. Ju Y-E, Larson-Prior L, Duntley S. Changing demographics in REM sleep behavior disorder: possible effect of autoimmunity and antidepressants. Sleep medicine 2011;12:278-283.
5. McCarter SJ, Tippmann-Peikert M, Sandness DJ, et al. Neuroimaging-evident lesional pathology associated with REM sleep behavior disorder. Sleep medicine 2015;16:1502-1510.
6. Limousin N, Dehais C, Gout O, Héran F, Oudiette D, Arnulf I. A brainstem inflammatory lesion causing REM sleep behavior disorder and sleepwalking (parasomnia overlap disorder). Sleep medicine 2009;10:1059-1062.
7. Geddes MR, Tie Y, Gabrieli JD, McGinnis SM, Golby AJ, Whitfield-Gabrieli S. Altered functional connectivity in lesional peduncular hallucinosis with REM sleep behavior disorder. Cortex 2016;74:96-106.
8. Haba-Rubio J, Frauscher B, Marques-Vidal P, et al. Prevalence and determinants of rapid eye movement sleep behavior disorder in the general population. Sleep 2018;41:zsx197.
9. Pujol M, Pujol J, Alonso T, et al. Idiopathic REM sleep behavior disorder in the elderly Spanish community: a primary care center study with a two-stage design using video-polysomnography. Sleep medicine 2017;40:116-121.
10. Postuma RB, Iranzo A, Hu M, et al. Risk and predictors of dementia and parkinsonism in idiopathic REM sleep behaviour disorder: a multicentre study. Brain 2019;142:744-759.
11. Gan-Or Z, Mirelman A, Postuma RB, et al. GBA mutations are associated with rapid eye movement sleep behavior disorder. Annals of clinical and translational neurology 2015;2:941-945.

12. Tan X, van Egmond L, Partinen M, Lange T, Benedict C. A narrative review of interventions for improving sleep and reducing circadian disruption in medical inpatients. Sleep medicine 2019;59:42-50.
13. Ehrminger M, Latimier A, Pyatigorskaya N, et al. The coeruleus/subcoeruleus complex in idiopathic rapid eye movement sleep behaviour disorder. Brain 2016;139:1180-1188.
14. Stefanis L. α-Synuclein in Parkinson's disease. Cold Spring Harb Perspect Med 2012;2:a009399.
15. Scott D, Roy S. α-Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis. Journal of Neuroscience 2012;32:10129-10135.
16. Braak H, Del Tredici K, Rüb U, De Vos RA, Steur ENJ, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiology of aging 2003;24:197-211.
17. Gan-Or Z, Alcalay RN, Rouleau GA, Postuma RB. Sleep disorders and Parkinson disease; lessons from genetics. Sleep medicine reviews 2018;41:101-112.
18. Stefanis L. α-Synuclein in Parkinson's disease. Cold Spring Harb Perspect Med 2012;2:a009399-a009399.
19. Singleton A, Hardy J. The evolution of genetics: Alzheimer's and Parkinson's diseases. Neuron 2016;90:1154-1163.
20. Nalls MA, Pankratz N, Lill CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nature genetics 2014;46:989.
21. Chang D, Nalls MA, Hallgrímsdóttir IB, et al. A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci. Nature genetics 2017;49:1511.
22. Nalls MA, Blauwendraat C, Vallerga CL, et al. Expanding Parkinson's disease genetics: novel risk loci, genomic context, causal insights and heritable risk. BioRxiv 2019:388165.
23. Foo JN, Chew EGY, Chung SJ, et al. Identification of Risk Loci for Parkinson Disease in Asians and Comparison of Risk Between Asians and Europeans: A Genome-Wide Association Study. JAMA neurology 2020.
24. Kilarski LL, Pearson JP, Newsway V, et al. Systematic review and UK-based study of PARK2 (parkin), PINK1, PARK7 (DJ-1) and LRRK2 in early-onset Parkinson's disease. Movement Disorders 2012;27:1522-1529.
25. Mata IF, Lockhart PJ, Farrer MJ. Parkin genetics: one model for Parkinson's disease. Human molecular genetics 2004;13:R127-R133.

26. Schneider SA, Alcalay RN. Neuropathology of genetic synucleinopathies with parkinsonism: review of the literature. Movement Disorders 2017;32:1504-1523.
27. Ambroziak W, Koziorowski D, Duszyc K, et al. Genomic instability in the PARK2 locus is associated with Parkinson's disease. Journal of applied genetics 2015;56:451-461.
28. La Cognata V, Morello G, D'Agata V, Cavallaro S. Copy number variability in Parkinson's disease: assembling the puzzle through a systems biology approach. Human genetics 2017;136:13-37.
29. Bonifati V, Rohe C, Breedveld G, et al. Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. Neurology 2005;65:87-95.
30. Djarmati A, Hedrich K, Svetel M, et al. Detection of Parkin (PARK2) and DJ1 (PARK7) mutations in early-onset Parkinson disease: Parkin mutation frequency depends on ethnic origin of patients. Human mutation 2004;23:525-525.
31. Kumazawa R, Tomiyama H, Li Y, et al. Mutation analysis of the PINK1 gene in 391 patients with Parkinson disease. Archives of neurology 2008;65:802-808.
32. Del Rey NL-G, Quiroga-Varela A, Garbayo E, et al. Advances in Parkinson's disease: 200 years later. Frontiers in neuroanatomy 2018;12:113.
33. Chenbhanich J, Sringean J, Bhidayasiri R. Beyond the classic Segawa disease, GCH1-associated neurodegenerative parkinsonism: practical considerations for physicians. Journal of movement disorders 2017;10:102.
34. Guella I, Sherman HE, Appel-Cresswell S, Rajput A, Rajput AH, Farrer MJ. Parkinsonism in GTP cyclohydrolase 1 mutation carriers. Brain 2015;138:e349.
35. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. science 1997;276:2045-2047.
36. Miller D, Hague S, Clarimon J, et al. α-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. Neurology 2004;62:1835-1838.
37. Roosen DA, Cookson MR. LRRK2 at the interface of autophagosomes, endosomes and lysosomes. Molecular neurodegeneration 2016;11:1-10.
38. Blauwendraat C, Reed X, Kia DA, et al. Frequency of loss of function variants in LRRK2 in Parkinson disease. JAMA neurology 2018;75:1416-1422.
39. Lesage S, Dürr A, Tazir M, et al. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. New England Journal of Medicine 2006;354:422-423.

40. Ozelius LJ, Senthil G, Saunders-Pullman R, et al. LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. New England Journal of Medicine 2006;354:424-425.
41. Kalia LV, Lang AE. Parkinson's disease. The Lancet 2015;386:896-912.
42. Klein C, Lohmann-Hedrich K, Rogaeva E, Schlossmacher MG, Lang AE. Deciphering the role of heterozygous mutations in genes associated with parkinsonism. The Lancet Neurology 2007;6:652-662.
43. Lesage S, Brice A. Parkinson's disease: from monogenic forms to genetic susceptibility factors. Human molecular genetics 2009;18:R48-R59.
44. Blauwendraat C, Nalls MA, Singleton AB. The genetic architecture of Parkinson's disease. The Lancet Neurology 2020;19:170-178.
45. Usenovic M, Tresse E, Mazzulli JR, Taylor JP, Krainc D. Deficiency of ATP13A2 leads to lysosomal dysfunction, α-synuclein accumulation, and neurotoxicity. Journal of Neuroscience 2012;32:4240-4246.
46. Conedera S, Apaydin H, Li Y, et al. FBXO7 mutations in Parkinson's disease and multiple system atrophy. Neurobiology of aging 2016;40:192. e191-192. e195.
47. Guo Y-p, Tang B-s, Guo J-f. PLA2G6-associated neurodegeneration (PLAN): review of clinical phenotypes and genotypes. Frontiers in Neurology 2018;9:1100.
48. Gan-Or Z, Liong C, Alcalay RN. GBA-associated Parkinson’s disease and other synucleinopathies. Current neurology and neuroscience reports 2018;18:44.
49. Krohn L, Ruskey JA, Rudakou U, et al. GBA variants in REM sleep behavior disorder: a multicenter study. medRxiv 2019:19010991.
50. Krohn L, Wu RY, Heilbron K, et al. Fine-Mapping of SNCA in Rapid Eye Movement Sleep Behavior Disorder and Overt Synucleinopathies. Annals of Neurology 2020;87:584-598.
51. Lippa C, Duda J, Grossman M, et al. DLB and PDD boundary issues: diagnosis, treatment, molecular pathology, and biomarkers. Neurology 2007;68:812-819.
52. Guerreiro R, Ross OA, Kun-Rodrigues C, et al. Investigating the genetic architecture of dementia with Lewy bodies: a two-stage genome-wide association study. The Lancet Neurology 2018;17:64-74.
53. Bras J, Guerreiro R, Darwent L, et al. Genetic analysis implicates APOE, SNCA and suggests lysosomal dysfunction in the etiology of dementia with Lewy bodies. Human molecular genetics 2014;23:6139-6146.

54. Labbé C, Heckman MG, Lorenzo-Betancor O, et al. MAPT haplotype H1G is associated with increased risk of dementia with Lewy bodies. Alzheimer's & Dementia 2016;12:1297-1304.
55. Fanciulli A, Wenning GK. Multiple-system atrophy. New England Journal of Medicine 2015;372:249-263.
56. Sixel-Döring F, Trenkwalder C. REM Sleep Behavior Disorder Associated with Parkinson's Disease and Multiple System Atrophy. In: Schenck CH, Högl B, Videnovic A, eds. Rapid-Eye-Movement Sleep Behavior Disorder. Cham: Springer International Publishing, 2019: 53-65.
57. Hara K, Momose Y, Tokiguchi S, et al. Multiplex families with multiple system atrophy. Archives of neurology 2007;64:545-551.
58. Vidal J-S, Vidailhet M, Derkinderen P, Tzourio C, Alpérovitch A. Familial aggregation in atypical Parkinson's disease: a case control study in multiple system atrophy and progressive supranuclear palsy. Journal of neurology 2010;257:1388-1393.
59. Sailer A, Scholz SW, Nalls MA, et al. A genome-wide association study in multiple system atrophy. Neurology 2016;87:1591-1598.
60. Mikasa M, Kanai K, Li Y, et al. COQ2 variants in Parkinson's disease and multiple system atrophy. Journal of Neural Transmission 2018;125:937-944.
61. Mitsui J, Matsukawa T, Sasaki H, et al. Variants associated with Gaucher disease in multiple system atrophy. Annals of clinical and translational neurology 2015;2:417-426.
62. Blauwendraat C, Reed X, Krohn L, et al. Genetic modifiers of risk and age at onset in GBA associated Parkinson's disease and Lewy body dementia. Brain 2020;143:234-248.
63. Bencheikh BOA, Ruskey JA, Arnulf I, et al. LRRK2 protective haplotype and full sequencing study in REM sleep behavior disorder. Parkinsonism & related disorders 2018;52:98-101.
64. Krohn L, Öztürk TN, Vanderperre B, et al. Genetic, structural, and functional evidence link TMEM175 to synucleinopathies. Annals of Neurology 2020;87:139-153.
65. Fernández-Santiago R, Iranzo A, Gaig C, et al. Absence of LRRK2 mutations in a cohort of patients with idiopathic REM sleep behavior disorder. Neurology 2016;86:1072-1073.
66. Li J, Ruskey JA, Arnulf I, et al. Full sequencing and haplotype analysis of MAPT in Parkinson's disease and rapid eye movement sleep behavior disorder. Movement Disorders 2018;33:1016-1020.

67. Gan-Or Z, Montplaisir JY, Ross JP, et al. The dementia-associated APOE ε4 allele is not associated with rapid eye movement sleep behavior disorder. Neurobiology of aging 2017;49:218. e213-218. e215.
68. Rudakou U, Futhey NC, Krohn L, et al. SMPD1 variants do not have a major role in REM sleep behavior disorder. Neurobiology of Aging 2020.
69. Hiersch L, Yaron Y. 26 - Expanded Carrier Screening. In: Pandya PP, Oepkes D, Sebire NJ, Wapner RJ, eds. Fetal Medicine (Third Edition). London: Content Repository Only!, 2020: 254-262.e252.
70. Behjati S, Tarpey PS. What is next generation sequencing? Archives of Disease in Childhood-Education and Practice 2013;98:236-238.
71. Tilak M-K, Botero-Castro F, Galtier N, Nabholz B. Illumina library preparation for sequencing the GC-rich fraction of heterogeneous genomic DNA. Genome Biology and Evolution 2018;10:616-622.
72. Guan P, Sung W-K. Structural variation detection using next-generation sequencing data: a comparative technical review. Methods 2016;102:36-49.
73. Yohe S, Thyagarajan B. Review of clinical next-generation sequencing. Archives of pathology & laboratory medicine 2017;141:1544-1557.
74. Zampieri S, Cattarossi S, Bembi B, Dardis A. GBA analysis in next-generation era: Pitfalls, Challenges, and Possible Solutions. The Journal of Molecular Diagnostics 2017;19:733-741.
75. Chardon JW, Beaulieu C, Hartley T, Boycott KM, Dyment DA. Axons to exons: the molecular diagnosis of rare neurological diseases by next-generation sequencing. Current neurology and neuroscience reports 2015;15:64.
76. Logsdon GA, Vollger MR, Eichler EE. Long-read human genome sequencing and its applications. Nature Reviews Genetics 2020:1-18.
77. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619-1622.
78. Chastain EC. Targeted Hybrid Capture Methods. Clinical Genomics: Elsevier, 2015: 37-55.
79. Absalan F, Ronaghi M. Molecular inversion probe assay. Comparative Genomics: Springer, 2007: 315-330.

80. Turner EH, Lee C, Ng SB, Nickerson DA, Shendure J. Massively parallel exon capture and library-free resequencing across 16 genomes. Nature methods 2009;6:315-316.
81. Vives L, Fu W, Phelps IG. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619-1622.
82. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome research 2013;23:843-854.
83. Mertes F, ElSharawy A, Sauer S, et al. Targeted enrichment of genomic DNA regions for next-generation sequencing. Briefings in functional genomics 2011;10:374-386.
84. O'roak B, Stessman H, Boyle E, et al. Recurrent de novo mutations implicate novel genes underlying simplex autism risk. Nature communications 2014;5:5595.
85. Cantsilieris S, Stessman HA, Shendure J, Eichler EE. Targeted Capture and high-throughput sequencing using molecular inversion probes (MIPs). Genotyping: Springer, 2017: 95-106.
86. Nalls MA, Pankratz N, Lill CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nature genetics 2014;46:989-993.
87. Rugbjerg K, Friis S, Ritz B, Schernhammer E, Korbo L, Olsen J. Autoimmune disease and risk for Parkinson disease: a population-based case-control study. Neurology 2009;73:1462-1468.
88. Li X, Sundquist J, Sundquist K. Subsequent risks of Parkinson disease in patients with autoimmune and related disorders: a nationwide epidemiological study from Sweden. Neurodegenerative diseases 2012;10:277-284.
89. Su X, Federoff HJ. Immune responses in Parkinson’s disease: interplay between central and peripheral immune systems. BioMed research international 2014;2014.
90. Reale M, Iarlori C, Thomas A, et al. Peripheral cytokines profile in Parkinson’s disease. Brain, behavior, and immunity 2009;23:55-63.
91. Scalzo P, Kümmer A, Cardoso F, Teixeira AL. Increased serum levels of soluble tumor necrosis factor-α receptor-1 in patients with Parkinson's disease. Journal of neuroimmunology 2009;216:122-125.

92. Dufek M, Hamanová M, Lokaj J, et al. Serum inflammatory biomarkers in Parkinson's disease. Parkinsonism & related disorders 2009;15:318-320.
93. Kannarkat GT, Boss JM, Tansey MG. The role of innate and adaptive immunity in Parkinson's disease. Journal of Parkinson's disease 2013;3:493-514.
94. Nissen SK, Shrivastava K, Schulte C, et al. Alterations in blood monocyte functions in Parkinson's disease. Movement Disorders 2019;34:1711-1721.
95. Liebner S, Dijkhuizen RM, Reiss Y, Plate KH, Agalliu D, Constantin G. Functional morphology of the blood–brain barrier in health and disease. Acta neuropathologica 2018;135:311-336.
96. Daneman R, Prat A. The blood–brain barrier. Cold Spring Harbor perspectives in biology 2015;7:a020412.
97. Ramirez AI, de Hoz R, Salobrar-Garcia E, et al. The role of microglia in retinal neurodegeneration: Alzheimer's disease, Parkinson, and glaucoma. Frontiers in Aging Neuroscience 2017;9:214.
98. Depboylu C, Stricker S, Ghobril J-P, Oertel WH, Priller J, Höglinger GU. Brain-resident microglia predominate over infiltrating myeloid cells in activation, phagocytosis and interaction with T-lymphocytes in the MPTP mouse model of Parkinson disease. Experimental neurology 2012;238:183-191.
99. Garretti F, Agalliu D, Lindestam Arlehamn CS, Sette A, Sulzer D. Autoimmunity in Parkinson's Disease: the role of α-synuclein-specific T cells. Frontiers in immunology 2019;10:303.
100. Stankovic ND, Teodorczyk M, Ploen R, Zipp F, Schmidt MH. Microglia–blood vessel interactions: a double-edged sword in brain pathologies. Acta neuropathologica 2016;131:347-363.
101. McGeer PL, Itagaki S, Boyes BE, McGeer E. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 1988;38:1285-1285.
102. Caggiu E, Arru G, Hosseini S, et al. Inflammation, infectious triggers, and Parkinson's Disease. Frontiers in neurology 2019;10:122.

103. Iranzo A. RBD Associated with Paraneoplastic Neurological Syndromes and Autoimmune Disorders. In: Schenck CH, Högl B, Videnovic A, eds. Rapid-Eye-Movement Sleep Behavior Disorder. Cham: Springer International Publishing, 2019: 93-106.
104. Mujcic H, Rzymski T, Rouschop KM, et al. Hypoxic activation of the unfolded protein response (UPR) induces expression of the metastasis-associated gene LAMP3. Radiotherapy and Oncology 2009;92:450-459.
105. de Saint-Vis B, Vincent J, Vandenabeele S, et al. A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. Immunity 1998;9:325-336.
106. Gan-Or Z, Dion PA, Rouleau GA. Genetic perspective on the role of the autophagy-lysosome pathway in Parkinson disease. Autophagy 2015;11:1443-1457.
107. Nagelkerke A, Sieuwerts AM, Bussink J, et al. LAMP3 is involved in tamoxifen resistance in breast cancer cells through the modulation of autophagy. Endocr Relat Cancer 2014;21:101-112.
108. Nguyen M, Wong YC, Ysselstein D, Severino A, Krainc D. Synaptic, mitochondrial, and lysosomal dysfunction in Parkinson's disease. Trends in neurosciences 2019;42:140-149.
109. Dehay B, Martinez-Vicente M, Caldwell GA, et al. Lysosomal impairment in Parkinson's disease. Movement Disorders 2013;28:725-732.
110. Senkevich K, Gan-Or Z. Autophagy lysosomal pathway dysfunction in Parkinson's disease; evidence from human genetics. Parkinsonism & Related Disorders 2019.
111. Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L. Wild type α-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. Journal of Biological Chemistry 2008;283:23542-23556.
112. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. α-Synuclein is degraded by both autophagy and the proteasome. Journal of Biological Chemistry 2003;278:25009-25013.
113. Rochet J-C, Hay BA, Guo M. Molecular insights into Parkinson's disease. Progress in molecular biology and translational science: Elsevier, 2012: 125-188.
114. Perier C, Vila M. Mitochondrial biology and Parkinson's disease. Cold Spring Harb Perspect Med 2012;2:a009332.
115. Gan-Or Z, Amshalom I, Kilarski LL, et al. Differential effects of severe vs mild GBA mutations on Parkinson disease. Neurology 2015;84:880-887.

116. Krohn L, Ruskey JA, Rudakou U, et al. GBA variants in REM sleep behavior disorder: a multicenter study. Neurology 2020.
117. Osellame LD, Duchen MR. Defective quality control mechanisms and accumulation of damaged mitochondria link Gaucher and Parkinson diseases. Autophagy 2013;9:1633-1635.
118. Ron I, Rapaport D, Horowitz M. Interaction between parkin and mutant glucocerebrosidase variants: a possible link between Parkinson disease and Gaucher disease. Human molecular genetics 2010;19:3771-3781.
119. Mazzulli JR, Xu Y-H, Sun Y, et al. Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. Cell 2011;146:37-52.
120. Pavlou MAS, Outeiro TF. Epigenetics in Parkinson's disease. Neuroepigenomics in Aging and Disease: Springer, 2017: 363-390.
121. Zhao A, Li Y, Niu M, et al. SNCA Hypermethylation in Rapid Eye Movement SleepBehavior Disorder Is a Potential Biomarker for Parkinson's Disease. Journal of Parkinson's Disease 2020:1-9.
122. Gan-Or Z, Mirelman A, Postuma RB, et al. GBA mutations are associated with Rapid Eye Movement Sleep Behavior Disorder. Annals of clinical and translational neurology 2015;2:941-945.
123. Nalls M, Blauwendraat C, Vallerga C, et al. Expanding Parkinson's disease genetics: novel risk loci, genomic context, causal insights and heritable risk. bioRxiv. Epub, 2019.
124. Chenbhanich J, Sringean J, Bhidayasiri R. Beyond the Classic Segawa Disease, GCH1-Associated Neurodegenerative Parkinsonism: Practical Considerations for Physicians. JMD 2017;10:102-104.
125. Guella I, Sherman HE, Appel-Cresswell S, Rajput A, Rajput AH, Farrer MJ. Parkinsonism in GTP cyclohydrolase 1 mutation carriers. Brain 2014;138:e349-e349.
126. Blauwendraat C, Nalls MA, Singleton ABJTLN. The genetic architecture of Parkinson's disease. 2019.
127. Usenovic M, Tresse E, Mazzulli JR, Taylor JP, Krainc D. Deficiency of ATP13A2 Leads to Lysosomal Dysfunction, α-Synuclein Accumulation, and Neurotoxicity. 2012;32:4240-4246.
128. Conedera S, Apaydin H, Li Y, et al. FBXO7 mutations in Parkinson's disease and multiple system atrophy. 2016;40:192. e191-192. e195.

129. Guo Y-P, Tang B-S, Guo J-F. PLA2G6-Associated Neurodegeneration (PLAN): Review of Clinical Phenotypes and Genotypes. Front Neurol 2018;9:1100-1100.
130. Gan-Or Z, Rouleau GA. Genetics of REM Sleep Behavior Disorder. In: Schenck CH, Högl B, Videnovic A, eds. Rapid-Eye-Movement Sleep Behavior Disorder. Cham: Springer International Publishing, 2019: 589-609.
131. Heckman MG, Soto-Ortolaza AI, Sanchez Contreras MY, et al. LRRK2 variation and dementia with Lewy bodies. Parkinsonism & Related Disorders 2016;31:98-103.
132. Bencheikh BOA, Ruskey JA, Arnulf I, et al. LRRK2 protective haplotype and full sequencing study in REM sleep behavior disorder. 2018;52:98-101.
133. Li J, Ruskey JA, Arnulf I, et al. Full sequencing and haplotype analysis of MAPT in Parkinson's disease and rapid eye movement sleep behavior disorder. 2018;33:1016-1020.
134. Dickson DW, Heckman MG, Murray ME, et al. APOE ε4 is associated with severity of Lewy body pathology independent of Alzheimer pathology. 2018;91:e1182-e1195.
135. Gan-Or Z, Montplaisir JY, Ross JP, et al. The dementia-associated APOE ε4 allele is not associated with rapid eye movement sleep behavior disorder. 2017;49:218. e213-218. e215.
136. Krohn L, Wu RYJ, Heilbron K, et al. Fine-Mapping of SNCA in Rapid Eye Movement Sleep Behavior Disorder and Overt Synucleinopathies. 2020;87(4):584-598.
137. Krohn L, Öztürk TN, Vanderperre B, et al. Genetic, Structural, and Functional Evidence Link TMEM175 to Synucleinopathies. 2020;87:139-153.
138. Thorpy MJJN. Classification of sleep disorders. 2012;9:687-701.
139. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. bioinformatics 2009;25:1754-1760.
140. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research 2010;20:1297-1303.
141. Li X, Patel JC, Wang J, et al. Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. J Neurosci 2010;30:1788-1797.
142. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016;536:285-291.

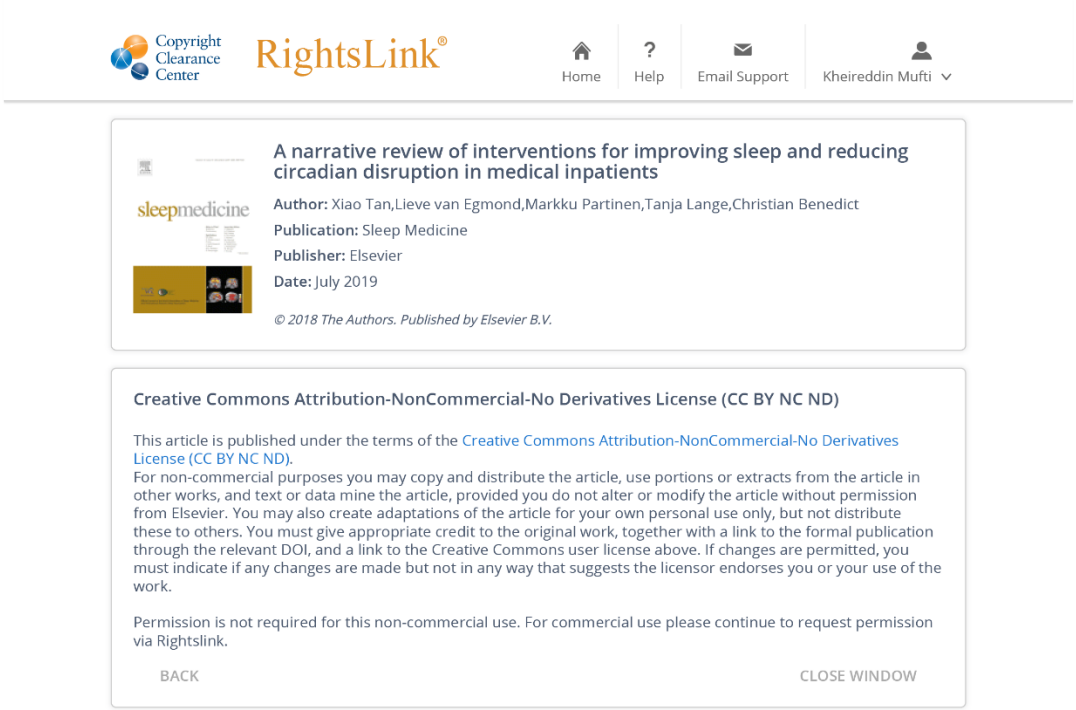
143. Lee S, Emond MJ, Bamshad MJ, et al. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. 2012;91:224-237.
144. Lücking CB, Dürr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. New England Journal of Medicine 2000;342:1560-1567.
145. Plagnol V, Curtis J, Epstein M, et al. A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. 2012;28:2747-2754.
146. Reed X, Bandrés-Ciga S, Blauwendraat C, Cookson MR. The role of monogenic genes in idiopathic Parkinson's disease. Neurobiology of Disease 2019;124:230-239.
147. Samaranch L, Lorenzo-Betancor O, Arbelo JM, et al. PINK1-linked parkinsonism is associated with Lewy body pathology. Brain 2010;133:1128-1142.
148. Takanashi M, Li Y, Hattori N. Absence of Lewy pathology associated with PINK1 homozygous mutation. Neurology 2016;86:2212-2213.
149. Fernández-Santiago R, Iranzo A, Gaig C, et al. Absence of LRRK2 mutations in a cohort of patients with idiopathic REM sleep behavior disorder. 2016;86:1072-1073.
150. Zhang J, Xu C-Y, Liu J. Meta-analysis on the prevalence of REM sleep behavior disorder symptoms in Parkinson's disease. BMC Neurol 2017;17:23-23.
151. Zhang X, Sun X, Wang J, Tang L, Xie A. Prevalence of rapid eye movement sleep behavior disorder (RBD) in Parkinson's disease: a meta and meta-regression analysis. Neurological Sciences 2017;38:163-170.
152. Jiang H, Huang J, Shen Y, et al. RBD and Neurodegenerative Diseases. Molecular Neurobiology 2017;54:2997-3006.
153. Lesage S, Condroyer C, Klebe S, et al. Identification of VPS35 mutations replicated in French families with Parkinson disease. 2012;78:1449-1450.
154. Kumar KR, Weissbach A, Heldmann M, et al. Frequency of the D620N Mutation in VPS35 in Parkinson Disease. Archives of Neurology 2012;69:1360-1364.
155. Gan-Or Z, Rouleau GA. Genetics of REM Sleep Behavior Disorder. Rapid-Eye-Movement Sleep Behavior Disorder: Springer, 2019: 589-609.

156. Gan-Or Z, Mirelman A, Postuma RB, et al. GBA mutations are associated with rapid eye movement sleep behavior disorder. Ann Clin Transl Neurol 2015;2:941-945.
157. Heckman MG, Soto-Ortolaza AI, Contreras MYS, et al. LRRK2 variation and dementia with Lewy bodies. Parkinsonism & related disorders 2016;31:98-103.
158. Mufti K, Rudakou U, Yu E, et al. A comprehensive analysis of dominant and recessive parkinsonism genes in REM sleep behavior disorder. medRxiv 2020.
159. Alcalay RN, Mallett V, Vanderperre B, et al. SMPD1 mutations, activity, and α-synuclein accumulation in Parkinson's disease. Movement Disorders 2019;34:526-535.
160. Gan-Or Z, Ozelius LJ, Bar-Shira A, et al. The p. L302P mutation in the lysosomal enzyme gene SMPD1 is a risk factor for Parkinson disease. Neurology 2013;80:1606-1610.
161. Dickson DW, Heckman MG, Murray ME, et al. APOE ε4 is associated with severity of Lewy body pathology independent of Alzheimer pathology. Neurology 2018;91:e1182-e1195.
162. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic acids research 2010;38:e164-e164.
163. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. The American journal of human genetics 2007;81:559-575.
164. Lee S, Emond MJ, Bamshad MJ, et al. Optimal unified approach for rare-variant association testing with application to small-sample case-control whole-exome sequencing studies. The American Journal of Human Genetics 2012;91:224-237.
165. Machiela MJ, Chanock SJ. LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. Bioinformatics 2015;31:3555-3557.
166. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nature genetics 2013;45:580-585.
167. Ishihara K, Hirano T. BST-1/CD157 regulates the humoral immune responses in vivo. Chemical immunology 2000;75:235-255.
168. Yamamoto-Katayama S, Ariyoshi M, Ishihara K, Hirano T, Jingami H, Morikawa K. Crystallographic studies on human BST-1/CD157 with ADP-ribosyl cyclase and NAD glycohydrolase activities. Journal of molecular biology 2002;316:711-723.

169. Liang F, Qi RZ, Chang CF. CD157 undergoes ligand-independent dimerization and colocalizes with caveolin in CHO and MCA102 fibroblasts. Cell Signal 2002;14:933-939.
170. Dominguez-Bautista JA, Klinkenberg M, Brehm N, et al. Loss of lysosome-associated membrane protein 3 (LAMP3) enhances cellular vulnerability against proteasomal inhibition. European journal of cell biology 2015;94:148-161.

APPENDIX

Copyright permissions



Copyright Clearance Center RightsLink®

Home | Help | Email Support | Kheireddin Mufti

A narrative review of interventions for improving sleep and reducing circadian disruption in medical inpatients

Author: Xiao Tan,Lieve van Egmond,Markku Partinen,Tanja Lange,Christian Benedict

Publication: Sleep Medicine

Publisher: Elsevier

Date: July 2019

© 2018 The Authors. Published by Elsevier B.V.

Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)

This article is published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND).
For non-commercial purposes you may copy and distribute the article, use portions or extracts from the article in other works, and text or data mine the article, provided you do not alter or modify the article without permission from Elsevier. You may also create adaptations of the article for your own personal use only, but not distribute these to others. You must give appropriate credit to the original work, together with a link to the formal publication through the relevant DOI, and a link to the Creative Commons user license above. If changes are permitted, you must indicate if any changes are made but not in any way that suggests the licensor endorses you or your use of the work.

Permission is not required for this non-commercial use. For commercial use please continue to request permission via Rightslink.

BACK CLOSE WINDOW

THE AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE LICENSE TERMS AND CONDITIONS

Jun 23, 2020

This Agreement between Mr. Kheireddin Mufti ("You") and The American Association for the Advancement of Science ("The American Association for the Advancement of Science") consists of your license details and the terms and conditions provided by The American Association for the Advancement of Science and Copyright Clearance Center.

License Number	4855060032912
License date	Jun 23, 2020
Licensed Content Publisher	The American Association for the Advancement of Science
Licensed Content Publication	Science
Licensed Content Title	Multiplex Targeted Sequencing Identifies Recurrently Mutated Genes in Autism Spectrum Disorders
Licensed Content Author	Brian J. O'Roak,Laura Vives,Wenqing Fu,Jarrett D. Egertson,Ian B. Stanaway,Ian G. Phelps,Gemma Carvill,Akash Kumar,Choli Lee,Katy Ankenman,Jeff Munson,Joseph B. Hiatt,Emily H. Turner,Roie Levy,Diana R. O'Day,Niklas Krumm,Bradley P. Coe,Beth K. Martin,Elhanan Borenstein,Deborah A. Nickerson,Heather C. Mefford,Dan Doherty,Joshua M. Akey,Raphael Bernier,Evan E. Eichler,Jay Shendure
Licensed Content Date	Dec 21, 2012
Licensed Content Volume	338

Licensed Content Issue	6114
Volume number	338
Issue number	6114
Type of Use	Thesis / Dissertation
Requestor type	Scientist/individual at a research institution
Format	Print and electronic
Portion	Figure
Number of figures/tables	1
Title	Targeted next-generation approach to study parkinsonism genes in REM sleep behavior disorder
Institution name	McGill University
Expected presentation date	Jun 2020
Portions	Figure 1 A
Requestor Location	Mr. Kheireddin Mufti university rue 3647 APT. 16 Montreal, QC H3A 2B3 Canada Attn: Mr. Kheireddin Mufti

Total 0.00 USD

Terms and Conditions

American Association for the Advancement of Science TERMS AND CONDITIONS

Regarding your request, we are pleased to grant you non-exclusive, non-transferable permission, to republish the AAAS material identified above in your work identified above, subject to the terms and conditions herein. We must be contacted for permission for any uses other than those specifically identified in your request above.

The following credit line must be printed along with the AAAS material: "From [Full Reference Citation]. Reprinted with permission from AAAS."

All required credit lines and notices must be visible any time a user accesses any part of the AAAS material and must appear on any printed copies and authorized user might make.

This permission does not apply to figures / photos / artwork or any other content or materials included in your work that are credited to non-AAAS sources. If the requested material is sourced to or references non-AAAS sources, you must obtain authorization from that source as well before using that material. You agree to hold harmless and indemnify AAAS against any claims arising from your use of any content in your work that is credited to non-AAAS sources.

If the AAAS material covered by this permission was published in Science during the years 1974 - 1994, you must also obtain permission from the author, who may grant or withhold permission, and who may or may not charge a fee if permission is granted. See original article for author's address. This condition does not apply to news articles.

The AAAS material may not be modified or altered except that figures and tables may be modified with permission from the author. Author permission for any such changes must be secured prior to your use.

Whenever possible, we ask that electronic uses of the AAAS material permitted herein include a hyperlink to the original work on AAAS's website (hyperlink may be embedded in the reference citation).

AAAS material reproduced in your work identified herein must not account for more than 30% of the total contents of that work.

AAAS must publish the full paper prior to use of any text.

AAAS material must not imply any endorsement by the American Association for the Advancement of Science.

This permission is not valid for the use of the AAAS and/or Science logos.

AAAS makes no representations or warranties as to the accuracy of any information contained in the AAAS material covered by this permission, including any warranties of

merchantability or fitness for a particular purpose.

If permission fees for this use are waived, please note that AAAS reserves the right to charge for reproduction of this material in the future.

Permission is not valid unless payment is received within sixty (60) days of the issuance of this permission. If payment is not received within this time period then all rights granted herein shall be revoked and this permission will be considered null and void.

In the event of breach of any of the terms and conditions herein or any of CCC's Billing and Payment terms and conditions, all rights granted herein shall be revoked and this permission will be considered null and void.

AAAS reserves the right to terminate this permission and all rights granted herein at its discretion, for any purpose, at any time. In the event that AAAS elects to terminate this permission, you will have no further right to publish, publicly perform, publicly display, distribute or otherwise use any matter in which the AAAS content had been included, and all fees paid hereunder shall be fully refunded to you. Notification of termination will be sent to the contact information as supplied by you during the request process and termination shall be immediate upon sending the notice. Neither AAAS nor CCC shall be liable for any costs, expenses, or damages you may incur as a result of the termination of this permission, beyond the refund noted above.

This Permission may not be amended except by written document signed by both parties.

The terms above are applicable to all permissions granted for the use of AAAS material. Below you will find additional conditions that apply to your particular type of use.

FOR A THESIS OR DISSERTATION
If you are using figure(s)/table(s), permission is granted for use in print and electronic versions of your dissertation or thesis. A full text article may be used in print versions only of a dissertation or thesis.

Permission covers the distribution of your dissertation or thesis on demand by ProQuest / UMI, provided the AAAS material covered by this permission remains in situ.

If you are an Original Author on the AAAS article being reproduced, please refer to your License to Publish for rules on reproducing your paper in a dissertation or thesis.

FOR JOURNALS:
Permission covers both print and electronic versions of your journal article, however the AAAS material may not be used in any manner other than within the context of your article.

FOR BOOKS/TEXTBOOKS:
If this license is to reuse figures/tables, then permission is granted for non-exclusive world rights in all languages in both print and electronic formats (electronic formats are defined below).

If this license is to reuse a text excerpt or a full text article, then permission is granted for non-exclusive world rights in English only. You have the option of securing either print or electronic rights or both, but electronic rights are not automatically granted and do garner additional fees. Permission for translations of text excerpts or full text articles into other languages must be obtained separately.

Licenses granted for use of AAAS material in electronic format books/textbooks are valid only in cases where the electronic version is equivalent to or substitutes for the print version of the book/textbook. The AAAS material reproduced as permitted herein must remain in situ and must not be exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit), except in the case of permitted textbook companions as noted below.

You must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher."

If your book is an academic textbook, permission covers the following companions to your textbook, provided such companions are distributed only in conjunction with your textbook at no additional cost to the user:

- Password-protected website
- Instructor's image CD/DVD and/or PowerPoint resource
- Student CD/DVD

All companions must contain instructions to users that the AAAS material may be used for non-commercial, classroom purposes only. Any other uses require the prior written permission from AAAS.

If your license is for the use of AAAS Figures/Tables, then the electronic rights granted herein permit use of the Licensed Material in any Custom Databases that you distribute the electronic versions of your textbook through, so long as the Licensed Material remains within the context of a chapter of the title identified in your request and cannot be downloaded by a user as an independent image file.

Rights also extend to copies/files of your Work (as described above) that you are required to provide for use by the visually and/or print disabled in compliance with state and federal laws.

This permission only covers a single edition of your work as identified in your request.

FOR NEWSLETTERS:
Permission covers print and/or electronic versions, provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit)

FOR ANNUAL REPORTS:
Permission covers print and electronic versions provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit)

FOR PROMOTIONAL/MARKETING USES:
Permission covers the use of AAAS material in promotional or marketing pieces such as information packets, media kits, product slide kits, brochures, or flyers limited to a single print run. The AAAS Material may not be used in any manner which implies endorsement or promotion by the American Association for the Advancement of Science (AAAS) or Science of any product or service. AAAS does not permit the reproduction of its name, logo or text on promotional literature.

If permission to use a full text article is permitted, The Science article covered by this permission must not be altered in any way. No additional printing may be set onto an article

copy other than the copyright credit line required above. Any alterations must be approved in advance and in writing by AAAS. This includes, but is not limited to, the placement of sponsorship identifiers, trademarks, logos, rubber stamping or self-adhesive stickers onto the article copies.

Additionally, article copies must be a freestanding part of any information package (i.e. media kit) into which they are inserted. They may not be physically attached to anything, such as an advertising insert, or have anything attached to them, such as a sample product. Article copies must be easily removable from any kits or informational packages in which they are used. The only exception is that article copies may be inserted into three-ring binders.

FOR CORPORATE INTERNAL USE:
The AAAS material covered by this permission may not be altered in any way. No additional printing may be set onto an article copy other than the required credit line. Any alterations must be approved in advance and in writing by AAAS. This includes, but is not limited to the placement of sponsorship identifiers, trademarks, logos, rubber stamping or self-adhesive stickers onto article copies.

If you are making article copies, copies are restricted to the number indicated in your request and must be distributed only to internal employees for internal use.

If you are using AAAS Material in Presentation Slides, the required credit line must be visible on the slide where the AAAS material will be reprinted

If you are using AAAS Material on a CD, DVD, Flash Drive, or the World Wide Web, you must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher." Access to any such CD, DVD, Flash Drive or Web page must be restricted to your organization's employees only.

FOR CME COURSE and SCIENTIFIC SOCIETY MEETINGS:
Permission is restricted to the particular Course, Seminar, Conference, or Meeting indicated in your request. If this license covers a text excerpt or a Full Text Article, access to the reprinted AAAS material must be restricted to attendees of your event only (if you have been granted electronic rights for use of a full text article on your website, your website must be password protected, or access restricted so that only attendees can access the content on your site).

If you are using AAAS Material on a CD, DVD, Flash Drive, or the World Wide Web, you must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher."

FOR POLICY REPORTS:
These rights are granted only to non-profit organizations and/or government agencies. Permission covers print and electronic versions of a report, provided the required credit line

appears in both versions and provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately.

FOR CLASSROOM PHOTOCOPIES:
Permission covers distribution in print copy format only. Article copies must be freestanding and not part of a course pack. They may not be physically attached to anything or have anything attached to them.

FOR COURSEPACKS OR COURSE WEBSITES:
These rights cover use of the AAAS material in one class at one institution. Permission is valid only for a single semester after which the AAAS material must be removed from the Electronic Course website, unless new permission is obtained for an additional semester. If the material is to be distributed online, access must be restricted to students and instructors enrolled in that particular course by some means of password or access control.

FOR WEBSITES:
You must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher."

Permissions for the use of Full Text articles on third party websites are granted on a case by case basis and only in cases where access to the AAAS Material is restricted by some means of password or access control. Alternately, an E-Print may be purchased through our reprints department (brocheleau@rockwaterinc.com).

REGARDING FULL TEXT ARTICLE USE ON THE WORLD WIDE WEB IF YOU ARE AN 'ORIGINAL AUTHOR' OF A SCIENCE PAPER

If you chose "Original Author" as the Requestor Type, you are warranting that you are one of authors listed on the License Agreement as a "Licensed content author" or that you are acting on that author's behalf to use the Licensed content in a new work that one of the authors listed on the License Agreement as a "Licensed content author" has written.

Original Authors may post the 'Accepted Version' of their full text article on their personal or on their University website and not on any other website. The 'Accepted Version' is the version of the paper accepted for publication by AAAS including changes resulting from peer review but prior to AAAS's copy editing and production (in other words not the AAAS published version).

FOR MOVIES / FILM / TELEVISION:
Permission is granted to use, record, film, photograph, and/or tape the AAAS material in connection with your program/film and in any medium your program/film may be shown or heard, including but not limited to broadcast and cable television, radio, print, world wide web, and videocassette.

The required credit line should run in the program/film's end credits.

FOR MUSEUM EXHIBITIONS:
Permission is granted to use the AAAS material as part of a single exhibition for the duration of that exhibit. Permission for use of the material in promotional materials for the exhibit must be cleared separately with AAAS (please contact us at permissions@aaas.org).

FOR TRANSLATIONS:
Translation rights apply only to the language identified in your request summary above.

The following disclaimer must appear with your translation, on the first page of the article, after the credit line: "This translation is not an official translation by AAAS staff, nor is it endorsed by AAAS as accurate. In crucial matters, please refer to the official English-language version originally published by AAAS."

FOR USE ON A COVER:
Permission is granted to use the AAAS material on the cover of a journal issue, newsletter issue, book, textbook, or annual report in print and electronic formats provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately

By using the AAAS Material identified in your request, you agree to abide by all the terms and conditions herein.

Questions about these terms can be directed to the AAAS Permissions department permissions@aaas.org.

Other Terms and Conditions:

v 2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Ethics certificate

MUHC REB – NEUPSY / CÉR CUSM – NEUPSY
3801, rue University, # 686
Montréal (Québec) H3A 2B4
Tel. 514.398.1046
reb.neuro@mcgill.ca
www.leneuro.ca

Annual renewal submission

Submit date: **2019-12-04 11:55**
Project's REB approbation date: **2016-12-01**
Project number: **2017-2944**
Form status: **Approved**

Submitted by: **Zaharieva, Vessela**
Nagano identifier: **RBD-PD**
Form: **F9 - 50014**

Administration

1. **MUHC REB Panel & Co-chair(s):**
   Neurosciences-Psychiatry (NEUPSY)
   Co-chairs: Judith Marcoux, Brigitte Pâquet

2. **REB Decision:**
   Approved - REB delegated review

3. **Comments on the decision:**
   Please note that this Annual Renewal Form was submitted prior to the REB approval expiry.

4. **Renewal Period Granted:**
   Until 2020-12-01

5. **Date of the REB final decision & signature**
   2019-12-04
   Signature

Ms. Brigitte Paquet, LL.B.
Brigitte Pâquet, LL.B.
Co-Chair, MUHC REB (NEUPSY Panel)

6. FWA 00000840 - FWA 00004545

7. **Local REB number**
IRB00010120

A. General information

1. **Indicate the full title of the research study**

The genetics of REM sleep behavior disorder, Parkinson's disease and other synucleinopathies

2. **If relevant, indicate the full study title in French**

3. **Indicate the name of the Principal Investigator in our institution (MUHC)**

Gan-Or, Ziv

From which department is the principal investigator?

Neurology

4. **Are there local co-investigators & collaborators involved in this project?**

Yes

List all the local co-investigators & collaborators involved in the research study

Rouleau, Guy

Department of co-investigators & collaborators

Neurology

Fon, Edward A

Department of co-investigators & collaborators

Neurology

Postuma, Ronald

Department of co-investigators & collaborators

Neurology

B. Project development

1. **Study start date:**
2016-12-19

2. **Expected ending date of the study:**

☐ Determined date
☑ Undetermined date

3. **Date of recruitment of the first participant?**

☑ 1st enrollment date is...
☐ No participant enrolled

1st participant enrollment date:
2016-12-21

4. **Add a brief statement on the study status**

The project is still on-going

5. **Information about the participants at this institution, since the beginning of the project**

Number of participants who have been recruited
2647
Number of participants who have not yet completed the study (still in progress)
0
Number of participants who've completed the study
2647
Number of participants who were recruited to the study, but who were then excluded or withdrawn:
0
Number of participants who dropped out (voluntary withdrawal):
0
Number of participants who died during the study
0

6. **Information about the participants at this institution (MUHC) since the previous REB approval**

Number of participants who have been recruited
566
Number of participants who have not yet completed the study (still in progress)
0
Number of participants who've completed the study
566
Number of participants who were recruited to the study, but who were then excluded or withdrawn:
0
Number of participants who dropped out (voluntary withdrawal):
0
Number of participants who died during the study
0

7. **Since the previous REB approval (annual renewal or initial approval):**

Were there any changes to the protocol (or to the databank management framework) ?

No

Specify the current version/date:

V3 2018-06-21

Date approved by the REB:

2018-06-21

Were there any changes to the information and consent form?

No

Specify the current version/date:

V1

REB approval date:

2016-03-14

Were there any reportable adverse events at this site (or, for multi-center projects, at an institution under the jurisdiction of our REB) that should be reported to the REB under section 5.2.1 of " SOP-REB- 404001 " ?

https://muhc.ca/cae/page/standard-operating-procedures-sops

No

Has there has been any new information likely to affect the ethics of the project or influence the decision of a participant as to their continued participation in the project ?

No

Were there any deviations / major violations protocol (life -threatening or not meeting the inclusion / exclusion criteria)?

No

Was there a temporary interruption of the project?

No

Have the project results been submitted for publication, presented or published?

No

Has the REB been notified of a conflict of interest - (apparent , potential or actual), of one or more members of the research team - that was not known when it was last approved project?

No

Do you want to bring any other info to the REB's attention?

No

8. **Is there a data safety monitoring committee analyzing data on the safety and efficacy of the treatment?**

No

C. Signature

1. **Comments**

All samples come from the RouBank that continues to increase throughout the study period. We need as many as possible samples and therefore we have not a target for number of participants

2. **I confirm that all information is complete & accurate.**

First & last name of person who completed the submission

Vessela Zaharieva