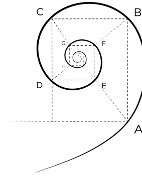

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Integrated genomic analysis of isolated CD14+ monocytes in patients with GBA-related Parkinson's disease

Dottorando: Giulietta Maria RIBOLDI

Matricola N° : R12009

TUTORE: Giacomo Pietro COMI

CO-TUTORE: Alessio Barnaba DI FONZO

COORDINATORE DEL DOTTORATO: prof. Michele SAMAJA

This thesis was evaluated by three independent Reviewers:

Prof. Stefano Duga, PhD
Humanitas University
Department of Biomedical Sciences
Milano (Italy)

Prof/Dr. Michela Deleidi, MD-PhD
University of Tübingen
German Center for Neurodegenerative Diseases
Tübingen (Germany)

Dr. Nicoletta Plotegher, PhD
University of Padova | UNIPD
Department of Biology
Padova (Italy)

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SOMMARIO

Introduzione: Mutazioni del gene codificante per la beta-glucocerebrosidasi umana (GCase), gene *GBA*, rappresentano il più frequente fattore di rischio genetico per lo sviluppo della malattia di Parkinson (MP). I meccanismi patogenetici associati a questa mutazione e i fattori modulanti responsabili della sua ridotta penetranza non sono ancora completamente noti. Allo stesso tempo, le funzioni della GCase si collocano ad un crocevia tra i meccanismi endolisosomiali e la risposta immunitaria, due possibili meccanismi chiave nei processi patogenetici della malattia di Parkinson.

Scopo: col presente lavoro ci proponiamo di caratterizzare i profili clinici di una popolazione di pazienti con MP e controlli sani (CTRL) con e senza mutazioni del gene *GBA* e di studiare i profili di trascrizione genica di monociti CD14+ purificati per l'identificazione di 1) meccanismi patogenetici associati con GBA-MP; 2) potenziali biomarcatori per la diagnosi precoce di portatori di mutazioni di *GBA* che svilupperanno malattia; 3) e di nuovi target terapeutici per approcci di terapie personalizzate.

Materiali e metodi: pazienti con MP e CTRL sono stati arruolati presso il Fresco Institute (NYU Langone Health, NY) e la Mount Sinai School of Medicine (NY). Abbiamo raccolto dati demografici, storia clinica e familiare, sintomi motori e non motori della MP. DNA e RNA da monociti purificati CD14+, plasma e cellule mononucleate da sangue periferico (PBMC) sono stati raccolti da una coorte di soggetti con MP e da CTRL con e senza mutazioni del gene *GBA*. Genotipizzazione (Illumina Global Screening Array con pannelli ad hoc per malattie neurodegenerative) e analisi di sequenziamento di

RNA (60M ribo-depleted, paired-end reads) sono stati effettuati. I dati sono stati poi analizzati tramite tecniche integrate di genomica utilizzando diversi metodi computazionali (espressione genica, modelli di interazione, analisi di outliers, e *trans*-eQTLs).

Risultati: l'analisi dei dati clinici di 19 MP/GBA+, 37 MP/GBA-, 37 CTRL/GBA-, 9 CTRL/GBA+ ha mostrato una maggiore incidenza di storia familiare e disordini cognitivi in MP/GBA+ e di sintomi non-motori in CTRL/GBA+. Analisi genomiche integrate di 56 MP/GBA-, 66 CTRL/GBA-, 23 MP/GBA+, e 13 CTRL/GBA+ hanno mostrato un ampio numero di geni differentemente espressi e di pathways deregolati nella popolazione MP/GBA+ rispetto a CTRL/GBA+ e MP/GBA-. In particolare, rispetto ai soggetti MP/GBA-, soggetti MP/GBA+ presentano una deregolazione dei livelli di alpha-synucleina e di pathways legati al metabolismo della beta-amiloide e ai processi di invecchiamento cellulare. Rispetto ai soggetti CTRL/GBA+, in soggetti MP/GBA+ vi è una deregolazione dei principali pathways legati alla MP. Le analisi di outliers e *trans*-eQTLs hanno confermato il coinvolgimento di target lisosomiali, legati al metabolismo delle membrane cellulari, e mitocondriali, identificando ulteriori geni target associati a questi meccanismi.

Discussione: l'analisi di dati clinici e demografici di soggetti con MP e CTRL con e senza mutazioni del gene *GBA* ha permesso di identificare distinte caratteristiche in queste coorti. Analisi genomiche di monociti CD14+ purificati hanno mostrato specifici bersagli molecolari e meccanismi alterati che possono contribuire alla comprensione dei meccanismi legati al ruolo di mutazioni di *GBA* nel contesto della MP.

ABSTRACT

Background: Genetic mutations of the gene encoding for the beta-glucocerebrosidase (GCase), *GBA* gene, represent the major genetic risk factor for Parkinson's disease (PD). The pathogenic mechanisms associated with these mutations and modifiers responsible for the reduced penetrance of this gene are not fully elucidated yet. However, the function of the *GBA* gene is at the crossroad between the endo-lysosomal pathway and the immune response, which are two main mechanisms involved in PD pathogenesis.

Aim: With the present work we aim to characterize the clinical features of a population of patients with PD and healthy controls (CTRL) with and without mutations of the *GBA* gene and characterize the transcriptomic profiles of purified CD14+ monocytes in order to identify 1) pathogenic mechanisms associated with GBA-PD; 2) potential biomarkers for earlier detection of *GBA* mutation carriers who will phenoconvert to a disease status; 3) new therapeutic targets for precision medicine approaches.

Material and methods: PD patients and CTRL were enrolled at the Fresco Institute (NYU Langone Health, NY) and Mount Sinai School of Medicine (NY). Demographic information, clinical and family history, as well as motor and non-motor symptoms of PD were collected. DNA and RNA from purified CD14+ monocytes, plasma and peripheral blood mononuclear cells (PBMC) were collected. Genotyping (Illumina Global Screening Array with custom Neurodegenerative disease panel) and RNAseq analysis (60M ribo-depleted, paired-end reads) was performed. Data were analyzed through integrative genomic analysis leveraging different

computational methods (differential expression, nested interaction model, outlier detection and *trans*-eQTLs).

Results: Statistical analysis comparing the clinical phenotypes of 19 PD/GBA+, 37 PD/GBA-, 37 CTRL/GBA-, 9 CTRL/GBA+ showed increased non-motor symptoms in CTRL/GBA+ and increased family history and cognitive impairment in the PD/GBA+ cohort. Integrative genomic analysis of a cohort of 56 PD/GBA-, 66 CTRL/GBA-, 23 PD/GBA+, and 13 CTRL/GBA+ identified a large number of differentially expressed genes and deregulated pathways in the PD/GBA+ compared to CTRL/GBA+ as well as PD/GBA- groups. In particular, PD/GBA+ showed deregulated alpha-synuclein-, amyloid- and aging-related processes compared to PD/GBA-. Compared to CTRL/GBA+, in manifesting carriers there was a deregulation of all the major pathogenic pathways previously associated with PD. Outliers and *trans*-eQTLs analysis confirmed a prominent involvement of lysosomal, membrane trafficking, and mitochondrial targets, identifying also additional related genes.

Discussion: Clinical and demographic analysis of PD patients and CTRL with and without *GBA* mutations highlighted characteristic features in the PD/GBA+ and CTRL/GBA+ cohort. Genomic analysis of isolated CD14+ monocytes identified specific molecular targets and deregulated pathways that can help understanding the pathogenic mechanisms associated with *GBA* mutations in the context of PD.

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1. List of symbols and figures

Symbols

ACD: acid citrate dextrose

ACOT9: Acyl-CoA Thioesterase 9

AD: autosomal dominant

ADe: Alzheimer dementia

ADRC: Alzheimer's Research Center

AIFM3: Apoptosis Inducing Factor Mitochondria Associated 3

AIP: Aryl Hydrocarbon Receptor Interacting Protein

AJ: Ashkenazy Jewish

ALG-2: Alpha-1,3/1,6-mannosyltransferase ALG2

ANGPT1: angiopoietin 1

AP2A1: Adaptor Related Protein Complex 2 subunit alpha-1

AP2B1: Adaptor Related Protein Complex 2 subunit beta-1

AP2S1: Adaptor Related Protein Complex 2 subunit sigma-1

APEX1: Apurinic/Apyrimidinic Endodeoxyribonuclease 1

AR: autosomal recessive

ARHGAP1: Rho GTPase activating protein

ATAD3B: ATPase Family AAA Domain Containing 3B

ATG7: Autophagy Related 7

ATP6V0C(1F): ATPase H+ Transporting V0 Subunit C(V1 Subunit F)

ATP13A2*:* ATPase Cation Transporting 13A2

ATP2A2: ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2

BIN1: Bridging Integrator 1

BLOC1S1: Biogenesis Of Lysosomal Organelles Complex 1 Subunit 1

BP: Biological Processes

BPMD: Bendheim Parkinson and Movement Disorders (Center at Mount Sinai School of Medicine (NY, US))
BST1: Bone marrow stromal cell antigen 1, ADP-ribosyl cyclase 2, *CD157*
BST2: Bone marrow stromal cell antigen 2
BTBD2: BTB Domain Containing 2
CAMTA2: Calmodulin Binding Transcription Activator 2
CBD: corticobasal degeneration
CC: Cellular Component
CCH: Center for Cognitive Health
CHCHD2: Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 2
CHID1: Chitinase Domain Containing 1
CHML: CHM Like Rab Escort Protein
CHSY1: Chondroitin Sufate Synthase 1
CLEC16A: C-Type Lectin Domain Containing 16A
COMT: catechol-O-methyltransferase
COX8A: Acyl-CoA Thioesterase 9
CPM: count per million
CSF: cerebrospinal fluid
CT: computed tomography
CTRL: control
CTSB: cathepsin B
CXCR2: C-X-C Motif Chemokine Receptor 2
DAT: dopamine transporter
DBS: deep brain stimulation
DJ-1: Protein/Nucleic Acid Deglycase
DLST: Dihydrolipoamide S-Succinyltransferase
DNAJC6: DnaJ Heat Shock Protein Family (Hsp40) Member C6
DNAJC13: DnaJ Heat Shock Protein Family (Hsp40) Member C13
DOCK1: Dedicator of Cytokinesis 1
DYRK1A: Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A

ECH1: Enoyl-CoA Hydratase 1

EIF4G1: Eukaryotic translation initiation factor 4 gamma 1

EL: endolysosomal

EOPD: early onset Parkinson’s disease

EP300: E1A Binding Protein P300

EPN1: Epsin 1

eQTLS: expression quantitative trait loci

ER: endoplasmic reticulum

ERLIN1: ER lipid raft associated 1

FAM161A: FAM161A Centrosomal Protein A

FBXO7: F-Box Protein 7GBA: glucocerebrosidase

FDR: False Discovery Rate

FILIP1L: Filamin A Interacting Protein 1 Like

FIS1: Fission Mitochondrial 1

FUCA2: Alpha-L-Fucosidase 2

GBA: glucocerebrosidase

GBAP: glucocerebrosidase pseudogene

GCase: beta-glucocerebrosidase

GCH1: GTP cyclohydrolase 1

GD: Gaucher’s disease

GIGYF2: GRB10 Interacting GYF Protein 2

GLA: alpha-galactosidase A

GO: Gene Ontology

GPT2: Glutamic--Pyruvic Transaminase 2

GSEA: Gene Set Enrichment Analysis

GTex: Genotype-Tissue Expression

GTPBP3: GTP binding protein 3, Mitochondrial

GWAS: genome wide association study

HEXA: hexosaminidase Subunit alpha

HEXB: hexosaminidase Subunit Beta

HIGD2A: HIG1 Hypoxia Inducible Domain Family Member 2A
HPSE: heparanase
HPS3: Hermansky-Pudlak Syndrome 3 Protein
IRAK4: Interleukin 1 Receptor Associated Kinase 4
IRB: Institutional Review Board
IST1: increased sodium tolerance 1 gene
KMT2B: lysine methyltransferase 2D
KMT2D: lysine methyltransferase 2D
ICAM1: Intracellular Adhesion Molecule 1
IL- : interleukin-
IMMT: Inner Membrane Mitochondrial Protein
IPA: Ingenuity pathway analysis
ITM2B: integral membrane protein 2B
JOPD: juvenile onset Parkinson’s disease
GATD3B: Glutamine Amidotransferase Like Class 1 Domain Containing 3B
GSA: Global Screening Array
H&Y: Hoehn and Yahr
HGNC: HUGO Gene Nomenclature Committee
HIFU: high intensity focused ultrasounds
HLA: human leukocyte antigen
LAMTOR2: Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 2
LARS2: Leucyl-TRNA Synthetase 2, Mitochondrial
LBD: Lewy Body Dementia
LD: linkage disequilibrium
LEDD: levodopa equivalent daily dose
LFNG: O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase
LITAF: Lipopolysaccharide Induced TNF Factor
LMNA: Lamin A/C
LPS: lipopolysaccharide

LRP10: LDL receptor related protein 10

LRRK1: leucine rich repeat kinase 1

LRRK2: Leucine-Rich Repeat Kinase 2

LS: Low Sample

LSD: lysosomal storage disorders

LYST: Lysosomal Trafficking Regulator

MAF: Minor allele frequency

MAOi: monoamine oxidase inhibitors

MAP4: Microtubule Associated Protein 4

MAPT: microtubule-associated protein tau

MARK2: Microtubule Affinity Regulating Kinase 2

MCI: mild cognitive impairment

MCP1: monocyte chemoattractant protein 1

MDS-UPDRS: Movement Disorder Society-Unified Parkinson’s Disease Rating Scale

ME2: Malic Enzyme 2

MF: Molecular Function

MIB1: Mindbomb E3 Ubiquitin Protein Ligase 1

MCCC1: Methylcrotonoyl-CoA Carboxylase 1

MNDA: Myeloid Cell Nuclear Differentiation Antigen

MOCA: MOntreal Cognitive Assessment

MPG: N-Methylpurine DNA Glycosylase

MRI: magnetic resonance imaging

MRPL4(12,23,49, 53): Mitochondrial Ribosomal Protein L4(12,23,49, 53)

mRNA: micro RNA

MSA: multiple system atrophy

MT-ATP6: Mitochondrially Encoded ATP Synthase Membrane Subunit 6

MT-ND(1, 2, 4): Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit (1, 2, 4)

mTORC1: mechanistic target of rapamycin complex 1

MRPS23: Mitochondrial Ribosomal Protein S23

MyND: Myeloid cells in Neurodegenerative Disease

NAGLU: N-Acetyl-Alpha-Glucosaminidase

NCBI: National Center for Biotechnology Information

NCSTN: Nicastrin

NDUFA5(S6): NADH:Ubiquinone Oxidoreductase Subunit A5 (S6)

NFATC(1, 3): Nuclear Factor of Activated T-cells, cytoplasmic (1, 3)

NIH: National Institutes of Health

NK: natural killer

NOTCH1: Notch receptor 1

NYU: New York University

NUDT13: Nudix Hydrolase 13

OMIM: Online Mendelian Inheritance in Man

OUTRIDER: OUTlier in RNA-seq fInDER

PBMC: peripheral blood mononuclear cells

PCA: posterior cerebral atrophy

PCCB: NADH:Ubiquinone Oxidoreductase Subunit S6

PD: Parkinson’s disease

PDCD6IP: Programmed Cell Death 6 Interacting Protein

PDHX: Propionyl-CoA Carboxylase Subunit Beta

PIGD: Postural Instability and Gait Difficulty

PINK1: PTEN induced putative kinase 1

PIK3R5: Phosphoinositide 3-kinase regulatory subunit 5

PLA2G6: Phospholipase A2 Group VI

PLBD1: Phospholipase B Domain Containing 1

PLBD2: Phospholipase B Domain Containing 2

POLG: DNA Polymerase Gamma

POLR2D: RNA polymerase II subunit D

PPARD: peroxisome proliferator activated receptor delta

PRKN: parkin

PSP: progressive supranuclear palsy

QC: quality control

RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted

RAB (5, 11B, 33B, 39A): Rab GTPase family (5, 11B, 33B, 39A)

RAP1GAP2: RAP1 GTPase activating protein 2

RARS2: Arginyl-TRNA Synthetase 2, Mitochondrial

RBD: sleep behavior disorder

REM: rapid eye movement

RIN: RNA integrity number

RNA-seq: RNA-sequencing

RPTOR: regulatory associated protein of MTOR complex 1

RSRP1: Arginine and Serine Rich Protein 1

RUNX3: Runt-related transcription factor 3

SCAMP4: Secretory Carrier Membrane Protein 4

SCO2: Pyruvate Dehydrogenase Complex Component X

SDC3: Syndecan 3

SDHC: Succinate Dehydrogenase Complex Subunit C

SERCA2: sarco/endoplasmic reticulum Ca2+-ATPase

SKIL: SKI Like Proto-Oncogene

SLC25A24: Solute Carrier Family 25 Member 24

SMPD1: acid-sphingomyelinase

SN: substantia nigra

SNCA: alpha-synuclein

SNP: single nucleotide polymorphism

SNRPC: Small Nuclear Ribonucleoprotein Polypeptide C

SORD: Sorbitol Dehydrogenase

SORT1: sortilin

SPECT: single photon emission computerized tomography

SPI1: Spi-1 Proto-Oncogene

SPPL2A: Signal Peptide Peptidase Like 2A

SRGAP1: SLIT-ROBO Rho GTPase activating protein 1
SVA: surrogate variable analysis
SYNJ1: Synaptojanin 1
SYNRG: synergin gamma
TD: tremor dominant
TIMM21(29): Translocase of Inner Mitochondrial Membrane 21(29)
TMEM230: Transmembrane Protein 230
TMLHE: Trimethyllysine Hydroxylase, Epsilon
TMM: Trimmed Mean of M-values
TNF: tumor necrosis factor
TOP1MT: DNA Topoisomerase I Mitochondrial
TPM: Transcripts Per Million
TRMU: TRNA 5-Methylaminomethyl-2-Thiouridylate Methyltransferase
TSC2: Tuberous Sclerosis Complex 2
TSG101: Tumor Susceptibility gene 101
UBA52: Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1
UBE2L3: Ubiquitin Conjugating Enzyme E2 L3
UCHL1: Ubiquitin C-Terminal Hydrolase L1
UCSC: University of California, Santa Cruz
UPR: unfolded protein response
UPSIT: University of Pennsylvania Smell Identification Test
UQCRC2: Ubiquinol-Cytochrome C Reductase Core Protein 2
UQCRHL: Ubiquinol-Cytochrome C Reductase Hinge Protein Like
UTY: Ubiquitously Transcribed Tetratricopeptide Repeat Containing, Y-Linked
VAMP7: Vesicle Associated Membrane Protein 7
VCF: Variant Call Format
VDR: vitamin D receptor
VPS (13C, 35, 41): vacuolar protein sorting-associated protein (13C,35, 41)
XIST: X-inactive specific transcript

WASF2: Wiskott-Aldrich syndrome protein family member 2

WES: Whole Exome Sequencing

WGS: Whole Genome Sequencing

ZNF180: Zinc Finger Protein 180

Iconography

Figures

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Table 1. Clinical characterization of study cohort.

Supplementary Table 1. Targeted pathway enrichment in PD/GBA+ vs CTRL/GBA+.

2. Research Integrity declaration

Results reported in this work comply with the four fundamental principles of research integrity of The European Code of Conduct for Research Integrity (ALLEA, Berlin, 2018):

- Reliability in ensuring the quality of research, reflected in the design, the methodology, the analysis and the use of resources;
- Honesty in developing, undertaking, reviewing, reporting and communicating research in a transparent, fair, full and unbiased way;
- Respect for colleagues, research participants, society, ecosystems, cultural heritage and the environment;
- Accountability for the research from idea to publication, for its management and organization, for training, supervision and mentoring, and for its wider impacts.

3 Introduction

3.1 Parkinson’s disease

Parkinson’s disease (PD) is the second most common neurodegenerative disease worldwide [1]. First described by Dr. James Parkinson in 1817 in his “Essay on the Shaking Palsy”, the understanding of this disorder has grown over the course of the years [1]. We now know that PD is caused by the progressive degeneration of the dopaminergic neurons in the substantia nigra in the midbrain [1]. Despite much being discovered about its pathologic hallmarks, molecular mechanisms and genetic predisposition, the pathogenesis of this disorder has not been yet fully elucidated, preventing the identification of curative treatments [1]. However, a better understanding of genetics, deregulated pathways and interaction with environmental factors and inflammation, are crucial for the clarification of the pathogenic mechanisms associated with this disease.

3.1.1 Parkinson’s disease: clinical features

PD is a clinical syndrome characterized by four cardinal features, consisting of resting tremor, cogwheel rigidity, bradykinesia, and postural instability [1]. Usually, the average age of disease onset is around 60 years. Subjects with onset before the age of 40 years, or according to some other literature before the age of 50 years, are referred as early or young-onset PD (EOPD), while cases with onset before the age of 20 years are defined as juvenile-onset PD (JOPD) [1]. PD is a degenerative disorder which gradually progresses over

time. Thanks to the symptomatic treatments available nowadays, this disorder has a less severe impact on life expectancy in the majority of the cases compared to the past. Although we used to consider PD as a pure movement disorder, we now know that a number of so called “non-motor symptoms” represent an important component of this disease [2]. These consist mainly in: dysautonomia (manifesting as constipation, urinary disfunction such as urinary urgency and increase frequency, orthostatic hypotension); psychiatric manifestations such as anxiety and depression as well as possible hallucinations or illusions especially in the most advanced phases of the disease; rapid eye movement (REM) sleep behavior disorder (RBD), due to the loss of muscle atonia during the REM phase of the sleep and manifesting as acting out of the dreams and/or talking, screaming during this phase of the sleep; cognitive impairment up to dementia in certain cases; and hyposmia [2]. Some of these symptoms have been recognized now to manifest up to many years before the onset of the motor symptoms, therefore referred as premotor manifestations [2]. However, the specificity of these symptoms is low. Thus, they are still not sufficient to guide an early diagnosis of PD, which would be instead crucial for early interventions once disease modifying treatments will be available. Indeed, we know that the motor manifestations of PD became evident when more than the 70-80% of the dopaminergic neurons in the substantia nigra have already died [1].

The non-motor symptoms seem instead to be caused by the degeneration of different systems (such as the serotoninergic, cholinergic and noradrenergic ones) as well as by the deposition of

alpha-synuclein, the hallmark protein of PD, in different regions of the brain stem and/or cortical regions (such as in subjects with dementia or hallucinations) as well as in the periphery, such as in the enteric system or autonomic terminations [3].

Motor and non-motor symptoms can be present in different combinations across patients. The study of large cohorts of subjects with PD over the past decades allowed the identification of different clinical phenotypes, suggesting that PD is probably a collection of different syndromes more than being a single entity [4]. Indeed, some patients may present with a tremor-dominant PD, with usually a more benign and slower progression of the symptoms and less involvement of the cognitive functions [5]. In other cases, tremor is never present throughout the whole course of the disease, while rigidity and postural instability are prominent, referred as Postural Instability and Gait Difficulty (PIGD) phenotypes [5]. In some patients, non-motor symptoms, such as RBD and dysautonomia, are prominent. Those cases are usually associated with a more severe progression of the disease and outcomes [6]. Finally, disease progression can be very slow for some, while being more tumultuous and aggressive for others [1].

The cause of these underlying different phenotypes is not completely elucidated yet. Some correlations have been identified with different genetic forms of PD. For examples, cases of duplication or triplication of the alpha-synuclein (*SNCA*) gene present with an earlier age of onset and a more severe and aggressive phenotypes, with rapid progression and cognitive impairment [7]. Other mutations, such as the ones in the Leucine-Rich Repeat Kinase 2 (*LRRK2*) are

characterized by a more classical phenotypes, with late onset, moderate rate of progression and mild non-motor manifestations [7]. On the other side, the exposure to environmental factors, such as toxic factors (chemicals, pesticides, toxins, among the others), smoke, coffee, head trauma, physical activity, as well as inflammation can affect the onset and/or rate of progression of the disease [1].

3.1.2 Parkinson's disease: diagnosis and treatment

The diagnosis of PD is based on clinical and neuropathological findings, leveraging the combination of the cardinal motor features (resting tremor, cogwheel rigidity, bradykinesia, and postural instability), as well as non-motor symptoms and the absence of atypical features that may lead to the diagnosis of atypical parkinsonism or other conditions [1]. Most of the literature in PD is still based on the UK Brain Bank Criteria [8]. The motor and non-motor symptoms can be evaluated and quantified also with the use of rating scales. The most commonly used in the clinical settings, but especially for clinical trials and research studies, is the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS), which provides a wide evaluation of motor and non-motor aspects of the disease through questionnaires and physical examination [9]. The Hoehn and Yahr (H&Y) scale is instead a 5 point scales which allows grading patients based on a score that reflects the degrees of involvement of the disease as well as disability [10]. The more widely used scale for the assessment of cognitive complains in PD is the MOntreal Cognitive Assessment

(MOCA scale) [11]. The University of Pennsylvania Smell Identification Test (UPSIT) is a validated test for the assessment of smell function [12]. Additional scales can be used instead to assess dysautonomia, psychiatric involvement, as well as RBD [13].
Imaging studies can be used to support the diagnosis of PD. Brain computed tomography (CT-scan) or magnetic resonance imaging (MRI) studies do not show specific abnormalities in PD. Therefore, these assessments are mostly used to rule out the presence of other abnormalities (such as deposition of minerals, like iron, calcium, manganese or copper, in the basal ganglia, as well as vascular, inflammatory or cancer lesions) that would suggest an alternative diagnosis [14]. Additional imaging techniques utilized in the context of PD aim to functionally characterize the dopaminergic disfunction. This is the case of the dopamine transporter (DAT) single photon emission computerized tomography (SPECT) imaging (better known as DatScan), which assesses the status of innervation of presynaptic dopaminergic terminals from the substantia nigra (SN) to the basal ganglia, thus reflecting the degeneration of the dopaminergic neurons. Although this test can be informative, its limited sensitivity and specificity make it suitable only for differentiating PD form other conditions (such as essential tremor or drug induced parkinsonism) while this is not required to establish a diagnosis of PD [15]. Other, more recent approaches, such as the fluorodopa PET scan, seem instead more promising and accurate and may soon become part of the clinical assessments [16]. Recently, a number of other tests have been proposed as supportive tools for diagnostic purposes in PD, such as, for example, the transcranial ultrasounds (showing

hyperechogenicity of the degenerating SN), as well as the seeding aggregation assays of alpha-synuclein on skin punch biopsies or other tissues [17].

The response to dopaminergic medications is an additional important aspect in the diagnosis of PD. The introduction of levodopa, a precursor of dopamine, which is the missing neurotransmitter in PD due to the degeneration of the dopaminergic neurons in the SN, revolutionized the outcome of this disorders [1]. This is administered orally, together with carbidopa to block the dopamine decarboxylase and limit peripheral levodopa metabolism and thus increasing the amount delivered to the brain. Levodopa is metabolized in the dopaminergic neurons in the SN. A number of different formulations of levodopa are available nowadays, as well as dopamine agonists. The latest are drugs that directly target the post-synaptic dopaminergic receptors in the basal ganglia, thus mimicking the effect of dopamine [18]. The response to dopaminergic medications, especially levodopa, is usually quick (within 30-40 minutes after its administration). Improvement of the motor symptoms in response to dopaminergic medications is a supportive element for the diagnosis of PD. A lack of response is usually suggestive of an alternative diagnosis [18].

Other medications used for the treatment of PD target the cholinergic system (such as the trihexyphenidyl), as well as the NMDA receptors (amantadine), or can increase the duration of a dopaminergic effect (such as the monoamine oxidase inhibitors (MAOi), and the catechol-O-methyltransferase (COMT) inhibitors) [18]. Surgical approaches, such as deep brain stimulation (DBS) and more recently also the

high intensity focused ultrasounds (HIFU), are also important therapeutic options in the scenario of PD [18].
Despite a growing knowledge of the clinical presentation of PD and the response to different therapeutic approaches, nowadays a definitive diagnosis of this disease can still only be achieved through neuropathology.

3.1.3 Parkinson's disease: neuropathology

The classical neuropathological hallmarks of PD are the loss of dopaminergic neurons and the accumulation of Lewy bodies (alpha-synuclein reactive aggregates) in specific brain regions [8]. In particular, classical PD is characterized by the loss of the ventro-lateral neurons of the substantia nigra pars compacta, while the loss of medial neurons is more common in cases associated with dementia [19]. Other regions that may present a neuropathological involvement in the brain of patients with PD encompass the medullary tegmentum and the dorsal motor nucleus of the vagus, the locus coeruleus, hypothalamus, amygdala and the basal nucleus of Meynert; moderate degree of involvement is found in the cortex (cingulate and temporal cortex); hippocampus, oculomotor complex, and pontine tegmentum (also raphe and pedunculopontine nuclei); a milder involvement can be found in the caudate nuclei, putamen, and superior frontal girus [19]. As described for other neurodegenerative conditions, also in PD the degeneration is progressive and can reflect the clinical stages of the disease, according to the rating system proposed by Braak et al [20]. According to this classification, neuropathological findings are first identified in the caudal region of

the brain stem (such as the dorsal motor nucleus of the vagus and the olfactory bulb) and then gradually progress rostrally, involving the locus coeruleus, substantia nigra and eventually the amygdala and the cortex [20]. In the areas where the neuronal degeneration is present, proliferation of microglia and astrocytes is reported as well most likely promoting scavenger activities of the debris from dying cells [19].

Many variants of the classical neuropathological findings detailed above have been reported in patients with PD. For example, in a number of genetic forms of PD, such as the ones associated with *LRRK2* and *Parkin* mutations, Lewy bodies and prominent alpha-synuclein deposition can be absent despite a classical pattern of neuronal degenerations [21].

3.2 Pathogenic mechanisms of Parkinson's disease

For the understanding of the molecular changes underlying PD, genetics studies as well as cellular and animal models have been crucial. From a macroscopic point of view, it is established that the loss of dopaminergic innervation from the SN affects the direct and indirect pathways between the basal ganglia (globus pallidus, corpus striatum, thalamus and subthalamus) and the motor cortex. As described above, other systems in the brain (i.e. the serotoninergic, cholinergic and noradrenergic ones) and in the periphery (according, for example, to the so called "gut-brain axis" where alpha-synuclein would initially deposit in the intestinal mucosa), are involved in PD [3]. However, we still have only a limited knowledge regarding the causative molecular dysfunction that eventually leads to the disease.

3.2.1 The genetic architecture of PD

About 5-10% of all cases of PD are considered familial and at least 5% show a pattern of inheritance consistent with a Mendelian trait [22].

Genetics was first involved in the scenario of PD in 1997, when a genetic mutation in the alpha-synuclein gene (*SNCA*) was identified in a large Italian kindred (named Contursi family because based in the town of Contursi in the South of Italy) with a strong family history of PD [23]. Since then, a growing number of genetic mutations have been associated with PD [24]. We now know that the genetic architecture of PD is complex and for sure not fully defined yet.

In particular, we recognize a number of monogenic forms (both autosomal dominant, autosomal recessive, and more rarely X-linked) as well as genetic risk factors and genetic variants that can increase the risk of developing PD [22]. The current guidelines for the nomenclature of the genetic forms of PD suggest to refer to those with the prefix "PARK-" followed by the name of the mutated gene, replacing the previous nomenclature system (consisting of the PARK- prefix followed by a crescent number based on the time of discovery of the different mutations) in order to reduce loci overlaps, missingness or errors of assignment [25].

Among the <u>autosomal dominant</u> (AD) genetic mutations associated with PD, the most frequent one is represented by the mutation of the *LRRK2* gene, which explains up to 10% of all the familial cases of PD as well as 1% of the sporadic cases [26,27]. Mutations of the *LRRK2* gene were initially identified in 2004 through the linkage

analysis of few large kindreds with a family history of PD [28,29]. As discussed above, mutations of this gene are associated with a phenotype that highly resembles the ones of idiopathic PD, although in some cases the classical Lewy bodies pathology is missing [30,31]. The *LRRK2* gene encodes for a kinase expressed and active in different tissues [32]. Because of its kinase activity, LRRK2 is active on a large number of downstream targets and thus can affect different molecular pathways, such as the vesicular and endolysosomal pathway, the autophagic and mitochondrial pathways, the immune response, and cytoskeleton metabolism [32]. Genetic mutations of the *LRRK2* gene associated with PD have been described across the entire gene. However, the far most common one, the Gly2019Ser (G2019S) mutation, is located in the kinase domain of *LRRK2* [32]. In patients with PD, mutations of LRRK2 are associated with an increase kinase activity, suggesting the possibility of a toxic gain of function effect [32]. This observation has very important therapeutic implications. Indeed, the majority of clinical trials targeting *LRRK2* mutations aim to silence the gene or its kinase activity in target tissues [33].

Other relevant AD forms of PD are the ones associated with mutations of the *SNCA* gene (PARK-SNCA, previously PARK1, caused by point mutations, duplications or triplications of the gene), and the vacuolar protein sorting-associated protein 35 (PARK-VPS35, previously PARK17) [24]. Mutations of these two genes are rare. Mutations of SNCA are usually associated with an earlier age of onset, especially in cases of duplications or triplication of the gene [24]. Mutations of VPS35 are instead commonly associated with late

onset, more classical forms of PD [24]. Other autosomal dominant forms of PD, which are even more rare than the ones associated with *SNCA* and *VPS35* mutations, are associated with mutations of the Phospholipase A2 Group VI (PLA2G6, previously PARK14) and F-Box Protein 7 (FBXO7, previously PARK15) [34–36]. Other rare and, in some cases, not definitely confirmed AD mutations are the following: *UCHL1* (PARK5), *GIGYF2* (PARK11), *DNAJC13* (PARK21), *CHCHD2* (PARK22), *POLG*, *LRP10*, *TMEM230*, *EIF4G1* [37,38].

Autosomal recessive (AR) forms of PD usually present with an earlier age of onset, either EOPD but also JOPD [24]. The most frequent AR form of PD is associated with the mutation of the *Parkin* gene (previously PARK2), which however is still rare, being identified in only 2.6% of EOPD [39,40]. These patients usually present with an onset of the disease before the age of 40 years, excellent response to low doses of levodopa, but they tend to manifest early onset of drug-related motor fluctuations and dyskinesia (involuntary movements caused by an imbalance in the activation of the basal ganglia circuitry due to a combination of the progression of the degenerative processes related to the disease and the stimulation induced by the dopaminergic medications) [41]. These patients usually present few non-motor symptoms and slow progression [41]. Late-onset cases associated with the mutation of this gene have been reported as well [42]. Similar phenotypes can be presented by subjects with other AR mutations associate with PD, such as the ones in the PTEN induced putative kinase 1 (*PINK1*, previously PARK6) and the Protein/Nucleic Acid Deglycase *DJ-1* (previously

PARK7), which are even more rare than *Parkin* mutations [41,43,44]. Other genes identified as AR causes of PD are instead usually associated with more atypical phenotypes. This is the case of *ATP13A2* (previously PARK9, which is the causative mutation of the Kufor-Rakeb disease, a complex syndrome characterized by early onset parkinsonism, spasticity, dysarthria, myoclonus, supranuclear gaze palsy, cognitive impairment and hallucinations), DNAJC6 (DnaJ Heat Shock Protein Family (Hsp40) Member C6, previously PARK19, described in few families with early onset and severe PD), SYNJ1 (Synaptojanin 1, previously PARK20, associated with cognitive impairment and more severe deterioration as well as seizure), VPS13C (vacuolar protein sorting-associated protein 13C, previously PARK23, associated with rapidly progressive and disabling PD) [45].
Other than the monogenic forms of PD, two important genetic risk factors of PD have been identified over the last two decades. These are mutations of the *LRRK2* and *GBA* genes [46]. Mutations of these genes are known to increase the risk of developing PD in carriers. They mostly have a reduced penetrance, except for certain mutations of *LRRK2* which are fully penetrant and considered AD [46]. Compared to the other identified monogenic forms of PD, *LRRK2* and *GBA* mutations are significantly more frequent across patients with PD. This makes these two genes very suitable targets for possible therapeutic approaches and gene therapy, since they will allow treating a greater number of PD patients. Moreover, an impaired activity of these two genes has been identified also in subjects with no genetic mutations of these genes, supporting their central role in the pathogenesis of PD as well as the possibility of

benefit a larger number of patients by targeting them [33]. *GBA* mutations and its relationship with PD are detailed below.

Other more rare genetic risk factors for the disease are the mutations in the microtubule-associated protein tau (*MAPT*) as well as certain mutations of *SNCA* [46].

In recent years, the study of sporadic cases of PD through the quickly evolving technologies of the whole exome (or genome) sequencing (WES and WGS respectively) and growingly larger Genome Wide Association Studies (GWAS) have elucidated a number of other loci associated with this condition [47–51]. Considering all the different GWAS performed with PD patients in the last decade, a total of 90 PD-associated loci have been identified [50]. Among those, some are close to regions encoding genes already associated with monogenic forms of PD or to risk factors for this disease. This is the case of *SNCA*, *MAPT*, *GCH1*, and *LRRK2* [47–51]. Additional loci have been identified close to genes that may have a relevant role in the pathogenesis of PD (such as *HLA-DQB1* because of its involvement in the inflammatory/immune response) [47–51]. However, variants identified through GWAS studies have two important implications that need to be considered. First, those are common variants, thus despite being more frequent in the PD population, they have reduced pathogenicity compared to the monogenic forms of PD. Therefore, their role in the architecture of PD pathogenesis and their usefulness as biomarkers for early diagnosis or as therapeutic targets has to be further explored. Second, GWAS detect regions frequently lying in non-coding regions of the genome. The study of expression quantitative trait loci (eQTL),

meaning the role of common variants in modulating the expression of coding genes that can be located close to the studied SNP (-cis eQTL) or more than 5Mb apart from it (trans-eQTLs), have showed that GWAS variants cannot necessarily be functionally relevant only for the gene located in their proximity, thus a more comprehensive assessment of the meaning of these variants is needed [52].

3.2.1 Molecular mechanisms of PD

In order to better understand the specific molecular alterations found in GBA-driven PD, it is important to mention the molecular pathways that have been deemed responsible for the pathogenesis of PD so far. In particular, evidences from the literature, based on genetics and preclinical studies, support the involvement of three main pathways: the endo-lysosomal (EL) and autophagic pathway, the mitochondrial pathways, and inflammation [53].

As further described below, the discovery of mutations of the *GBA* gene in PD has pointed out the role of ER stress and vesicle trafficking abnormalities in PD. Other than *GBA*, other genetic variants causative for PD and parkinsonism belong to these pathways, such as *LRRK2*, *ATP13A2*, *VPS35*, *VPS13C*, and *SYNJ1*. The impairment of the EL pathway can also be responsible for the failure of autophagic pathways described in PD and both these mechanisms are linked to the accumulation of toxic substrates and to the mitochondrial disfunction, as detailed below, which have been described in this disorder [54].

Mitochondrial impairment in the context of PD was initially suggested by the findings of a deficit of the complex 1 in PD mouse models

[55,56]. In the following years, genetic mutations responsible for early onset PD have also been identified in the mitochondrial genes *PRKN*, *PINK1*, and PARK7 (or *DJ-1*), further suggesting the involvement of these organelles and their function in PD. Mechanisms linking mitochondrial dysfunction and PD pathogenesis can be related to impaired mitochondrial homeostasis and mitophagy, impaired oxidative stress – that can be toxic for host cells and particularly for dopaminergic neurons -, impaired calcium homeostasis, and mutation of the mitochondrial DNA [57,58].

On the other side, genetics seems also to support a role of lipid metabolism in PD pathogenesis. In fact, an increased frequency of common variants associated with the lysosomal storage disorders (LSD) has been reported in PD patients compared to controls [59]. Moreover, a higher incidence of PD has been reported also in patients with different forms of LSD, such as Gaucher disease (associated with biallelic mutations of *GBA*), Niemann-Pick disease type A and B due to mutations of the acid-sphingomyelinase (*SMPD1*) gene, as well as Fabry disease, due to mutations of the alpha-galactosidase A (*GLA*) gene [60,61]. Interestingly, reduced levels of cathepsin D, another lysosomal protein, have been reported also in autoptic brains of PD and LBD patients [62]. These works supported a role for testing lysosome enzyme activities in PD patients as possible biomarkers for the disease [63].

A role of inflammation and of the immune system in the pathogenesis of PD has been supported nowadays by a number of different works, as more and more interesting findings related to these mechanisms continue to emerge (as extensive summarized in [64]). The

multifaceted involvement of inflammation in PD will be detailed below (see paragraph 3.4 “Inflammation, immune system and Parkinson’s disease”). It is important to mention that both the innate and the adaptive immune system have been described in PD-related mechanisms and that both the central nervous system and the periphery show characteristic impairments in patients with PD [64]. Inflammation has been reported in many neurodegenerative disorders, such as Alzheimer disease and Amyotrophic Lateral Sclerosis, where it is still not clear whether the activation of this response represents a causative or a reactive mechanism in the pathogenesis of these conditions [65]. Intriguingly, the involvement of inflammation and of the immune system in PD is supported by genetics, biochemical, histological and epidemiological aspects, further suggesting the importance of looking at those mechanisms in order to better understand PD and its pathogenesis [64].

While considering the pathogenic mechanisms underlying PD, it is also important to mention the possible role of alpha-synuclein. As reported above, this was the first genetic mutation associated with PD and soon after, accumulation of this protein in the so called Lewy Bodies was reported [68]. Since then alpha-synuclein aggregated have been considered the hallmark of PD, so much so that PD and few other conditions (such as Multiple System Atrophy - MSA) are referred as alpha-synucleinopathies. Interestingly, it is not yet fully understood whether alpha-synuclein precipitation and aggregation in the dopaminergic neurons and the other cells involved in the disease, represent a causative mechanism or a secondary event. Interestingly, though, alpha-synuclein is also involved in all the

previously mentioned PD-related mechanisms, such as the mitochondrial and the EL pathway disfunction, as well as neuroinflammation. Indeed, in the context of a mitochondrial dysfunction, alpha-synuclein has been reported to interfere with the stability of mitochondrial membrane, homeostasis, and dynamics, as well as the mitochondrial mediated energetic metabolisms, mostly through its interaction with complex I [69,72,73]. On the other side, alpha synuclein certainly plays a role in vesicle trafficking, while lysosomal dysfunctions, such as in the context of *GBA* mutations and reduced GCase activity, can cause increased alpha-synuclein phosphorylation, which favors the aggregation and precipitation of the protein [75,77,79]. Finally, alpha-synuclein can activate an inflammatory response, both mediated by the innate immune system, though microglial cells, as well as the innate immune system by behaving as an antigen for the activation of T-cell mediated response [81,83,195].

3.3 GBA and Parkinson's disease

3.3.1 *GBA* and the glucocerebrosidase

GBA is the gene that codes for the beta-glucocerebrosidase (GCase) [66]. Located on the long arm of the chromosome 1, at position 1q21, the gene consists of a total of 12 exons. This gene is located in a complex region presenting different genes and pseudogenes. *GBA* itself owns a pseudogene, called *GBAP1*, which shares about 96% of homology with *GBA* [66,67]. Because of that, the sequencing of the *GBA* gene using chip, arrays, or next generation sequencing

technique and customized panels can be difficult and misleading, thus requiring dedicated sequencing and approaches [70].
The GCase is a lysosomal enzyme responsible for the degradation of glucosylceramide in glucose and ceramide [71]. The activation of this enzyme is possible at specific pH levels, as the ones found in the lysosome. Before being release to the lysosome, the GCase undergoes multiple post-translational modifications, such as glycosylation, in the trans-Golgi network [74]. *GBA* is expressed ubiquitously across tissues (according to Genotype-Tissue Expression (GTEx) Project – GTex, phs000424.vN.pN).

3.3.2 *GBA* and Gaucher disease

Mutations of the *GBA* gene have been classically associated with Gaucher's disease (GD), which belongs to the family of the lysosomal storage disorders (LSD) [76]. GD is an autosomal recessive condition that can present with both systemic as well as neurological symptoms. Three different forms of Gaucher disease have been described, called GD type 1, GD type 2 and GD type 3 [78]. While GD type 1 is mostly a metabolic disorder (presenting with hepatosplenomegaly, osteopenia, thrombocytopenia and anemia, as well as possible monoclonal gammopathy, failure to growth and increased risk of tumor), GD type 2 and 3 are instead the two neuronopathic forms (acute infantile neuronopathic (GD type 2) and chronic neuronopathic (GD type 3)) and can encompass seizure, early developmental delay, as well as spasticity, in addition to the systemic manifestations.

At present, more than 300 *GBA* mutations have been found in patients with GD [80]. Mutations of *GBA* have been classified in mild or severe mutations according to their association with GD type 1 or 2 and 3, respectively. For example, the N370S mutation (or N409S according to the new nomenclature) is associated only with the systemic form (GD type 1), both in homozygous or compound heterozygous states [76,82]. Other mutations, such as the L444P (or L483P according to the new nomenclature) is instead associated with the most severe forms of GD, namely GD type 2 and 3 [76,82].
GCase dysfunctions, caused by the mutations of *GBA*, are responsible for a deficit of the degradation of the glucosylceramide, lysosome failure and accumulation of aberrant lysosomes in the macrophages, which are the resident component of the innate immune system in the reticuloendothelial compartment of the target tissues (such as liver, spleen, bones, and brain among the others) [78]. These aberrant macrophages are called Gaucher's cells and represent the pathogenic hallmark of this disease. GD is also characterized by a chronic state of inflammation and increased release of cytokines. More recently, complement activation has been postulated to play a role in the pathogenic mechanisms of GD, particularly involving the C5a and C3a components and their receptors, as well as a number of downstream pathways, such as the mitochondrial and the lysosomal pathways [84].
At present, a number of treatments are available for the systemic form of GD (type 1), such as the substrate reduction therapy (SRT) or the enzyme replacement therapy (ERT). These are very effective in treating the systemic manifestations of GD type 1 but they are not

beneficial on the neurological symptoms of GD type 2 and 3, since they are not able to cross the blood brain barrier [85].

3.3.3 *GBA* and Parkinson's disease: an overview

The first report suggesting a link between *GBA* mutations and PD was in 2004 [86]. Prior to that, an increased incidence of PD in patients affected with GD type 1 as well as in their family members who were carriers of mutations of *GBA* was noticed in some works from the literature [87–92]. Larger population studies in the following years confirmed these initial observations [93–96]. The incidence of *GBA* mutations in PD patients was estimated across different populations around the word describing variable incidences in different ancestries [93–96]. Similarly to mutations of the *LRRK2* gene, the penetrance of *GBA* mutations is reduced. It has been estimated that the monoallelic and biallelic mutations of *GBA* can increase the risk of developing PD by 5 to 10 times compared to the rest of the population, with an incidence ranging from 2 to 30% across populations [97]. Interesting, the majority of the first multicenter and large population studies targeted only those mutations of *GBA* that were found to be more frequently associated with PD, such as the N370S and L444P [93–96]. More recently, the discovery of a growing number of mutations of the *GBA* gene associated with PD lead to a series of new studies at different latitudes were, instead, the entire gene was screened in patients with PD and controls [98–101]. This allowed the identification of an even higher frequency of *GBA* mutations among patients with PD, in both familial and sporadic cases, compared to previous data. For

example, in a large study in an Italian cohort of PD patients the incidence of *GBA* mutations was estimated to be 14.3%, in a study from the Netherlands it was 15%, in an Irish cohort 8.3%, and in patients of Ashkenazy Jewish (AJ) ancestry from Israel and New York of 18% [98–101]. Therefore, *GBA* mutations are now considered, together with mutations of the *LRRK2* gene, the major genetic risk factor for PD.

At present, more than 60 mutations of *GBA* have been identified in PD [82]. As already reported above, some of these mutations are more common in PD patients, such as the N370S and L444P, accounting for up to 70-80% of the reported mutations in this disease [102]. Some mutations are significantly more frequent across specific populations and ancestries, such as the N370S, E326K, R496H and 84GG among AJ, while the L444P is found in non-AJ European populations, the K198E in Columbian subjects, and the R120W in Asian populations [102,103].

Mild and severe mutations, as defined above in the context of GD, can be associated with different PD phenotypes in terms of penetrance and severity of clinical presentation, where severe mutations seems to be associated with more aggressive phenotypes and higher penetrance of the mutation [104].

As expected, different mutations have different functional impacts on the function and residual activity of the GCase, possibly explaining the variable phenotypical manifestations associated with different mutations [105–109].

GBA mutations described in PD patients mostly consist in point mutations, but complex rearrangement (also between *GBA* and its

pseudogene), insertion and deletion have been reported as well [82]. Interestingly, there have been ongoing discussions among experts in the fields regarding the pathogenic role in PD of two of these mutations of *GBA*, the p.E326K (or E365K according the new nomenclature) and T408M (or T396M according to the previous nomenclature). Indeed, these mutations are not associated with GD and the initial studies in PD cohorts were inconsistently proving a significant higher incidence of these mutations in PD patients [101,110–112]. However, later analysis eventually supported their association with PD [113].

3.3.4 Clinical phenotype of GBA-PD and other alpha-synucleinopathies

A number of studies in the literature tried to assess a possible phenotype-genotype correlation between *GBA* mutations and PD [104]. Other than the association of a more aggressive phenotypes in carriers of severe mutations of *GBA*, as reported above, a number of other characteristic clinical traits related to this genetic mutation were defined. In particular, subjects with PD and *GBA* mutations usually manifest an earlier age of onset (between 3 to 11 years earlier compared to idiopathic PD patients) [95,102,103,114]. Motor and non-motor symptoms can be more severe too. Particularly, cognitive impairment and hallucination as well as RBD are more frequent in GBA-related PD, with a 3-fold increased risk of dementia in respect to idiopathic PD [95,103,115]. Also, dysautonomia, anxiety and depression are found more commonly in symptomatic and preclinical phases of GBA-related PD [95,103,116–118]. The motor symptoms

are usually characterized by a predominance of the rigid-akinetic presentation, with more frequent therapy-related motor complications, such as motor fluctuations and dyskinesia [103,108,119]. Interestingly, even if *GBA* mutations are associated with a more severe PD phenotype, *GBA* carriers seem to show milder impairment at DatScan imaging compared to iPD in the early stages of the disease [120]. Unfortunately, despite the identification of these phenotypic traits it is still difficult to identify carriers of these mutations across PD patients only based on their clinical presentation.

Other than PD, *GBA* mutations have been reported to be more frequent also in subject with RBD (without PD), Lewy body dementia, and Multiple System Atrophy [121–133]. All these conditions are referred as “alpha-synucleinopathy”, being characterized by the deposition of this protein in target regions of the brain and in certain cases also systemically. This observation supports the idea of an important role of GCase disfunctions and *GBA* mutations in alpha-synuclein deposition and thus related pathology.

3.3.5 *GBA* and Parkinson’s disease: neuropathology

There are a number of studies in the literature reporting neuropathological findings from patients with GD and heterozygous carriers [21,86,97,131–139]. Despite a limited characterization of the neuronal loss in the brains of these patients, the present of Lewy bodies, and thus of alpha-synuclein accumulation, was consistent across cases, with a prominent cortical localization [21,86,97,131–139]. In some cases of GD type 1, Gaucher cells in the brain

parenchyma were identified as well [91,136,140]. In few cases, Alzheimer disease (AD) pathology, characterized by deposition of beta amyloid and Tau, was reported [133,134,136]. Interestingly though, despite these findings and despite the increased association between GBA-related PD and dementia, previous screening of *GBA* mutations in AD cohorts failed to identify an increased frequency of these mutations in those subjects [130,141].

3.3.6 *GBA* and Parkinson's disease: molecular mechanisms

Because of the high frequency of *GBA* mutations in PD patients and its possible role at a crossroad of different pathogenic mechanisms associated with PD, the *GBA* gene and the GCase function have been extensively studied in clinical and preclinical works which helped elucidating some of its disease-associated pathways. Nevertheless, a number of questions are still not yet answered.

The molecular mechanisms associated with *GBA* mutations in the pathogenesis of PD described so far could be summarized in the following categories:

1. *Reduced GCase activity and alpha synuclein accumulation*. Reduced levels of GCase activity has been reported in both peripheral blood (dried blood spot and monocytes), cerebrospinal fluid, and brain tissues, especially the substantia nigra, of subjects with mono- or biallelic mutations of *GBA* [62,142–147]. Interestingly, decreased GCase activity was also found in brains of subjects with PD with no detected mutations in the *GBA* gene, further stressing the central role of this gene and its enzyme in the pathogenesis of PD [145]. Only few studies have instead explored the levels of

expression of the *GBA* gene in the brain reporting contrasting results [145,146]. On the other side, different *GBA* mutations are associated with different levels of residual GCase activity, possibly explaining the phenotype-genotypes correlation observed in mild versus severe *GBA* variants [148].

In patients with PD and *GBA* mutations, together with reduced levels of GCase activity, increased levels of alpha-synuclein in cellular models from induced pluripotent stem cells, brain tissues, and animal models have been reported and those can be restored through the correction of the GCase activity [149–153]. On the contrary reduced levels of apha-synuclein were reported in the cerebrospinal fluid of patients with PD and *GBA* mutations [154,155].

Different hypothesis have been suggested to define the relationship between GBA mutations, reduced GCase activity and alpha-synuclein accumulation. It is also important to note that increased levels of alpha-synuclein seems to reduce GCase activity themselves, culminating in a vicious circle between the two proteins that can be deleterious for the hosting cells [156].

Most likely, alpha-synuclein accumulation is caused by an impairment of the lysosomal activity associated with GCase disfunctions as well as by a failure of different cellular degrading systems related the autophagy–lysosomal pathway (i.e. macroautophagy, chaperone mediated autophagy and microautophagy). On one side, the failure of these systems can disrupt alpha-synuclein metabolism and degradation causing its accumulation [157–159]. On the other side, these events can also cause an impairment of the lipid metabolism which can itself interfere

with alpha-synuclein homeostasis and increase its deposition in the cells [156,160]. Reduced GCase activity has also been reported to be related with an increased release of alpha-synuclein from the cells, possibly facilitating the spreading of the disease from cell to cell [161,162]. Moreover, according to a recent work, impaired GCase activity seems to determine the neuronal susceptibility to alpha-synuclein accumulation [163].

2. *Impairment of the endolysosomal pathway and lipid metabolism*. The lysosomal pathway seems to play a central role for different mechanisms involved in PD [164–166]. In *GBA* mutation carriers impairment of the endolysosomal and vesicular trafficking pathways has been reported [163,167]. Indeed, the lysosome represent a central hub for this system and its disfunction can be responsible for a deregulation of the entire pathway. Lysosomal functions and membrane trafficking can affect lipid metabolism. In fact, distinctive lipidomic profiles have been reported in GBA mutations carriers compared to controls, possibly connected to the GCase disfunction [168,169]. Despite that, the role of the accumulation of glucosylceramide, the substrate of GCase, in the pathogenesis of PD is still debated in the literature. On the other side, impaired vesicular trafficking can cause impaired release of exosomes in *GBA* carriers, as reported in animal models and in GD patients [167,170].

3. *Cells stress response*. Another important mechanism in GBA-related PD is associated with stress cell response in the cells. A number of works have showed in different animal models that mutations in the *GBA* gene would affect the conformation and proper folding of the GCase in the endoplasmic reticulum (ER) [171]. Once

transcribed, GCase is normally processed in the trans-Golgi network to reach maturation through a chain of post-translational modifications, and finally reach the lysosomes. According to this model, mutated GCase are trapped in the ER and this accumulation triggers a stress cell response (unfolded protein response, UPR) that can be detrimental for the host cells [172–176]. These mechanisms can be reverse by using chaperons molecules, such as Ambroxol, that are indeed already under evaluation in a number of clinical trials for the treatment of the GBA-related synucleinopathy [174].

6. *Immune response and GBA mutations*. Finally, GBA mutations have also been associated with aberrant monocyte/macrophage mediated inflammatory response [177]. In particular, in patients with PD and *GBA* mutations, a distinct pattern of inflammation and cytokines were described [177]. At the same time, microglia activation has been observed in the brain of transgenic animal models for the *GBA* gene [178,179]. This is not surprising, as we know that one of the major hallmark of GD is the presence of the so called Gaucher cells, which consist of aberrant macrophages engulfed with non-functional lysosome, as well as increased inflammation.

3.3.7 *GBA* and Parkinson's disease: genetic modifiers

As reported above, *GBA* mutations have a reduced penetrance in PD. For this reason, one of the main focuses in the research related to GBA-PD is the identification of possible modifiers that may affect the penetrance and pathogenicity of *GBA* mutations and that can be tackle for therapeutic interventions or leveraged as diagnostic

biomarkers. In the literature few works suggest the presence of possible modifiers of *GBA*.

By analyzing GWAS data from a large cohort of *GBA* carriers affected with PD, it was reported that Parkinson's disease genetic risk score, mostly mediated by hits in the proximity of the cathepsin B (CTSB) and in the SNCA loci, can affect the age of onset and the penetrance of *GBA* mutations in patients with PD [180]. At the same time, common variants in proximity of the *GBA* gene have been reported to have a modulatory effect on the expression levels of *GBA* itself across different tissues as well as on other genes that can interact with *GBA* and thus function as modifiers [181].

The role of GBAP1 as possible modifiers has been studied as well [182]. In particular, it has been showed that miR-22-3p can target both *GBA* and *GBAP1*, given the high homology between the two genes. In the proposed model, GBAP1 functions as a competing-endogenous RNA, whose levels can affect the silencing effect of miR-22-3p on GBA and GBAP1 and, as a consequence, the expression levels of *GBA* across tissues [182].

Other two genes that were described as possible modifiers of the pathogenicity of the mutations of the *GBA* gene are Metaxin 1 (*MTX1*) and Bridging Integrator 1 (*BIN1*) [183,184]. Indeed, the homozygous mutation c.184T>A (p.S63T) of *MTX1*, which is located in close proximity to the locus of *GBA* on chromosome 1, were found to be more frequent in *GBA* mutation carriers and it is enriched in early onset PD [184]. On the opposite, the presence of the minor allele rs13403026 of *BIN1* gene, also described as a risk factor for

Alzheimer disease, was associated with a later age of onset of PD in *GBA* mutations carriers [183,185].

Finally, a number of works explored the clinical and molecular consequence of the possible interaction between *LRRK2*, the other major genetic risk factor for PD, and *GBA* [186–188]. In particular, the interplay of these two mutations seem to affect alpha-synuclein spreading and *GBA* specific molecular mechanism [187,188]. Indeed, lysosomal dysfunctions and increased inflammatory response in *GBA* transgenic mice were normalized after inhibition of LRRK2 activity, supporting a connection between the activities of these two proteins [187].

3.4 Inflammation, immune system and Parkinson's disease

Inflammation and immune system represent the defensive compartment that our organism utilizes to protect its integrity from dangerous interaction with external or internal stimuli. This response is achieved though the activation of a cellular and a molecular (such as cytokines) mechanisms. The immune response can be divided in an innate immune response and an adaptive immune response. The innate immune response is the first line of defense that is activated once an external organism is encountered for the first time by our system. This compartment consists of circulating monocytes and their resident counterpart, namely macrophages, histocytes, microglia, or osteoclast among the others, according to the host tissues. The circulating monocytes are divided as well in different sub-population based on surface markers and differences in activations in response to exogenous insults. In particular, the

following sub-classes can be recognized: classic (CD14+/CD16-), intermediate (CD14+/CD16+) and non-classical (CD14dim/CD16+) monocytes. In the brain, both resident specialized microglia, as well as circulating monocytes, or monocytes-derived microglia cells have been identified.
The adaptive response is instead the second line of defense which activates and produces a more specific response through the production of antibodies. This is mediated by different subtypes of T cells.
The activation of the complement, important for the opsonization and thus recognition and degradation of pathogens, and well as cytokines release, that modulates the inflammatory response and different cells types activation, are also important in the context of the inflammatory response.

3.4.1 Immune response and inflammation in PD

The first report of activated microglial cells in brains of subjects affected with PD is from 1988 [189]. Since then a number of further evidences have stressed a possible role of inflammation in PD pathogenesis. On one side, the association between PD and certain inflammatory or autoimmune disorders was described [190]. Moreover, different works in the literature have recently reported an increased activation of the peripheral and central immune response in PD patients. Neuropathology and PET studies showed that patients with PD have an increased microglial activation, which it still not clear whether this represents a causative or a reactive consequence to the neurodegenerative processes [191,192]. Indeed,

increased microglial activation can be a compensatory mechanism trigger by the failure of the scavenger activity of microglial cells themselves for the removal alpha-synuclein or other toxic proteins, possibly due to an impairment of the endolysosomal compartment, as described above. On the other side, damaged or dying neurons can trigger microglia activation through the release of specific cytokines [193]. Microglia has also been described to interact with astrocytes modulating the balance between the protective and neurotoxic state of these cells [194]. I adjunct, recent works have also reported a possible interesting role of the adaptive immune response in the brain of subjects with PD [195]. The authors showed that dopaminergic neurons can function as antigen presenting cells, exposing specific alpha-synuclein peptides able to trigger a T cell response in the brain of patients with PD [195].

A number of works have reported an increased inflammatory response in patients with PD compared to aged matched controls. Levels of IL-1β, IL-2, IL-4, IL-6, IL-10, TNF, MCP1, RANTES, and MIP1α are increased in the peripheral blood and/or in the CSF of patients with PD [196–198]. Interestingly, distinct cytokine and interleukin profiles in the peripheral blood has been described in PD patient with the *GBA* and *LRRK2* mutations [177,199,200]. The inflammatory S100b protein is also upregulated in brain tissues of patients with PD and in their CSF [201]. Moreover, monocytes from patients with PD show an increased reactivity to lipopolysaccharide (LPS), a marker of the response to bacterial infection, compared to controls [202].

An interesting recent work also showed that the inhibition of the entrance of peripheral monocytes in the central nervous system by silencing chemotactic receptors is associated with a more limited degeneration of the dopaminergic neurons in a transgenic mouse model overexpressing alpha-synuclein [203]. This works nicely links the peripheral and central inflammatory response, also suggesting a role for the monocytes-derived microglia in the pathogenic mechanisms of PD.

Finally, genetics as well support a link between PD and inflammation and immune response. For example, other than with PD, the *LRRK2* gene is associated also with Crohn disease, a systemic inflammatory disorder [204]. Moreover, GWAS studied identified a number of loci associated with PD which are in close proximity with genes related to the immune and inflammatory response, such as the HLA regions [205].

Interestingly, genomic, cellular, and molecular studies in the context of PD pointed out as well at the crucial role of the innate immunity, whose expression profile is also enriched with PD-causative genes [206–209]. When considering genetic variants associated with PD it is interesting to notice that the majority of these genes have a relevant role in cells belonging to the immune system. Additionally, a polarization of the cis-regulatory effect of common variants associated with PD was identified in the innate immune compartment compared to the adaptive response, opposite to other conditions already established to be associated with the adaptive immune response, such as the rheumatoid arthritis [206]. Moreover, common variants associated with PD in the *LRRK2* gene showed to affect the

expression of this gene more significantly in peripheral CD14+ monocytes compared to brain tissues [210]. This reinforces again a possible role of these cells in PD pathogenesis that would be worth further exploring in order to better define these mechanisms in the disease.

3.4.2 Transcriptional signatures of human myeloid cells in PD

By assessing the transcriptomic profiles of CD14+ monocytes and microglia cells from a large cohort of subjects with PD and healthy controls (230 subjects total for monocytes and 55 brains for microglia isolation) we were able to start investigating distinctive expression profiles in these cells [211]. This work showed that cells of the innate immune system in the periphery and in the central nervous system (i.e. monocytes and microglia, respectively) in patients with PD presented very distinctive signatures. In particular, the lysosomal and the mitochondrial pathways appear to be significantly deregulated. Even more interestingly, the mitochondrial genes involved in the respiratory chain presented opposite pattern of expression between the periphery and the brain, suggesting a very specific activation in the two compartments. Single cell analysis further showed that the mitochondrial signature related to the respiratory chain, that we detected, was particularly represented in the intermediate subpopulation of peripheral monocytes (CD14++/CD16+). In adjunct, we showed that a significant number of PD risk alleles are specifically expressed in monocytes or can affect target gene expression or splicing in monocytes.

3.5 Innovative treatments, future prospective and open questions

Gene therapy and precision medicine are now a days a reality in many field of medicine and in PD too. With the discovery of a growing number of genetic mutations associated with the disease and a better definition of the associated molecular pathways, the identification of therapeutic targets is gaining growing interest in the field [212].

Interestingly, as previously showed, *GBA* is at the crossroads between some of the major possible PD causative mechanisms: the endo-lysosomal pathway, monocyte-mediated immune response, and the genetics of PD.

Because of its central role in the pathogenesis of PD and the relative high frequency of these mutations among PD patients, a number of clinical trials are already ongoing or are in the pipeline also for the treatment of GBA-related PD, leveraging small molecules, substrate inhibitors and gene therapy strategies [212].

However, few important questions are still waiting for answers that would be important for a better definition of effective therapeutic approaches, early intervention and genetic counseling of these patients. For example, given the reduced penetrance of these mutations in PD, what is actually driving the disease in subjects who will phenocovert compared to non-manifesting carriers? How can this inform us on the pathogenic mechanisms of PD? Are there distinctive clinical and/or molecular features in pre-manifesting carriers that will help to identify them earlier and allow early intervention that will prevent excessive loss of dopaminergic neurons in those subjects?

Can these markers be used for better counseling on the risk of disease in carriers and their family members? And lastly, because of the role of GBA in PD pathogenesis as well as in the innate immune response, how these two aspects are linked together?

4. Aim of the work

Purpose/Objective: The aim of this work is the identification of genomic traits that can help elucidating the pathogenic mechanisms associated with GBA-related PD. To achieve this goal, we will study a population of subjects with PD and controls with and without mutations of the *GBA* gene comparing the clinical traits and integrated transcriptomic profiles from CD14+ monocytes. The final goal of our analysis will be the identification of new therapeutic targets and new diagnostic biomarkers that can be used for the early diagnosis of *GBA* carriers making them suitable for pre-symptomatic targeted treatments, once available.

5. Material and Methods

5.1 Patients recruitment

5.1.1 Clinical centers and recruitment strategies

Subjects participating in the study were enrolled at The Marlene and Paolo Fresco Institute for Parkinson's and Movement Disorders at New York University (New York), the Bendheim Parkinson Movement Disorders Center at Mount Sinai (BPMD), the Alzheimer's Research Center (ADRC) and at the Center for Cognitive Health (CCH) at Mount Sinai Hospital (New York). Each Institution's Institutional Review Board approved the study protocol and the related procedures for subjects recruitment, as well as data and samples collection.

Subjects were enrolled only after obtaining a written consent. Participation in the study was only on a voluntary basis.

Subjects were enrolled during a dedicated research visit conducted by qualified research personnel.

5.1.2 Inclusion and exclusion criteria for subjects enrollment

As part of this study we enrolled subjects with a history of Parkinson's disease, atypical parkinsonism and other neurodegenerative conditions seen at the referring clinical centers.

Enrolled subjects were between the age of 18 and 100 years.

For Parkinson's disease, the diagnosis was established by a qualified movement disorder specialist, according to the United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria [213].

Healthy controls were defined as aged and gender-matched non-affected subjects, who didn't have a known diagnosis or evidence of Parkinson's disease or other neurological conditions at the time of evaluation. Non-affected subjects were enrolled among participants' partners and family members or among subjects who contacted the clinic expressing the willingness to participate as control subjects after knowing about the study through IRB approved initiatives (fliers, webpage portal).

5.1.3 Clinical assessments of recruited subjects

For the subjects enrolled at the Mount Sinai Hospital's Centers the following information was collected: demographic information (age, gender, self-reported ancestry), diagnosis (PD, non-affected subject, other), starvation status, medication history.

At the Fresco Institute for Parkinson's and Movement Disorders at New York University (NYU) during the research visit the following assessments were collected by qualified research and clinical personnel for each enrolled subject: demographic information (age, gender, self-reported ancestry), hand dominance, extended family history of PD or other relevant neurodegenerative or neurological conditions exploring the maximum number of generations the subject was able to report about, medication history, previous medical and surgical history – especially inflammatory diseases -, questionnaire for self-reported motor and non-motor PD-related symptoms (dysautonomia features - constipation, urinary symptoms, symptomatic orthostatic hypotension -, subjective loss of sense of smell, rapid eye movement (REM) sleep behavior disorders,

psychiatric manifestations - anxiety, depression, hallucinations -, motor complications related to dopaminergic treatments - dopamine-related impulse control disorders, motor fluctuations, dyskinesia). Only for subjects in the PD group, these additional data were also collected: age at the reported onset of motor symptoms, presenting motor symptoms, PD motor subtype (defined as postural instability and gait difficulty (PIGD) or tremor dominant (TD)).

The following rating scales were performed for each enrolled subject: Unified Parkinson's disease Rating Scale (UPDRS), Hoehn and Yahr (H&Y) rating scale, Montreal Cognitive Assessment (MoCA). At the Fresco Institute (NYU) only subjects with cognitive capacity, defined as scores of the MoCA scale equal or greater to 22 were included.

The collected data were all deidentified and they were stored in an IRB approved password-protected database.

5.2 Samples collection and processing

Blood samples were collected fresh on the day of the research visit by dedicated research personnel. We collected about 35 mL of peripheral blood from each subject. Samples were collected in Vacutainer blood collection tubes with acid citrate dextrose (ACD) (BD Biosciences). Blood samples were collected in the morning to reduce variability of samples components and cell activation. Samples were processed within 2-3 hours from collection at Dr. Towfique Raj's laboratory at Mount Sinai School of Medicine (NY).

5.2.1 DNA extraction and plasma collection

DNA was extracted from whole blood (0.5 ml) using the QiAamp DNA Blood Midi kit (Qiagen) according to the manufacturer's instructions. Nanodrop was utilized to assess DNA quality and concentration. Whole blood was aliquoted and stored at -80°C for subsequent DNA extraction. For plasma extraction samples were centrifuged at 1,500 g for 15 mins and the plasma fraction was then stored at -80 °C.

5.2.2 Sample processing and monocytes isolation

Sample processing consisted in isolation of peripheral blood mononuclear cells (PBMC) and subsequent CD14+ monocytes purification. For PBMC isolation, SepMate tubes (StemCell Technologies) were used. After dilution in 2-fold PBS (Gibco) tubes were filled with 15 ml of Ficoll-Plaque PLUS (GE Healthcare) and centrifuged at 1,200 g for 15 mins, followed by wash with PBS. Monocyte isolation was performed through sorting of 5 million PBMCs utilizing the AutoMacs sorter with CD14+ magnetic beads (Miltenyi) according to manufacturer's instructions. Sorted monocytes were stored at -80 °C in RLT buffer (Qiagen) + 1% 2-Mercaptoethanol (Sigma Aldrich).

5.2.3 RNA isolation

Isolated monocytes stored in RLT buffer were first thawed on ice. RNA was isolated with the RNeasy Mini kit (Qiagen) according to manufacturer's instructions, including the DNase I optional step. RNA was then stored at -80 °C until library preparation. RNA integrity

number (RIN) was assessed with TapeStation using Agilent RNA ScreenTape System (Agilent Technologies). RNA concentration was obtained with Qubit.

5.2.4 RNA library preparation

Part of the samples were processed in house for RNA library preparation. TruSeq Stranded Total RNA Sample Preparation kit (Illumina), with the Low Sample (LS) protocol, was utilized for library preparation according to the manufacturer’s instructions.

For the rest of the samples RNA library was prepared by commercial service (Genewiz Inc.). RNA was shipped and processed according to Standard RNA-seq protocol.

Ribo-depletion strategy to remove rRNA was utilized for both samples processed in house and at Genewitz Inc.

5.3 Sequencing and genotyping

5.3.1 Genome screening array

Genotyping of the DNA of the samples was performed with the Illumina Infinium Global Screening Array (GSA). This consists of a genome-wide backbone of 642,824 common variants and custom disease SNP content of about 60,000 SNPs.

5.3.2 *GBA* and *LRRK2* screening

Screening for the most common genetic mutations of the *GBA* and *LRRK2* genes associated with Parkinson’s disease and more frequent among the Ashkenazy Jew ancestry was performed through

targeted genotyping at Dr. William Nichols' laboratory at the Cincinnati Children's Hospital.

In particular, for the *LRRK2* gene the G2019S variant was screened; for the *GBA* the following 11 variants were analyzed: IVS2+1, 84GG, E326K, T369M, N370S, V394L, D409G, L444P, A456P, R496H, RecNcil.

The percentage of each mutation across the entire population and within manifesting and non-manifesting carriers was calculated for both *LRRK2* and *GBA* mutations. Minor allele frequency (MAF) was calculated as well for each screened variant.

5.3.3 RNA sequencing

All samples were sequenced at Genewiz Inc. on Illumina HiSeq 4000 platform with 150-bp paired-end reads and 60 million depth. Sequencing was performed in four independent batches.

5.3.4 Genotyping analysis

GSA data were then processed to ensure high quality data. The following quality control metrics were applied: minor allele frequency (MAF) >5%, SNP and samples call rate >95%, Hardy-Weinberg equilibrium (HWE) P-Value > 1 x 10-6. PLINK program was utilized to identify duplicated/related samples using pairwise IBD (identity-by-descent) estimation (PLINK PI_HAT values 0.99-1), and these were removed. Files were converted to Variant Call Format (VCF) with VCFTools.

The Michigan Imputation Server v1.0.4 (Minimac 3) was used for genomic data imputation [214]. The 1000 genomes phase 3 v5

mixed reference panel was eagle v2.3 phasing in quality control and imputation mode. Imputed VCF files were lifted over to hg38 with the hg19toHg38.over.chain.gz liftover chain file from UCSC (University of California, Santa Cruz) browser and liftoverVCF from Picard.

Post-imputation analysis filters were applied, consisting in MAF > 5% and HWE P-Value > 1 x 10-6, removing multi-allelic single nucleotide polymorphisms (SNPs) and indels, considering SNPs with imputation R2 > 0.3.

The final count of 5,951,770 variants was eventually retained after listed QC.

5.4 Analysis of clinical data

Clinical data were analyzed according to standard statistical methods. Normal distribution of the data for each trait was established using the Shapiro test. Mean and standard deviation were calculated for age, age of onset and rating scales (UPDRS, UPDRS part III, HY, UPSIT, MOCA) as well as for the levodopa equivalent daily dose (LEDD, calculated according to standard rate of conversion for the following dopamine-related medications: carbidopa/levodopa, carbidopa/levodopa extended release, monoamine oxidase (MAO) inhibitors, catecol-O-methyltransferase (COMPT) inhibitors, amantadine, dopamine agonist – pramipexole, rotigotine, ropinirole). For normally distributed data (age, age of onset, UPSIT score, MOCA score) t-test was performed. For not-normally distributed continue scores (UPDRS, UPDRS part III, HY, LEDD) Mann-Whitney U test was used. Z-score of proportion was used to compare categorical variables.

5.4.1 Ancestry analysis

PLINK program was utilized to confirm genetic ancestry of samples through principal components analysis [215] and comparing multidimensional scaling (MDS) of the values of the study cohort with data from the Phase 3 of 1000 Genome Project samples.

For the Ashkenazi Jewish (AJ) only, analyses were repeated using a custom panel as a reference.

5.5 Bioinformatic analysis

5.5.1 Data normalization

FASTQ files were processed utilizing the RAPiD-nf pipeline as previously described [216]. RAPiD-nf was implemented in the NextFlow framework ("Nextflow - A DSL for Parallel and Scalable Computational Pipelines" n.d.).

To assess quality of the sequences and technical metrics SAMtools (v1.9) and Picard (2.20) ("Picard Tools - By Broad Institute" n.d.) were utilized prior to and after alignment with FASTQC (0.11.8) ("Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data" n.d.) [217].

Using RAPiD we obtained automated alignment, quantification, and quality control of each RNA-seq sample. First, data were processed with trimmomatic (v0.36) for adapter trimming [218]. Afterwards, upon creating indexes from GENCODE (v30) ("GENCODE - Human Release 30" n.d.), STAR (2.7.2a) was utilized for aligning the samples to the human reference genome hg38 build

(GRCh38.primary_assembly) [219]. Quantification of gene expression was obtained with RSEM (1.3.1) [220].

Quality control of the data was performed running FASTQC (in the RAPiD3.0 pipeline). Results were visualized with MultiQC and by assessing estimated Transcripts Per Million (TPM), counts and Counts Per Million (CPM), as well as TMM-voom normalizations. The following thresholds were used for initial filtering of the data: > 20% of reads mapping to coding regions, > 20 Million passed reads, and ribosomal rate < 30%.

Sex mismatch was assessed by comparing reported sex with the expression of genes UTY and XIST, which didn't identify any sex mismatch in our cohort.

TPM and transcript level counts for individual genes were generated. CPM was instead calculated with the edgeR packing in R (cpm() function). Genes with < 1 count per million in at least 30% of the samples were considered low expression genes and they were excluded from the downstream analysis. The above processing led to a total of 13,711 genes.

5.5.2 Linear models for data regression

For downstream analysis TMM values obtained from edgeR and voom transformation were normalized with a linear model of regression. Initial design accounted for the majority of available technical and phenotypic variables (rna_batch + Sex + PCT_USABLE_BASES + PCT_RIBOSOMAL_BASES + AJ_gsa_assignment + RIN + PCT_CODING_BASES + PCT_INTERGENIC_BASES + MEDIAN_5PRIME_BIAS +

TOTAL_READS + PF_ALIGNED_BASES + PF_MISMATCH_RATE + C1 + C2 + C3 + C4 + C5 + C6 + C7 + C8 + C9 + C10 + PCT_INTRONIC_BASES + PCT_ADAPTER + next_day + C1_AJ + C2_AJ + C3_AJ + C4_AJ + C5_AJ + C6_AJ + C7_AJ + C8_AJ + C9_AJ + C10_AJ + MEDIAN_CV_COVERAGE + PCT_ADAPTER + Diagnosis). However, principal component and MDS analysis showed the persistence of samples outliers with an impact on the downstream analysis. Therefore, to reduce error rate and increase reproducibility of the data, these were then processed with the sva package in R for Surrogate Variable Analysis [221]. This package allows the identification of surrogate variables to be built directly from a high-dimensional dataset. Estimating variability of our data, based on genetic status (presence of *GBA* mutations) and phenotype (subjects with a diagnosis of Parkinson's disease or control groups), in order not to remove these variables from our data, a set of 13 surrogate variables were estimated in our set of data. Surrogate variables were built in the design for linear regression of the data. The contribution of known technical and phenotypical variables to the surrogate variables was obtained by linear regression between the surrogate variables and the covariates file and visualized with heatmap in R.

5.5.3 Differential expression pairwise comparison and nested analysis

A list of differential expressed genes was obtained with the limma package in R by combining expression data (after TMM normalization and voom transformation in R) and surrogate variable.

Differential expression analyses were pursued with two different approaches. For both approaches, the R package limma version 3.38.3 was used to fit a linear model and provide P-Value upon performing Bayesian moderated t-test. Multiple testing correction with Benjamini-Hochberg False Discovery Rate (FDR) was obtained leveraging the function in the limma package.

The cohort of subjects was subdivided into subgroups based on the disease status (subjects with PD vs controls (CTRL)) and *GBA* genetic mutation status (subjects carrying at least one *GBA* mutations (GBA+) and subjects with no *GBA* mutations (GBA-)). Four groups were thus obtained: PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA-.

In the first set of analysis we compared each pair of our cohorts separately, meaning PD/GBA+ vs PD/GBA-, PD/GBA+ vs CTRL/GBA+, PD/GBA+ vs CTRL/GBA-, PD/GBA- vs CTRL/GBA+, PD/GBA- vs CTRL/GBA-, CTRL/GBA+ vs CTRL/GBA-. A threshold of FDR < 0.15 was considered for identifying significant results.

In the second set of analysis we utilized a nested manually designed interaction term to analyze expression data in reference to the variable of interest, which consisted in disease status and GBA-mutation status. The interaction contrast was design as follow: [(InteractionPDGBA – InterationCTRLGBA) – (InteractioPDnone – InteractionCTRLnone) = PDCTRL_inGBAcarrier – PDCTRL_inGBANoncarrier]. This model was used to run a linear model of our analysis. Results from the comparison of each pair of groups were then extracted. A threshold of FDR < 0.05 was utilized for the selection of significant results.

5.5.4 Pathway enrichment analysis

Pathway enrichment analysis was performed utilizing the set of differentially expressed genes from the nested interaction model analysis considering genes with FDR < 0.05. Pathway enrichment analysis was run considering upregulated and downregulated genes separately in order to better characterize our set of differentially expressed genes.

Pathway enrichment was obtained utilizing different methods and platforms.

1. Gene Set Enrichment Analysis (GSEA) was used to analyze different terms from the Gene Ontology (GO) list (specifically: Cellular Component (CC), Molecular Function (MF), and Biological Processes (BP)) [222]. Pathways with enrichment with FDR < 0.01 or 0.05 (as specified in the results) were considered. Filters were set for pathways with less than 2000 genes. We analyzed up to the first 20 significant enriched pathways.

For the dataset obtained from analysis with OUTRIDER tool (see below) the additional following tools were utilized:

2. g-profiler (https://biit.cs.ut.ee/gprofiler/gost), a web server for functional enrichment analysis. Input data were the list of up and downregulated genes separately.

3. Ingenuity pathway analysis (IPA). Canonical data analysis for pathway enrichment was performed. Statistically significant pathways with P-value < 0.05 were accounted for in the final results.

The results from the different tools were then combined together based on P-values after multiple correction.

5.5.5 Curated pathway analysis

Pathway enrichment analysis was also performed to assess the enrichment in our sets of differentially expressed genes of curated pathways relevant to our research questions. The curated gene list that were considered were the following: myeloid markers (71 genes) based on literature search considering specific markers for monocytes, CD16-Monocytes, CD4-T cells, CD8-T cells, B cells, natural killer (NK) cells, dendritic cells, megakaryocytes; lysosomal gene list: 435 genes from The Human Lysosome Gene Dataset; lysosomal storage disease causative gene (LSD list) (54 genes) classified as sphingolipidoses, neuronal ceroid lipofuscinosis, mucolipidosis/oligosaccharides diseases; mitochondrial gene list from [223] (315 genes), classified in distinct mitochondrial pathways as reported in the cited paper, such as mitonuclear cross-talk, mitochondrial dynamics, and OXPHOS; ubiquitin-related gene list (428 genes) from ubiquitin-like modifier activating enzymes and ubiquitin conjugating enzymes E2 (HUGO Gene Nomenclature Committee (HGNC) dataset), and ubiquitin ligase E3.

Fisher exact test was run to assess the enrichment of curated pathways in the differential expressed gene lists. A threshold of P-Value < 0.15 was considered for significance.

5.5.6 Hypothesis driven pathway analysis

Differentially deregulated genes between the (PD/GBA+ vs PD/GBA- and PD/GBA+ vs CTRL/GBA+) were manually classified according to their functions and localization reported in the literature and in

available databases (UniProt, GeneCards, NCBI, OMIM, Genetic Home Reference NIH) [224–227]. For the PD/GBA+ vs CTRL/GBA+ groups, genes (129 up-regulated and 124 down-regulated) were then classified in five categories relevant for Parkinson's disease pathogenesis (mitochondrial pathway, endo-lysosomal pathway, genetics of PD, epigenetic, cytoskeleton, inflammation) and "others".
Moreover, enrichment for gene sets related to membrane and vesicle trafficking was assessed through Fisher-exact tests within up- and down-regulated genes (considered separately) between the PD/GBA+ vs CTRL/GBA+ groups. Gene sets were selected from the GO terms database [228,229]. The following gene sets were considered: "vesicle-mediated transport" GO:0016192; endolysosome GO:0036020; lysosomal membrane GO:0005765; vacuolar membrane GO:0005774; mitochondrial membrane GO:0031966; Golgi membrane GO:0000139; autophagosome membrane GO:0000421; vesicle membrane GO:0012506; endosome membrane GO:0010008; membrane invagination GO:0010324; membrane biogenesis GO:0044091; membrane assembly GO:0071709; membrane docking GO:0022406; phagocytic vesicles membrane GO:0030670; ER membrane GO:0005789; peroxisomal membrane transport GO:0015919; mitochondrial membrane fusion GO:1990613; synaptic vesicles membrane GO:0030672; secretory granule membrane GO:0030667; mitochondria outer membrane GO:0005741; mitochondrial inner membrane GO:0005743; coated vesicles membrane GO:0030662; endocytic vesicles membrane GO:0030666; cytoplasmic vesicles membrane GO:0030659; transport vesicles membrane GO:0030658;

autophagosome membrane docking GO:0016240; peroxisome membrane biogenesis GO:0016557; exocytic vesicles membrane GO:0099501; mitochondrial membrane fission GO:0090149; early endosome membrane GO:0031901; late endosome membrane GO:0031902; early phagosome membrane GO:0036186; complement activation GO:0006956; extracellular exosomes: GO:0070062; exosomes (RNA complex): GO:0000178; extracellular exosomes complex: GO:1990563; extracellular exosomes biogenesis: GO:0097734; extracellular exosomes macropinocytosis: GO:0061707; extracellular exosomes assembly: GO:0071971; cytoplasmic exosomes: GO:0000177; trans-synaptic signaling via exosomes: GO:0099157; regulation of extracellular exosomes assembly: GO:1903551; clathrin dependent exosomes assembly: GO:1990771; positive regulator of exosome assembly: GO:1903553; negative regulator of exosome assembly: GO:1903552; RNA polymerase exosomes dependent: GO:0030847, exosomal secretion: GO:1990182; regulation of exosomal secretion: GO:1903541; negative regulation exosomal secretion: GO:1903542; positive regulation exosomal secretion: GO:1903543.

Based on the deregulated pathways identified with the GSEA pathways enrichment analysis, enrichment for the following pathways according to the Fisher exact test was assessed as well: NOTCH1 signaling pathway GO:0007219; senescence associated vacuoles: GO:0010282 (plant); cell signaling via exosome: GO:0099156; cellular senescence: GO:0090398; lipid storage: GO:0019915, GO:0006869; lipid transport GO:0032594; tau protein binding GO:0048156; regulation Tau kinase activity GO:1902947,

GO:1902949, GO:1902948; Golgi related pathways: GO:0048211, GO:0005795, GO:0005794, GO:0005796, GO:0051645, GO:0006895, GO:0035621, GO:0055107, GO:0006888.

5.6 Genetic outliers

RNA-seq data can be also used to identify expression outliers within each single sample that may be expression of underlying genetic mutations, especially in regulatory regions, or compensatory/deregulated mechanisms. Different tools have been reported in the literature to explore this approach, based on Z-score distribution or a combination of Z-scores and the negative binomial distribution, respectively [230,231]. These tools presented some limitations such as the lack of specific statistical tests to compare the expression data and the lack of regression for known and unknown covariates that can greatly affect gene expression profiles. OUTRIDER is an additional tool that, instead, utilizes autoencoders to control for variation linked to unknown factors for data normalization. Single genes and single individuals outliers are then detected by comparing univariate cases with the distribution of each gene across the population, by calculating the negative binomial distribution of each single sample compared to all samples [232]. Autoencoders are also discharging samples with an excess of outliers genes, that may be related to other causes than having a biological relevance [232].

Counts of > 1 million in more than 30% of the samples were implemented in the tools. Data were normalized leveraging autoencoders (“OUTRIDER - OUTlier in RNA-Seq fInDER”).

Normalized dispersion and mean were then fitted in a binomial model followed by computation of two-sided p-value. The significance threshold was set at an FDR adjusted P-value adjust cut-off of 0.05 and z-score threshold of 2.

5.7 Trans-eQTLs

The trans-eQTL analysis was performed considering the SNPs within 1MB from the transcription starting site (TSS) of the GBA gene. The SNP for the N370S variant (N409S according to the new nomenclature) was genotypes separately and added manually. Association tests were performed between SNPs and all expressed genes (n=18,431) using QTLtools in "trans full pass" mode. Estimates were adjusted for the following covariates: age, gender, sequencing batch, % of usable base, % of ribosomal bases, and the 4 first genetic PCs.

After associations, SNPs were filtered based on linkage disequilibrium (LD) score considering r2 = 0.5 and MAF = 0.1 using the SNPclip Tool (https://ldlink.nci.nih.gov/?tab=snpclip). Targeted genes of the residual SNPs were analyzed. FDR < 0.15 was considered as the threshold for significance.

Results

6.1 Phenotypical characterization

6.1.1 Demographic and incidence of motor and non-motor symptoms

We compared demographic and phenotypical features in a cohort of 19 PD/GBA+, 37 PD/GBA-, 37 CTRL/GBA-, 9 CTRL/GBA+ subjects enrolled at the Fresco Institute, NYU Langone Health, NY. We performed pairwise comparisons between PD groups (GBA+ vs GBA-) and CTRL subjects (GBA+ vs GBA-).

PD/GBA+ vs PD/GBA-

Comparison between subjects with PD showed that GBA- subjects presented older age compared to PD/GBA+ subjects (69.8 +/- 9.49 vs 59.8+/-10.6 respectively with p = 0.000361), older age of onset (61.8 +/- 14.3 vs 53.9 +/- 11.6, p = 0.04181), and lower percentage of female subjects (43% in GBA- and 68% in GBA+, p = <0 .00001). The comparison of the single traits between groups is reported in **Figure 1** (**A and B**). In particular, motor scores (UPDRS, UPDRS part III, and HY) were not significantly different between PD/GBA+ and PD/GBA-, except for the freezing of the gait that was more frequent in the PD/GBA- group. For the non-motor scores, in the PD/GBA- cohort there was an increased frequency of RBD, orthostatic hypotension (OH), constipation and urinary symptom, and depression. LEDD and dyskinesia were comparable, while there was a slight but significant increased incidence in motor fluctuation in PD/GBA- vs PD/GBA+ subjects (35% vs 32% in PD/GBA- vs

PD/GBA+, p = <0 .00001) (not showed in the figure). Interestingly, in the PD/GBA- groups there was a significantly increased incidence of inflammatory diseases compared to the PD/GBA+ group (38% vs 12% respectively, p = <0 .00001) and of subjects on aspirin (24% vs 5% respectively, p = 00634). In the PD/GBA+ group anxiety (58% vs 24% in the PD/GBA+ vs PD/GBA- resepctively, p = <0 .00001) and cognitive impairment (6% vs 0% in the PD/GBA+ vs PD/GBA- respectively, p = <0 .00001) were more frequent.

CTRL/GBA+ vs CTRL/GBA-

Within controls, there were no significant differences in terms of age while there was an over-representation of female subjects in the CTRL/GBA- group compared to the CTRL/GBA+ group (65% vs 33% respectively, p = <0 .00001). Mean, standard deviation and percentage of continuous and binary data and statistical analysis are reported in **Figure 1** (**C and D**). In the CTRL/GBA+ compared to the CTRL/GBA- subjects there was a higher frequency of RBD symptoms (11% vs 5% respectively, p = <0 .00001), anxiety (44% vs 22% respectively, p = < 0.00001), and depression (22% vs 16% respectively, p = <0 .00001), but less frequent constipation (0% vs 14% respectively, p = 0.0271), and urinary symptoms (0% vs 16% respectively, p = 0.01468). in the CTRL/GBA+ cohort a significantly increased incidence of inflammatory diseases (11% vs 3% in the CTRL/GBA+ vs CTRL/GBA- respectively, p = <0 .00001) and use of aspirin (22% vs 8% in the CTRL/GBA+ vs CTRL/GBA- respectively, p = <0 .00001) was reported.

6.1.2 Family history

Because of the reduced penetrance of *GBA* mutations, we carefully examined the family history of our cohort of subjects to assess any difference in terms of concurrent conditions and heritability (**Figure 1, B and D**). In both the PD/GBA+ and CTRL/GBA+ groups family history was more frequent compared to the PD/GBA- and CTRL/GBA- groups, as expected (42% vs 35% in the PD/GBA+ vs PD/GBA- groups respectively, $p < 0.00001$; 67% vs 27% in the CTRL/GBA+ vs CTRL/GBA- groups respectively, $p < 0.00001$). Within PD subjects, maternal and paternal family history of PD were both more frequent in the GBA+ compared to the GBA- groups (26% vs 14% for the maternal family history and 26% vs 24% for the paternal family history in the PD/GBA+ vs PD/GBA- groups respectively) (**Figure 1B**). Within the CTRL groups, a maternal family history was more frequent across GBA+ subjects (33% vs 14% in CTRL/GBA+ vs CTRL/GBA- respectively, $p < 0.00001$) while a family history of PD on the paternal side of the family was slightly more common in the CTRL/GBA- group (22% vs 24% in the CTRL/GBA+ vs CTRL/GBA- respectively) (**Figure 1D**). Paternal and maternal family history has never been assessed in the literature to our knowledge. Thus, validation of these observations in a larger cohort of subjects may offer important insights in this context.

We also compared the frequencies of positive family history of tremor and dementia, as these two symptoms can be present in GBA-related PD phenotypes. We didn't identify any significant difference in the frequency of a family history of tremor between GBA+ and GBA- subjects within the PD and CTRL cohort. Statistical analysis

showed a significant difference in terms of family history of dementia in the PD cohorts. Interestingly, in the PD/GBA+ group there was a higher frequency of history of tremor compared to the CTRL/GBA+ group, although not significant (5% vs 0 respectively, p = 0. 08914). Instead, between the same two groups, a family history of dementia was significantly more frequent in the PD/GBA+ vs CTRL/GBA+ group (11% vs 0 respectively, p = 0.00338).

6.2 Genetic characterization of our cohort

Genetic screening for the most common *GBA* mutations associated with PD (as listed above in the Methods) in the AJ ancestry and the G2019S mutation of the *LRRK2* gene were screened in a larger cohort of subjects enrolled at the Fresco Institute (NYU Langone, NY) and Icahn School of Medicine (Mount Sinai). A total of 715 subjects were analyzed, consisting of subjects with PD (247), CTRL (235), subjects with Lewy Body Dementia (LBD, 2), atypical parkinsonism – multiple system atrophy (MSA, 5), atypical tauopathy (corticobasal degeneration and progressive supranuclear palsy, CBD and PSP respectively, 16) -, Alzheimer dementia (ADe, 52), mild cognitive impairment (MCI, 52), posterior cerebral atrophy (PCA, 1), other movement disorders (myoclonus, dystonia, Huntington disease, ataxia, tremor, 10), other (undefined diagnosis, 95). As expected, the incidence of *GBA* mutations was higher in the PD population compared to the CTRL cohort (17.41% vs 7.23% respectively, p = 0.00072 - Chi-squared test) considering both monoallelic and biallelic carriers (**Figure 2A**). The proportion of subjects with pathogenic *GBA* mutations was also higher compared

to the other classes of neurodegenerative conditions, such as AD, MCI, and atypical parkinsonism (7.69%, 3.85% and 0, respectively), confirming a specificity of this mutation for PD. We couldn't address the implication of *GBA* in LBD because we only had two samples. At the same time, the only subject included with PCA was found to be a carrier of a mutation in the *GBA* gene. However, being a single case this does not allow us to drive any conclusion in the context of this disease and *GBA* mutations.

The incidence of the *LRRK2* mutation G2019S was also increased in the PD population compared to CTRL (5.67% vs 0.85% respectively, $p = 0.00318$ - Chi-squared test). Across the other groups, one carrier of this mutation was found only in the population of subjects with a diagnosis of MCI (**Figure 2A**).

Finally, in the cohort of subjects with PD, 2 subjects were found carrying both a mutation in the *GBA* and one in the *LRRK2* gene (**Figure 2A**).

6.2.1 Incidence of different *GBA* mutation across PD patients and controls

We then calculated the frequency of the different mutations of the *GBA* gene that were tested across carriers, in both the PD and the CTRL groups (**Figure 2B**).

The majority of the carriers presented the N370S (or N409S according to the new nomenclature) mutation. MAF was 0.0543 in the PD cohort and 0.0252 within CTRL. The variants R496H, T369M, and IVS2+1 were found only in the PD group (with MAF 0.0027, 0.0054, and 0.0027, respectively). The mutation E326K has been

long debated in the literature and it was eventually considered to be significantly associated with an increased risk of developing PD, while it is not found among the pathogenic mutations associated with Gaucher's disease [101,110–113]. In our population we found a similar MAF of this mutation across PD and CTRL subjects (0.0082 and 0.0084) respectively. It can be argued whether these findings may suggest the possibility that some among the non-manifesting carriers of this mutation will develop PD in the future, thus re-establishing the expected proportion between carriers of this mutation in PD and CTRL subjects. Surprisingly, instead, for some other mutations (84GG and RecNcil) we found an increased frequency across CTRL compared to PD subjects (MAF of 0.0042 and 0.0027 in CTRL and PD respectively, for both mutations). Since those two mutations have been repeatedly reported in the literature as significantly associated with a risk of PD, the higher incidence across CTRL in our cohort may be related to the power of our analysis.

It is well established that the N370S and the L444P (or L483P according to the new nomenclature) are the two most common *GBA* mutations in the PD population (reviewed in [82]). The fact that our cohort was significantly enriched with carriers of the N370S variant while no subjects with the L444P variant were identified is very informative about the genetic background of our cohort. Indeed, the N370S mutation has been reported with higher frequency in the subjects with AJ ancestry, while the L444P is more common in the non-AJ Caucasian population (reviewed in [82]). Self-reported

ancestry data and genetic ancestry evaluation (see below) confirmed that the great majority of our cohort was European of AJ ancestry.

6.3 Study cohort for bioinformatic analysis

A subpopulation of PD and CTRL subjects with and without *GBA* mutations was then selected for the study of the transcriptomic profiles of CD14+ monocytes, based on availability of blood samples, good quality of extracted RNA and sequencing, according to procedures detailed above, as well as self-reported Caucasian ancestry, in order to limit variability due to genetic background architecture related to ancestry (**Figure 3**). The final population consisted of 56 PD/GBA-, 66 CTRL/GBA-, 23 PD/GBA+, and 13 CTRL/GBA+ subjects (**Table 1**). The majority of the carriers presented the N370S (N409S according to the new nomenclature) *GBA*-mutation (74% in the PD/GBA+ cohort, and 46% in the CTRL/GBA + cohort). Few subjects across the different cohorts also carried the G2019S mutation of the *LRRK2* gene (7% in the PD/GBA+, 4.3% in the PD/GBA-, and 1.5% in the CTRL/GBA- cohorts).

In terms of demographic data, females were more numerous in the PD/GBA+ and CTRL/GBA- cohorts (61% and 67% respectively) compared to the PD/GBA- and CTRL/GBA+ groups (30% and 54% respectively). The mean age was slightly higher in the GBA- subjects for both PD and CTRL compared to the GBA+ groups (PD/GBA+ = 60.3, PD/GBA- = 68.7, CTRL/GBA+ = 58.3, CTRL/GBA- = 67).

6.3.1 Ancestry

Genetic ancestry was determined as detailed above (Material and Methods). As expected, based on the self-reported information, the great majority of the selected subjects presented overlap with the European ancestry (**Supplementary Figure 1A**). Only one subject overlapped with the African ancestry, and 10 subjects with American ancestry.

When we further analyzed the subjects with European ancestry we were able to appreciate that they were almost equally distributed between AJ and non-AJ ancestry (**Supplementary Figure 1B**). This supported an enrichment of our population for subjects with AJ heritage, as previously suggested by the frequency of specific genetic variants of the *GBA* gene that were identified in our cohort.

6.4 Transcriptomic analysis of isolated CD14+ monocytes

RNA-expression data were obtained from 56 PD/GBA-, 23 PD/GBA+, 66 CTRL/GBA-, and 13 CTRL/GBA+ subjects. After rigorous QC and data normalization as previously discussed (Material and methods section) we compared a total of 13711 genes, considering only genes with expression higher than 1 CPM in 30% of the samples (**Supplementary Figure 2)**.

6.4.1 Targeted differential expression profiles

We first tested our samples for the enrichment of monocyte specific markers, as previously described. Although our samples presented increased expression also for some of the markers of the B cells and

dendritic cells among the leukocytes biomarkers, they clearly showed an upregulation for all the monocytes biomarkers that were expressed (**Supplementary Figure 3**), confirming our purification processes.

We then compared the level of expression of the *GBA* gene. Interestingly, there were no significant differences in the expression of our target gene across the four groups (**Figure 4A**). This is particularly relevant especially in light of a number of recent reports that showed that the GCase activity is reduced in monocytes of patients with PD, especially in subjects with PD carrying *GBA* mutations, and that the reduced activity persists as the disease progresses [142]. The presence of *LRRK2* mutations in some of the samples does not seem to drive *GBA* expression levels, as *LRRK2* carriers were not outliers for the levels of expression of *GBA* in any of the sub-groups. *GBA* mutations considered severe (because associated with GD type 2 and/o 3, as described in the Introduction), such as the 84GG and V394L, presented instead lower levels of expression (**Figure 4A**).

We then compared the expression profiles of the genes associated with the Lysosomal Storage Disorders. Previous works reported an enrichment of variants in these genes in patients with PD compared to non-affected subjects [233]. The co-existence of variants in genes involved in the same pathways may have a possible modulatory effect on the penetrance of a mutation. Distinct expression pathways of these genes were identified in the four groups (**Figure 4B**). The PD/GBA+ and PD/GBA- groups showed some similarities compared to the two CTRL groups, which instead have an almost opposite

profile of expression of these genes. There was no enrichment of different patterns of expression for any of the specific subtypes of the LSD (**Supplementary Figure 4**).

6.4.2 Differential expression analysis: overview

Differential expression analysis between each pair of groups was obtained with two different approaches, as described above (Material and Methods section) (**Figure 5A**). Considering significant genes at FDR < 0.05 with the nested interaction model analysis, we obtained 512 differentially expressed genes (383 downregulated and 129 upregulated) between PD/GBA+ and CTRL/GBA+ subjects; 1543 differentially expressed genes (1030 downregulated and 513 upregulated) between PD/GBA- and CTRL/GBA- subjects; 5 differentially expressed genes (4 downregulated and 1 upregulated) between PD/GBA+ and PD/GBA- subjects (and 44 differentially expressed genes considering FDR < 0.15); 0 differentially expressed genes between CTRL/GBA+ and CTRL/GBA- subjects (**Figure 5B**). The comparison between PD/GBA+ and CTRL/GBA- subjects was not considered for further analysis since it was not specific to our research questions.

Using the group-specific method (subsamples analysis) and FDR < 0.15 as a significant threshold, we obtained: 0 differentially expressed genes between PD/GBA+ and CTRL/GBA+ subjects; 2750 differentially expressed genes (1069 upregulated and 681 downregulated) between PD/GBA- and CTRL/GBA- subjects; 11 differentially expressed genes (9 upregulated and 2 downregulated)

between PD/GBA+ and PD/GBA- subjects; 0 differentially expressed genes between CTRL/GBA+ and CTRL/GBA- subjects (**Figure 5B**). These results show that the nested interaction model increased the power of our analysis, allowing the identification of differentially expressed genes also with a smaller samples size (such as the PD/GBA+ and CTRL/GBA- cohorts), that are instead probably underpowered for the limma/voom subsampled analysis. Moreover, the data showed a larger number of differentially expressed genes when comparing the subjects with PD vs CTRL than when comparing the same disease status (PD or CTRL) but different genetic status (GBA+ vs GBA-) (**Figure 5B)**.

6.4.3 Differential expression analysis: PD vs CTRL

The comparison between PD/GBA- and CTRL/GBA- subjects was not part of the aim of this work. However, this comparison was pursued to assess reproducibility of our analysis. The results were compared with our recent work [211] where transcriptomic profiles of CD14+ monocytes from a large cohort of PD and CTRL subjects (MyND cohort) were compared (**Supplementary Figure 5**). Some of the samples were overlapping between the two cohorts. Despite using different methods of normalization (linear regression of selected variables compared to surrogate variables in our analysis), there was an overlap of 98 out of 302 significantly differentially expressed genes (considering $FDR < 0.05$) which also showed a consistent directionality of expression ($r^2 > 0.9$), except for one gene (Autophagy Related 7, *ATG7*) (**Supplementary Figure 5**). This observation confirmed the consistency of our previous results despite

analyzing a smaller cohort and with a different method of normalization of our data.

6.4.4 Differential expression analysis: PD/GBA+ vs CTRL/GBA+

The comparison between manifesting and non-manifesting *GBA* mutations carriers can potentially elucidate disease related mechanisms that are deregulated in the PD group compared to the CTRL subjects. We considered the list of genes differentially expressed according to the nested interaction model analysis. Of the 512 differentially expressed genes, 197 were shared with the genes differentially expressed between PD and CTRL with no *GBA* mutations with whom they also shared the same directionality of expression, except for one gene. 315 genes were instead specific for the comparison between PD and CTRL within *GBA* carriers (**Figure 6)**.

Pathway analysis was performed with GSEA, as detailed above. We analyzed up and downregulated genes (129 genes up-regulated, 383 genes down-regulated) separately. The upregulated genes that were mostly driving the pathway enrichment included: *MNDA* (Myeloid Cell Nuclear Differentiation Antigen), *BST1* (Bone marrow stromal cell antigen 1, ADP-ribosyl cyclase 2, *CD157*), *FUCA2* (Alpha-L-Fucosidase 2), *HEXB* (hexosaminidase Subunit Beta), *ATG7* (Autophagy Related 7), and *RAB33B* (Rab GTPase family). Interestingly, *BST1* is also a GWAS hit [49], while *FUCA2* and *HEXB* are involved in senescence [234]. Considering only genes that were specific for this comparison and not shared with the PD/GBA- vs CTRL/GBA- comparison, among the upregulated genes we found

enrichment at FDR < 0.05 of pathways related to the immune response as well as of exocytosis and excitatory granules (considering BP, CC and MF) (**Figure 7**).

Across the enriched downregulated pathways, over-represented genes included: *EP300* (E1A Binding Protein P300, histone acetyltransferase), *VDR* (vitamin D receptor), *PPARD* (peroxisome proliferator activated receptor delta), *NOTCH1* (Notch receptor 1), *KMT2D* (lysine methyltransferase 2D), *ERLIN1* (ER lipid raft associated 1), *RPTOR* (regulatory associated protein of MTOR complex 1), *KMT2B* (lysine methyltransferase 2B), *LRRK1* (leucine rich repeat kinase 1), *RAP1GAP2* (RAP1 GTPase activating protein 2), *SRGAP1* (SLIT-ROBO Rho GTPase activating protein 1), *ARHGAP1* (Rho GTPase activating protein). Downregulated genes specific for the comparison PD/GBA+ vs PD/GBA- only, including BP, CC, and MF, were enriched for transcription/RNA-metabolism related pathways, signal transduction (synapses and calcium mediated signal transmission), kinase activity, as well as membrane trafficking and vesicle secretion although to a less extent compared to the upregulated genes (**Figure 7**).

Since vesicle trafficking and exocytosis processes may involve many different compartments and pathways in the cells, in order to further dissect the mechanisms underlying our set of genes we performed a targeted enrichment analysis. We selected those pathways whose GO terms were associated with “membrane”, “lysosome”, “endocytosis”, “exocytosis”. We selected a total of 32 pathways. We also considered curated pathways for lysosomal, ubiquitin, and proteasomal pathways, based on data reported in the literature

(**Supplementary Table 1**). Enrichment analysis of differentially up and downregulated genes, separately, between subjects PD/GBA+ and CTRL/GBA+ showed significant enrichment at P-value < 0.15 only for the GO terms "phagocytic vesicles membrane" (GO:0030670), "vesicle membrane" (GO: 0012506), "cytoplasmic vesicle membrane" (GO: 0030659) (**Figure 8, Supplementary Table 1)**. Moreover, no significant enrichment was identified also for the list of membrane trafficking genes from Bandres-Ciga et al., 2019 [235].

To further characterize the deregulated pathways, we performed a manually curated enrichment analysis of the up- and down-regulated genes as detailed above (Material and Methods section) (**Figure 9**). Interestingly we found an enrichment for almost all the selected pathways (endo-lysosomal and autophagy pathway, epigenetic, genetic of PD, immune response, mitochondria, calcium homeostasis) in both sets of genes, while cytoskeleton related genes were identified only within downregulated genes. Among the list of genes that belonged to each of these categories we identified genes related to some recurrent functions/pathways. In particular, we selected genes related to aging (*FUCA2* and *HEXB*), to the *LRRK2* gene (*RAB11B, WASF2, FILIP1L*), to the *ATP13A2* gene (*TSC2, VAC14*), to *NOTCH1* gene (*MIB1, EP300, RUNX3, EPN1, LFNG*, and *NOTCH1* itself), to TAU-processing (*RSRP1, IST1, MAP4, RSRP1, MARK2, EP300, DYRK1A*) (**Figure 9-10, Supplementary Figure 6**).

6.4.5 Targeted gene and network analysis

ATP13A2 and PD

The P5-ATPase ATP13A2 is a multi-transmembrane domain protein that is important in the lysosome and late endosome pathways [236]. Biallelic mutations of the *ATP13A2* genes cause the Kufor-Rakeb syndrome, a condition characterized by early onset parkinsonism, spasticity, dysarthria, myoclonus, supranuclear gaze palsy, cognitive impairment and hallucinations [237]. Heterozygous mutations of these genes have also been described in cases of PD, suggesting that this mutation may represent a risk factor for PD [238–243]. Interestingly, among the differentially expressed genes between the PD/GBA+ and CTRL/GBA+ groups *ATP13A2* was upregulated in the CTRL/GBA+ group compared to both the PD/GBA+ and also PD/GBA- groups ($p = 0.0098$ and $p = 0.0026$ respectively, FDR between PD/GBA+ and CTRL/GBA+ = 0.075 - Mann- Whitney U test) (**Figure 9-10, Supplementary Figure 6**). Across the differentially expressed genes with FDR < 0.05, a set of genes associated with *ATP13A2* were detected, such as *VAC14* and *TSC2*, suggesting a deregulation of the entire ATP13A2 related pathway. In particular, *VAC14* has been reported in cases of autosomal recessive PD and dystonia [244,245]. VAC14 is one of the components of the PIKfyve complex which produces the lipid phosphatidylinositol-3,5-bisphosphate (PI(3,5)P2) [246,247]. Interestingly, the interaction between PI(3,5)P2 and the N-terminal of ATP13A2 is important for the activation of the latter stimulating

protective mechanisms during mitochondrial stress and possibly alpha-synuclein toxicity [248]. This mechanism has been reported to be regulated by the Amyloid Precursor Protein (APP) as well [246]. The Tuberous Sclerosis Complex 2 (TSC2) is a regulator of mTORC1 and mediates the regulatory activity of ATP13A2 on the autophagy–lysosome pathway [249].

LRRK2 and GBA

A number of genes related to the activity of the *LRRK2* gene were found to be deregulated when comparing the expression profiles of the PD/GBA+ vs CTRL/GBA+ cohorts, such as *FILIP1L, RAB11B, WASF2, ATP2A2, as well as AP2A1* and *CHML*. *FILIP1L* and *LRRK2*, as well as *SNCA*, have been all reported to be all targeted by the miRNA-1224, suggesting a possible functional network between these proteins (**Figure 9-10, Supplementary Figure 6**) [250,251]. The protein encoded by the *AP2A1* gene is a subunit of the adaptor protein 2 (AP2) complex which modulates the recruitment of LRRK2 in the trans-Golgi network and its autophosphorylation activity, linking LRRK2 and the Clathrin-mediated endocytosis [252]. LRRK2 interacts with SERCA2/ATP2A2 to regulate calcium transport across the ER membrane through ER Ca^{2+} ATPase. PD-related mutations of *LRRK2* affect this pathway and increase Ca-overload mediated mitochondrial dysfunction [253]. LRRK2 phosphorylates and thus regulates the activity of WASF2 (or WAVE2) in actin remodeling during phagocytosis [254]. Vesicles trafficking, and in particular endocytosis, is modulated by LRRK2 also through the interaction with Rab11, Rab5 and VPS35 (another

gene responsible for a monogenic autosomal dominant form of PD) [255]. CHML participates in post-translational modification processes of geranylgeranylation, a form of prenylation, which regulate Rab proteins, which are also modulated by LRRK2 [256].

In our data *LRRK2* was upregulated in the PD/GBA+ group compared to the CTRL/GBA+ cohort. This is particularly relevant as we know that LRRK2 toxicity is mediated by an increased kinase activity of this protein. Increased expression levels of the gene may determine increased activity of the protein and thus a detrimental interaction between *GBA* mutations and the activity of LRRK2.

TAU-related genes and PD

Tau protein (encoded by the *MAPT* gene) has been implicated in a number of neurodegenerative diseases, such as Alzheimer's disease and certain types of atypical parkinsonism, such as the Progressive Supranuclear Palsy and Corticobasal Degeneration. The following genes, which are linked to Tau metabolism and activity, were significantly differentially expressed in our dataset: *IST1, RSRP1, MARK2, MAP4, EP300, DYRK1A* (**Figure 9**).

MARK2 is important for detaching TAU from microtubules through phosphorylation and it has already been associated with neurodegeneration [257]. *MARK2* is involved in mitochondrial homeostasis and also regulates *PINK1*, which is responsible for autosomal recessive forms of PD [258]. Tau aggregation, as observed in certain neurodegenerative processes, has been shown to reduce the expression of *IST1* which is a modulator of the Endosomal Sorting Complex Required for Transport (ESCRT) complex, affecting autophagosome-lysosome fusion [259]. DYRK1A

is involved in Tau phosphorylation [260]. Interestingly, it was also reported that DYRK1A can affect dopaminergic neurons survival in animal models [261].

NOTCH1 and PD

Other than *NOTCH1* itself, a number of genes related to the function of this gene were significantly deregulated between PD/GBA+ and CTRL/GBA+ subjects. Those were represented by *MIB1, EPN1, LFNG, EP300, RUNX3* (**Figure 9-10, Supplementary Figure 6**). These genes are involved in different compartments of the NOTCH-related pathways. Notably, *LFNG* is also the top differentially expressed gene (downregulated) in this comparison.

NOTCH is a highly conserved transmembrane domain protein that is involved in different cellular processes (such as cell proliferation, differentiation and apoptotic processes) [262]. Of note, membrane trafficking is significantly affecting NOTCH1 metabolism and availability. On the other side, NOTCH1 is cleaved by gamma-secretase, similarly to APP [263]. NOTCH1 is also involved in the modulation of the secretome dynamics in the cells. Secretomes consist of a set of proteins (such as cytokines, signal proteins, and growth factors, among the others) that can be secreted by the cells and modulate cellular interactions. Their dynamics are also affected by vesicle trafficking in the cells. During senescence, NOTCH1 can mediate a switch from the senescence-associated pro-inflammatory secretome to a TGF-beta rich secretome [264]. Among genes differentially expressed between GBA+ PD and CTRL subjects, all the genes involved in the endosomal sorting complexes required for the transport (ESCRT) machinery (i.e. *TSG101, ALG-2* interacting

protein, *Alix-PDCD6IP, SCAMP4*) were all down regulated although not all at a significant level.
Moreover, genes involved in the senescence processed (such as *FUCA2* and *HEXB*) are upregulated in PD/GBA+ subjects compared to the CTRL/GBA+ group, as observed during senescence processes (**Figure 9-10, Supplementary Figure 6**) [234].

6.4.6 Differential expression analysis: PD/GBA+ vs PD/GBA-

The number of significantly differentially expressed genes between the subjects with PD with and without mutation of the *GBA* gene was more limited compared to the previous comparison (PD/GBA+ vs CTRL/GBA+) (**Figure 5B and Figure 11**). When considering the nested interaction model analysis, we were able to identify 5 genes at FDR < 0.05 (1 upregulated and 4 downregulated) and 44 genes at FDR < 0.15. The pairwise comparison upon limma/voom normalization showed instead 11 genes at FDR < 15% (9 upregulated and 2 downregulated), of which 8 were overlapping with the genes identified by the nested interaction model analysis at FDR < 0.15.
Given the reduced size of this list of gene differential expressed between these two groups we were not able to perform a pathway enrichment analysis. However, considering the list of significantly deregulated gene we were able to detect interesting findings. In fact, alpha-synuclein gene (*SNCA*) was upregulated in the PD/GBA+ group compared to both the PD/GBA- subjects (p = 3.7 e-5) as well as compared to the CTRL/GBA- subjects (p = 6.4 e-5) and to the CTRL/GBA+ subjects although to a less extent (p = 0.028) (Mann-

Whitney U test) (**Figure 11C**, **Supplementary Figure 7**). Interestingly, two additional genes among the ones significantly differentially expressed, *POLR2D* and *NFATC3*, were also related to SNCA processing and metabolism (**Figure 11D**).

Among the other genes, two were related to amyloid regulation (*ITM2B* and *NCSTN* genes), maybe suggesting an involvement of the aging processes. Interestingly, the most deregulated gene between PD/GBA+ vs PD/GBA- subjects was *LMNA* which is responsible for the progeria syndrome, and thus related to aging as well [265].

Few other genes were instead associated with other known PD causative genes, such as *LMNA* itself (which interact with *LRRK2* on nuclear envelope integrity), *APEX1* (which is degraded by *Parkin* gene) or *MRPL4* (reported to be a rare variant associated with PD) (**Figure 11**) [266–268].

Finally, we found a deregulation of *LAMTOR2*, which is an amino acid sensing and activator of mTORC1 by recruiting it to the lysosome where it is activated (**Figure 11**).

Taken together, these observations suggest a deregulation of pathways associated with alpha-synuclein, aging and PD-related genetics in the PD/GBA+ vs PD/GBA- group.

6.4.7 Differential expression analysis: interaction term

When considering the genes that were differentially expressed for the interaction term in the nested interaction model analysis, there were no significant hits at FDR < 0.05, but 6 DE genes at FDR < 0.15 (**Figure 12**). This analysis highlights genes that have a different

directionality of expression in the PD/GBA+ group compared to both the PD/GBA- and the CTRL/GBA+ groups, thus that are specifically impaired in the PD/GBA+ subjects. This list encompassed the following genes: *ANGPT1* (angiopoietin gene involved in vascular development), *FILIP1L* (which is also one of the genes differentially expressed between the PD/GBA+ and CTRL/GBA+ groups and it may be coregulated with *LRRK2* and *SNCA* by miRNA-1224), *AC138028.2* (novel transcript, antisense to *PIEZO1* gene encoding for a mechanosensitive ion channel of which certain variants may attenuate the Plasmodium Falciparu’s infection), *SRGAP1* (Slit-Robo GTPase-activating protein 1, involved in neuronal migration), *PIK3R5* (Phosphoinositide 3-kinase regulatory subunit 5, responsible for Ataxia with Oculomotor-Apraxia type 3), *HPS3* (Hermansky-Pudlak Syndrome 3 Protein, biogenesis of lysosomal organelle complex 2 subunit 1) (**Supplementary Figure 8**). This last gene, *HPS3*, is particularly interesting in this context. This is a gene implicated in the biogenesis of lysosome-related organelles complex-2 (BLOC-2). Biallelic mutations of this gene are responsible for the Hermansky-Pudlak syndrome type 3. This is a systemic disorder characterized by oculocutaneous albinism and platelet storage deficiency [269]. This condition, other than being related to the lysosomal pathway, is also similar to the Chediak-Higashi syndrome caused by mutations of the lysosomal Trafficking Regulator (*LYST*) gene and that has been already reported to be associated with a levodopa-responsive parkinsonism with degeneration of the dopaminergic neurons of the substantia nigra [270]. So far, an increased incidence of

parkinsonism has not been reported in patients with Hermansky-Pudlak syndrome type 3 but this would be worth further exploring.

Because of the small number of genes identified by this analysis, we were not able to perform pathway enrichment analysis for this set of hits.

6.5 Genetic outliers

6.5.1 Normalization and total number of outliers

Count data of our four cohorts (PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA-) were processed according to the OUTRIDER pipeline as detailed above (**Supplementary Figure 9**) [232]. A total of 13711 genes in 158 total subjects were considered. This lead to the identification of 493 outlier genes distributed across the cohorts as follows: 41 genes in the PD/GBA+ group, 208 genes in the PD/GBA- group, 44 genes in the CTRL/GBA+ group, 200 genes in the CTRL/GBA- group. This corresponded to 125 subjects with at least one outlier gene (19 PD/GBA+ subjects, 45 PD/GBA- subjects, 8 CTRL/GBA+ subjects, 53 CTRL/GBA- subjects) (**Supplementary Figure 9**).

6.5.2 Characterization of outliers

No significant enrichment for the number of outlier genes or outlier subjects was detected across our four cohorts after Fisher exact test (% outlier genes per group P-value = 0.479, % outlier subjects per group P-value = 0.456), as well as there was no difference in

proportion of significantly up or down regulated across our cohorts (p = 0.06).

Enrichment for lysosomal and mitochondrial genes was also assessed. For the lysosomal genes, the list of genes in the GO term "lysosome" was considered (667 total genes). Although there was an increased percentage of lysosomal genes in the PD groups and especially in the GBA+ group (7% in PD/GBA+, 6% in the PD/GBA-, 4% in the CTRL/GBA-, and 2% in the CTRL/GBA- groups) this was not significant with Fisher exact test (p = 0.6808). Significantly enriched lysosomal genes encompassed *HEXA, VPS41* and *AP2B1* in the PD/GBA+ group; *HEXA, VAMP7, GLA, AP2S1, SPPL2A, PLBD2, PLBD1, UBA52, CLEC16A, BLOC1S1, CXCR2* in the PD/GBA- group; *ATP6V0C* in the CTRL/GBA+ group; *HEXB, GLA, NAGLU, BST2, SDC3, HPSE, CHID1, RAB39A, LITAF* in the CTRL/GBA- group (**Figure 13A**). Of note, the PD/GBA- subjects where *HEXA* was identified as an outlier is a known carrier of a monoallelic mutation in this gene, with no personal or family history of Tay-Sachs disease.

For the mitochondrial genes, a total of 1311 genes were considered from a curated gene list as detailed above. This analysis showed an increased number of outliers in the mitochondrial genes in the CTRL/GBA+ group (16% CTRL/GBA+, 8% CTRL/GBA-, 8% PD/GBA+, 12% PD/GBA- groups). Fisher's exact test showed p = 0.4461. Outlier genes in each group are reported in **Figure 13A**. Significantly enriched mitochondrial genes were: *MT-ND4, MT-ND2, MT-ND1, MT-ATP6, MRPS23, ECH1, AIP* in the CTRL/GBA+ group; *UQCRC2, TOP1MT, TMLHE, TIMM29, SORD, SDHC, RARS2,*

NDUFA5, MRPL12, MPG, ME2, LARS2, HIGD2A, FIS1, DLST, COX8A, ACOT9 genes in the CTRL/GBA- cohort; *SLC25A24, NUDT13, MRPL23, GATD3B* in the PD/GBA+ group; *UQCRHL, UBE2L3, UBA52, TRMU, TIMM21, SLC25A6, SCO2, PDHX, PCCB, NDUFS6, NDUFA, MRPL53, MRPL49, MRPL23, MCCC1, IMMT, GTPBP3, GPT2, FAM161A, BLOC1S1, ATAD3B, AIFM3* in the PD/GBA- group.

6.5.3 Pathway enrichment of genetic outliers

Pathway enrichment analysis was performed considering the list of outlier genes in the PD/GBA+ and the CTRL/GBA+ group, using GSEA, g-profiler, and IPA tools.

In the PD/GBA+ group, significant enrichment was detected with g-profiler and IPA for pathways related to the following: Neuroinflammation_signaling_pathway, Membrane_bounded_organelle, ERK/MAPK_signalling, Authophagy (**Figure 13B**). The genes that were greatly enriched in the listed pathways were: *ICAM1* (encoding for a leukocyte binding protein), *NFATC1* (transcription factor involved in immune response and cytokine production), *IRAK4* (signaling of innate immune response), *DOCK1* (encoding the Dedicator of cytokinesis protein 1 which is involved in cytoskeletal rearrangement for phagocytosis of apoptotic cells and mobility of the cells), and *VPS41* (involved in vesicular trafficking).

In the CTRL/GBA+ group pathway enrichment analysis identified the following pathways all related to mitochondrial functions: Oxidative_phosporilation,

Inner_mitochondrial_membrane_protein_complex, Thermogenesis, NADH_dehydrogenase_complex, Mitochondrial_respiratory_chain_complex_I. The major driver for pathway enrichment in this group were represented by the following genes: *MT-ATP* (component of the ATP synthase or mitochondrial complex 5, responsible for the final step of oxidative phosphorylation), *MT-ND, MT-ND2, MT-ND4* (all components of the NADH dehydrogenase or mitochondrial complex I) (**Figure 13B**). Two important observations can be extracted from this last set of data. First, those are all genes encoded by the mitochondrial DNA. Second, these outlier genes were identified in one subject who is the discordant monozygotic twin of another subject enrolled in our study affected with PD (**Figure 13C**). They are both carriers of the N370S *GBA* mutation.

When comparing hits from the OUTRIDER analysis and DE between PD/GBA+ and CTRL/GBA- we found 9 genes overlapping the DE data in these two cohort an in the outliers genes of the CTRL/GBA+ group (*ARF5* – involved in vesicular trafficking and activators of phospholipase D-, *ATP6V1F* – vacuolar *ATPase* (V-ATPase), *BTBD2, CAMTA2, SKIL, SNRPC, SPI1, ZNF180, HPS3* – described above as also found in the interaction term comparison) and one gene in common with the outliers of the PD/GBA+ group (*NFATC1*) (**Supplementary Figure 10**).

6.6 Tras-eQTL

Considering SNPs within 1 Mb of the *GBA* transcription starting site and manually adding the N370S SNP, we selected a total of 2494 SNPs (**Figure 14**).

After filtering of SNPs in linkage disequilibrium, based on a threshold of MAF = 0.1 and r2 = 0.5 as previously reported [271], a total of 265 SNPs within our targeted region were selected. These corresponded to 43138 target SNPs-gene pairs across the genome targeting 15055 unique genes.

After filtering for FDR < 0.15 we were able to select three final targeted tran-eQTLs: sortilin (*SORT1*, corresponding to the *GBA* SNP chr1:154489884), synergin gamma (*SYNRG*, corresponding to the *GBA* SNP chr1:154245167), Chondroitin Sufate Synthase 1 (*CHSY1*, corresponding to the *GBA* SNP chr1:154818004) (**Figure 14**).

6.6.1 Significant eQTLs and enrichment in our cohort

Interestingly, the three significant trans-eQTLs were all downregulated in PD/GBA+ vs CTRL/GBA+ cohorts as follow: for *SORT1* logFC (log fold change) = - 0.16 and FDR = 0.32; for *SYNRG* logFC = -0.06 and FDR = 0.66; for *CHSY1* logFC = -0.18 and FDR = 0.22 (**Figure 14**).

Functionally, these three eQTLs are all components of relevant pathways for PD/GBA pathological mechanisms. SORT1 acts as a receptor for different proteins to be transported across the Golgi apparatus to the lysosomes, including granulin which is encoded by the progranulin gene and is involved in a number of neurodegenerative conditions [272]. SYNRG is implicated in

endocytosis processes as well as membrane trafficking at the trans-Golgi network (TGN) [273]. Finally, CHSY1 has a beta-1,3-glucuronic acid and beta-1,4-N-acetylgalactosamine transferase activity and it has been implicated in the modulation of NOTCH signaling [274].

Enrichment analysis through Fisher exact test, however, didn’t show an enrichment of specific genotypes of the three trans-eQTLs either within PD vs CTRL subjects or in carriers vs non-carriers of *GBA* mutations.

7. Discussion

The involvement of the immune system in the pathogenesis of Parkinson's disease, encompassing the innate immune system in the periphery and in the brain - through he activation of the microglia -, and the adaptive immune system, has attracted growing attention over the last few years [190–192,194–199,201–203]. Genetics as well suggest a role of the immune response in the pathogenic mechanisms of PD, as showed by GWAS hits and by the enrichment of PD-related variants in the innate immune compartment [50,206]. Of note, GCase, the enzyme encoded by *GBA*, one of the most common genetic risk factors for PD, is as well particularly important for the metabolic processes of scavenger cells such as monocytes and macrophages. Interestingly, as we explored the clinical profiles of our cohorts of PD and CTRL subjects with and without mutations of the *GBA* gene, other than confirming specific phenotypic traits of these cohorts previous reported in the literature, we also found that the percentage of subjects on aspirin, an anti-inflammatory drug, was significantly less in subjects with PD without *GBA* mutations while among CTRL it was higher in *GBA*-mutation carriers, compared to subjects PD/GBA+ and CTRL/GBA-, respectively (**Figure 1**) [104]. Consistently with our results, a recent study showed as well a possible correlation between the use of anti-inflammatory drugs and the modulation of penetrance of mutations of the *LRRK2* gene, the other common genetic risk factor for PD, possibly further suggesting a role of inflammatory processes in PD-related mechanisms [275].

Starting from these observations, we decided to explore the effects of *GBA* mutations in monocytes from affected and non-affected

subjects. We analyzed the expression and genetic profiles of these cells leveraging different computational approaches and we were able to detect important novel data that can contribute to the understanding of the role of the mutation of *GBA* in the architecture of the pathogenesis of PD.

First, we observed that the expression profiles of patients with PD and *GBA* mutations were significantly different from the ones of PD patients without mutations of this gene as well as from non-manifesting carriers. The set of differentially expressed genes between the PD/GBA+ and CTRL/GBA+ cohorts showed only a partial overlap between the genes differentially expressed in PD vs CTRL subjects without GBA mutations, suggesting that specific pathogenic pathways are altered in the presence of this genetic mutation **(Figure 6**). Importantly, we didn't notice any difference between CTRL subjects with and without mutations of the *GBA* gene (**Figure 6**). This may support the hypothesis that *GBA* mutations by themselves are not enough to produce pathology, while additional factors are required to trigger disease mechanisms in these subjects. On the contrary, despite the differences in the expression profiles among our four cohorts, we didn't observe significant changes of the expression levels of the *GBA* gene itself (**Figure 4A**). This will be interesting to further explore especially in light of the fact that inconsistent results have been previously reported regarding the *GBA* expression levels in the few studies exploring target brain tissues (particularly the substantia nigra) of patients with PD, while reduced levels of GCase activity have been showed in monocytes

from patients with PD with and without *GBA* mutations compared to controls [142,143].

A closer look at the specific pathways and deregulated genes in manifesting carriers compared to the other cohorts showed an overlap with a number of molecular mechanisms previously associated with PD pathogenesis in other cellular or animal models, such as the endo-lysosomal pathway, the mitochondrial pathway, inflammation as well as a set of genes related to rare genetic forms of PD or previously reported in large PD GWAS studies (such as *BST1* and *SNCA*) [49–51,276]. This means that monocytes mirror a number of processes reported in better characterized cellular and animal models of this disease as well as dopaminergic neurons. Whether this suggests an active role of the innate immune compartment in causing the disease or represents just a response to the disease status cannot be inferred at the moment form our data, but it would be worth further exploring. Nevertheless, monocytes may represent a good platform to recapitulate and study PD-associated pathogenic mechanisms. Even more so, despite the small number of subjects in our cohorts – which, at the best of our knowledge, still represents the largest transcriptomic profiles analysis in patient with PD carrying mutations of the *GBA* gene - we were able to identify a large number of differentially expressed genes. This suggests the importance of considering homogenous cells types, such as isolated CD14+ monocytes, to dramatically reduce the variability due to background noise signals, as it can be the case by using whole blood.

Interestingly, we observed that monocytes of PD/GBA+ subjects compared to non-manifesting carriers showed a clear impairment of pathways related to membrane and vesicle metabolisms and sorting (**Figure 7**). This was confirmed not only by RNAseq analyses, but also by the assessment of outlier genes (that highlighted genes like *DOCK1* and *VPS41* in the PD/GBA+ cohort) and through the analysis of *GBA* trans-eQTL (which identified genes like *SORT1* and *SYNRG*) (**Figure 13 and 14).** Given the prominent impairment of this biological mechanism in these cells, it would be worth validating whether an early detection of the impairment of the vesicle trafficking and/or exocytosis in carriers of *GBA* mutations can be leveraged as possible biomarker for this disease. A pilot analysis for the study of the ultrastructural changes of isolated monocytes from our four cohorts, with a particular focus on vesicles and the endoplasmic compartment, is currently ongoing aiming to identify detectable characteristic changes related to the GBA- and disease-status.

Consistent with the findings from our unbiased pathway enrichment analysis that showed an impairment in the vesicle/membrane trafficking and the endo-lysosomal pathways, we indeed were able to identify a deregulation of known sets of genes related to these mechanisms. *ATP13A2* is implicated in the transport of late endosomes, and it has been already described in clinical conditions associated with parkinsonism, such as the Kufor-Rakeb syndrome [277]. Interestingly, we recently described a case of a patient with early onset PD who carried a pathogenic mutation in the *GBA* (p.Asn370Ser) and *ATP13A2* (c.3057delC, p.Tyr1020Thrfs*3) genes (Riboldi et al., *submitted*). This was a 28 year-old woman who

developed PD at the age of 23 year first manifesting with left hand tremor, associated with rigidity and bradykinesia on the same side. Dat-Scan confirmed a reduced uptake in the posterior and mid-putamen, with no alteration of the brain MRI. She was of AJ descent and had no family history of PD or other neurological conditions. We suspected that the concomitant mutation in these two genes both affecting the endo-lysosomal pathway may have been responsible for the early onset disease in this patient, supporting the hypothesis that second hits and additional genetic variants can affect the penetrance of *GBA* mutations. Targeting ATP13A2 dysfunction to modulate disease pathology in *GBA* carriers may represent an alternative or complementary strategy to tackle the disease in *GBA* carriers. Interestingly, it has been reported that small-molecules acting on GCase activity can reverse *ATP13A2* related alpha-synuclein pathology in cellular models even in the absence of mutations of the *GBA* gene, supporting a tight interplay between these two proteins [278]. Further validations in neuronal tissues, particularly in dopaminergic neurons and microglia, will be important to further support our observations in circulating monocytes.

In the context of the impairment of the endolysosomal-pathway, *LRRK2* and a number of related genes (i.e. *RAB11B*, *WASF2*, *ATP1A2*) have been found to be significantly deregulated as well (**Figure 9 and 10**). This is particularly interesting as *LRRK2* mutations represent the other common genetic risk factor for the development of PD [279]. In our cohort of PD/GBA+ patients we found increased expression levels of the *LRRK2* gene (**Figure 10).** Consistently with this observation, the majority of the mutations of

LRRK2 responsible for PD are associates with increased kinase activity [280]. Previous works in the literature supported an interaction between GCase and LRRK2 and between their genes [186–188]. The validation of these results may open the way of considering using the LRRK2-related therapeutic strategies currently tested in ongoing clinical trials also in the large portion of subjects with PD and *GBA* mutations, as suggested above for the *ATP13A2* gene. Thus, a growing understanding about the mechanisms concurring to *GBA*-related PD pathogenesis can really contribute to fine-tune therapeutic interventions.
The central role of the impairment of the endo-lysosomal pathways and membrane-trafficking in the pathogenesis of PD can also explain while this condition usually presents in adult age, except for very rare cases of aggressive genetic forms [1]. These pathways are very important in neurons, particularly in the dopaminergic cells, as well as other cellular types of the brain, and they usually undergo a normal process of senescence. Indeed, they can become less efficient as age progresses, manifesting, for example, with the accumulation in the brain of lipofuscin, which is indeed considered a marker of senescence. Genetic mutations of proteins involved in these pathways can accelerate aging of these systems. Interestingly, we identified a significant deregulation of *NOTCH1* and of a number of related genes, such as *RUNX3*, *EP300*, *EPN1*, *LFNG*, *MIB1* [262]. NOTCH1 is involved in a number of different molecular pathways in the cells and its turnover and metabolism is very much dependent on vesicle trafficking [281]. At the same time, *NOTCH1* can regulate compartments of the vesicular trafficking related to aging processes

in the cells, such as by modulating the switch between the inflammatory versus the aging secretomes, which can accelerate an aging response in the cells and thus their degeneration [264]. In our analysis, aging-related pathways and genes were deregulated between subjects with PD with and without mutations of the gene *GBA* (**Figure 11**). This is the case for example of the *LMNA* gene, which is responsible for the progeria syndrome, a conditions associated with accelerated aging, as well as a number of genes associate with the beta-amyloid and its metabolisms, such as *ITM2B* and *NCSTN* [265]. Whether these mechanisms are also deregulated in the dopaminergic neurons, the microglia or other brain cells in subject with *GBA*-related PD would be worth exploring in future analysis. The question remains whether an accelerated aging processes in PD can be further accelerated in carriers of *GBA* mutations, or whether the pathogenic variants of this gene are instead responsible in the first place for the activation of pathways that can cause accelerating aging.

Interestingly, we also reported that monocytes of subjects with PD and *GBA* mutations showed an increased expression of the *SNCA* gene, the hallmark protein of PD (**Figure 11 and Supplementary Figure 7**). We know that in brains of subjects with *GBA*-related PD there is a robust deposition of this protein, which could explain the more aggressive phenotype in carriers of these mutations compared to idiopathic PD in terms of earlier age of onset and increased frequency of cognitive impairment and non-motor symptoms [104]. It can be speculated that increased expression levels of *SNCA* can represent a compensatory mechanism to its aberrant accumulation

also aggravated by decreased GCase activity, as previously described [149–152]. However, *SNCA* upregulation could also represent a triggered mechanism in predisposed subjects, such as carriers of mutations affecting specific cellular pathways, like *GBA*.

Finally, we identified a deregulation of mitochondrial-related genes. In our previous work assessing expression profiles of circulating monocytes and microglia in idiopathic PD patients, we identified an opposite deregulation of the mitochondrial signature in the immune cells in the periphery compared to the central nervous system in PD patients [211]. Impaired mitochondrial genes were also detected as outliers among CTRL/GBA+ subjects, suggesting and involvement and a possible modulatory effect of these genes in the *GBA*-related pathogenesis (**Figure 13**).

To conclude, our results showed that cells of the innate immune system can be informative in the assessment of disease mechanisms associated with PD. We identified a set of genes and molecular pathways that are specific for *GBA*-related PD and that will be worth exploring and further validate for the identification of new disease biomarkers and innovative therapeutic targets.

8. Conclusions

With the present work we clinically characterized a population of patients with Parkinson's disease and control subjects with and without mutations of the *GBA* gene. This analysis confirmed the increased incidence of several clinical features in the PD/GBA+ cohort compared to subjects with idiopathic PD, such as cognitive impairment, anxiety, a more frequent family history, and an earlier age of onset, as previously reported in the literature [104]. Non-manifesting *GBA* carriers also presented an increased incidence of some of the non-motor symptoms associated with PD, such as depression, anxiety, RBD. As expected, a family history of PD was more common among GBA carriers, compared to non-carriers, interestingly mostly on the maternal side, worth to be further explored to assess whether this is confirmed in other cohorts.

Integrated genomic analysis consisting in expression profiles analysis and genotyping of isolated monocytes elucidated some important deregulated pathways and gene sets across the different cohorts. In particular, we found that PD/GBA+ subjects presented a deregulation of exocytosis and membrane trafficking pathways, of the immune response, as well as RNA processing. In particular, pathways related to LRRK2, ATP13A2, TAU and NOTCH1 were more significantly affected. The deregulation of the membrane processing and endo-vesicular pathways in the PD/GBA+ was also detected by the study of outliers genes within single subjects. In the future, it will be worth exploring possible polymorphisms or genetic mutations in these genes that may represent disease modifiers of the

penetrance of the *GBA* gene in PD. *trans*-eQTLS of the *GBA* gene identified three significant deregulated genes related to the membrane trafficking and trans-Golgi network as well as to the NOTCH1 pathway. In the future, it will be interesting to further explore these genetic targets with additional genomic assessments, such as epigenetic changes and chromatin access profiles. Validation of top candidate genes and pathways is currently ongoing in an independent cohort. Moreover, we are currently exploring the expression and deregulation of top candidate genes also in brain tissues of subjects with PD and *GBA* mutations. Additionally, the identification of a deregulation of exocytosis and membrane trafficking suggest a possible role for extracellular vesicles as possible biomarkers for this condition.

Finally, by comparing subject with PD with and without mutations of the *GBA* gene we instead identified a deregulation of pathways related to alpha-synuclein, amyloid metabolism and aging. It will be worth exploring whether *GBA* mutations can be responsible for accelerated aging processes in the cells or whether other factors causing accelerated aging are responsible for disease onset in predisposed subjects, such as the carriers of *GBA* mutations.

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10. Iconography

10.1 Figures

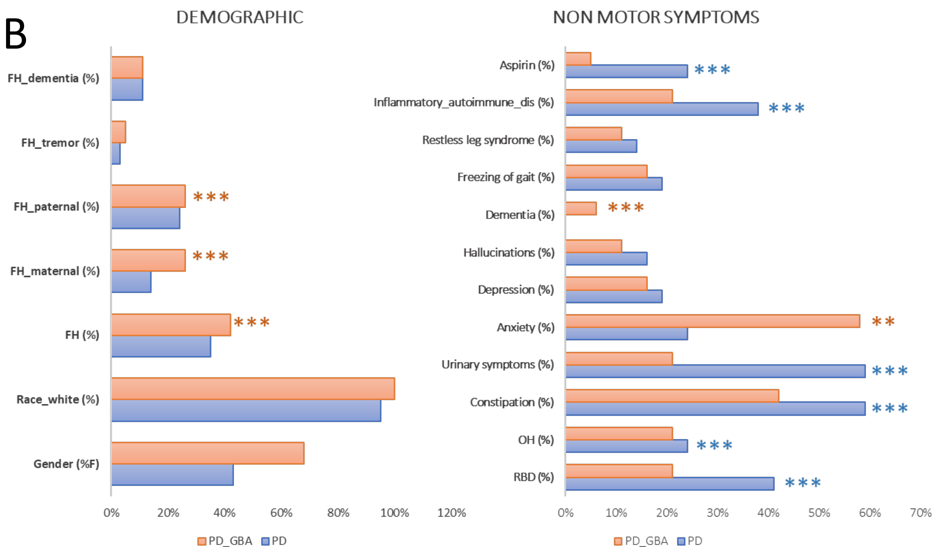
A

PD (37) vs PD/GBA+ (19)

	PD	PD_GBA	p.Value
Age (mean (sd))	69.8 (9.49)	59.8 (10.6)	** 0.000361[a]
Age_onset (mean (sd))	61.8 (14.3)	53.9 (11.6)	* 0.04181[a]
UPDRS (mean (sd))	28.3 (14.5)	25.4 (14.6)	0.61006[c]
Updrs_3 (mean (sd))	16.0 (10.1)	14.8 (9.25)	0.61006[c]
HY (mean (sd))	1.86 (0.585)	1.53 (0.612)	0.61006[c]
LEDD (mean (sd))	445 (277)	258 (188)	0.61006[c]
UPSIT (mean (sd))	20.0 (8.06)	20.1 (7.23)	0.485618[a]
MOCA (mean (sd))	26.8 (2.34)	27.1 (2.85)	*0.354174[a]*

[a] t-test
[b] z-score of proportion
[c] Mann-Whitney U test

* < 0.05
** < 0.005
*** < 0.00001



C

CTRL (37) vs CTRL/GBA+ (9)

	CTRL	CTRL_GBA	p.Value
Age (mean (sd))	56.2 (16.2)	56.3 (10.8)	*0.490005*[a]
Age_onset (mean (sd))	–	–	–
UPDRS (mean (sd))	3.84 (4.60)	4.89 (3.62)	*0.14007*[c]
Updrs_3 (mean (sd))	1.86 (2.16)	3 (3.43)	*0.14007*[c]
HY (mean (sd))	0	0.111 (0.333)	*0.30153*[c]
LEDD (mean (sd))	–	–	–
UPSIT (mean (sd))	33.3 (5.11)	30.9 (6.94)	*0.17361*[a]
MOCA (mean (sd))	27.7 (2.21)	27.7 (2.21)	*0.246786*[a]

[a] t-test
[b] z-score of proportion
[c] Mann-Whitney U test

*	< 0.5
**	< 0.05
***	< 0.00001

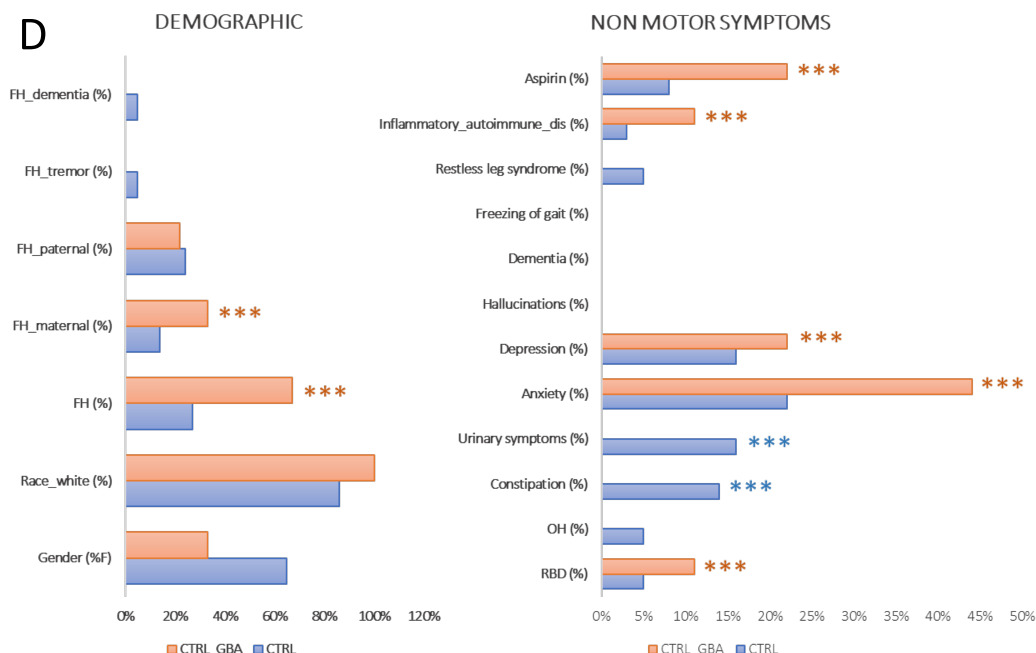


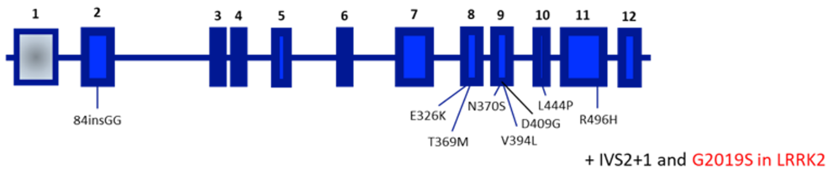
Figure 1 – Characterization of the clinical cohort of subjects (PD/GBA+ (19 subjects), PD/GBA- (37 subjects), CTRL/GBA+ (9 subjects), CTRL/GBA- (37 subjects)).

Statistical comparison of demographic and motor and non-motor scores between subjects PD/GBA+ and PD/GBA- (A), and subjects CTRL/GBA+ and CTRL/GBA- (C). For each continuous variable (table A and C) mean and standard deviations are reported. Bar plots (B and D) report percent values or binary data for the two comparisons (PD/GBA+ vs PD/GBA- and CTRL/GBA+ vs CTRL/GBA-).
Statistical analysis were conducted using t-test ([a]), Z-score of proportion ([b]), and Mann-Whitney U test ([c]), as indicated by corresponding apex reported next to each result. Asterixis were used to indicate the degree of significance (* < 0.05, ** < 0.005, *** < 0.00001).

A

	CTRL	PD	MSA	LBD	Atypical Tau*	AD	MCI	PCA	Other**	UNKN	Total
tot	235	247	5	2	16	52	52	1	10	95	715
GBA	15	41	0	0	0	4	2	1	0	4	67
GD	2	2	0	0	0	0	0	0	0	0	4
LRRK2	2	14	1	0	0	0	1	0	0	0	18
GBA/LRRK2	0	2	0	0	0	0	0	0	0	0	2
%GBA	7.23	17.41	0	0	0	7.69	3.85	100	0	4.21	9.93
%LRRK2	0.85	5.67	20	0	0	0	1.92	0	0	0	2.52

*ATYPICAL TAU = CBD, PSP
**OTHER = MYOCLONUS, DYSTONIA, HD, ATAXA, TREMOR



B

+ IVS2+1 and G2019S in LRRK2

MAF	84GG	IVS2+1	E326K	T369M	N370S	V394L	D409G	L444P	A456P	RecNcil	R496H
CTRL	0.0042	0	0.0084	0	0.0252	0	0	0	0	0.0042	0
PD	0.0027	0.0027	0.0082	0.0054	0.0543	0	0	0	0	0.0027	0.0027

Figure 2. Genetic screening of a large population of subjects with PD, other neurodegenerative conditions and CTRLs.
A total of 715 subjects were sequenced for the G2019S variant of the LRRK2 gene and for the following variants of the *GBA* gene: IVS2+1, 84GG, E326K, T369M, N370S, V394L, D409G, L444P, A456P, R496H, RecNcil (as reported in the figure below the table). Proportion of carriers for *GBA* and/or *LRRK2* variants is reported in (A). In (B), MAF for each variant across PD and CTRL subjects in the two cohorts (the one enrolled at NYU Langone Health, Fresco Institute, and the one enrolled at Mount Sinai school of Medicine) are reported.

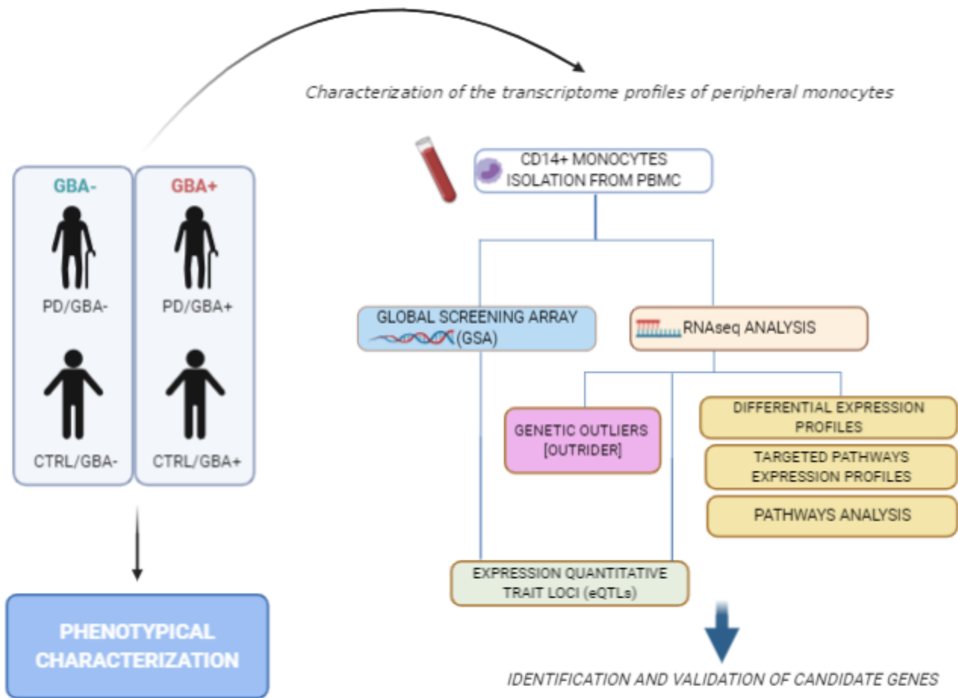
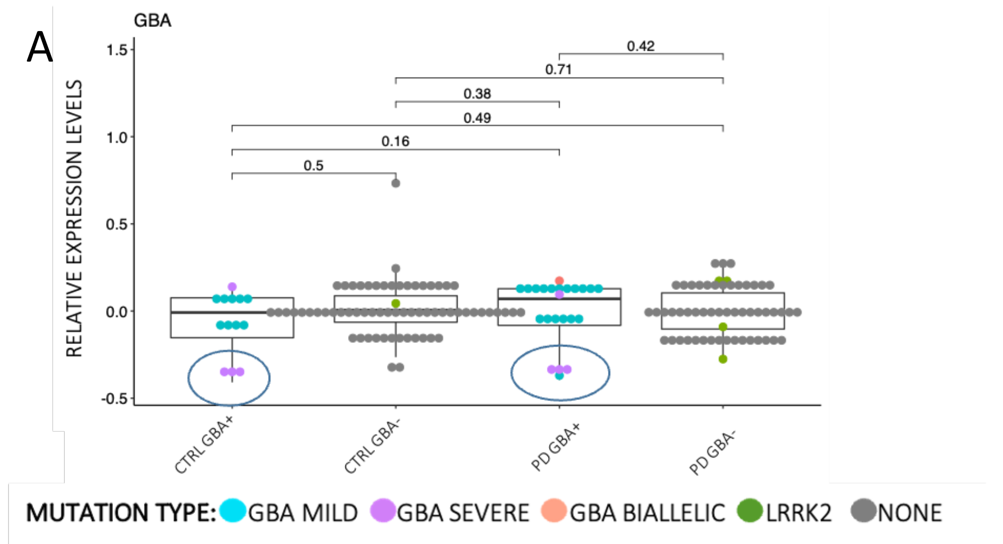


Figure 3. Project design schematic representation.
Schematic representation of project design for the collection and analysis of clinical data and biological samples. On the right: pipeline for the integrated genomic analysis.



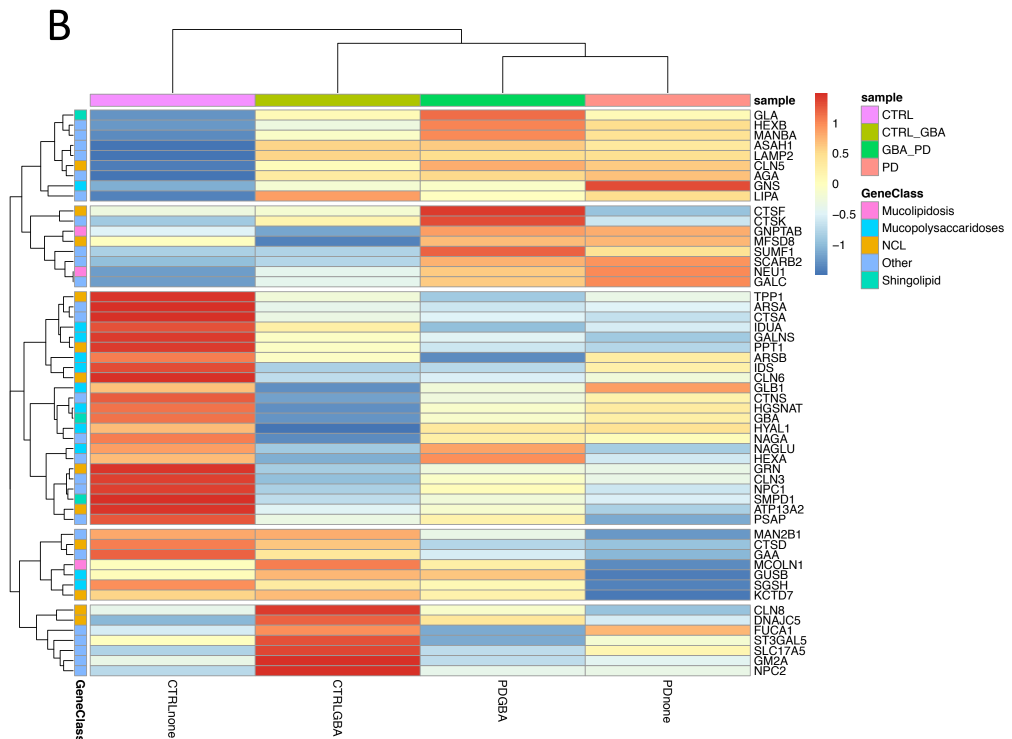


Figure 4. Targeted analysis of isolated CD14+ monocytes for the *GBA* gene and Lysosomal Storage disorders genes.

A) Levels of expression of the *GBA* gene across four cohorts (PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA-) are reported. Mann-Whitney U-test

was utilized to calculate statistical difference of the levels of expression across the four groups. Each dots represent one subject. Dots are colored based on *GBA* and *LRRK2* (G2019S) mutations (as reported in the legend below the boxplot: *GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). Subjects with lower levels of expression of *GBA* and with more severe *GBA* mutations are highlighted (circled) in the PD/GBA+ and CTRL/GBA+ groups.
B) TMM-normalization followed by voom transformation of expression count of the genes associated with Lysosomal Storage Disorders (LSD) across the four cohorts of subjects (PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA-, columns). Rows are color-coded according to different subgroups of LSD, as reported in the legend (Gene Class: Mucolipidosis, Mucopolysaccharidosis, Neuronal Ceroid lipofuscinosis (NCL), Sphingolipidoses, Others).

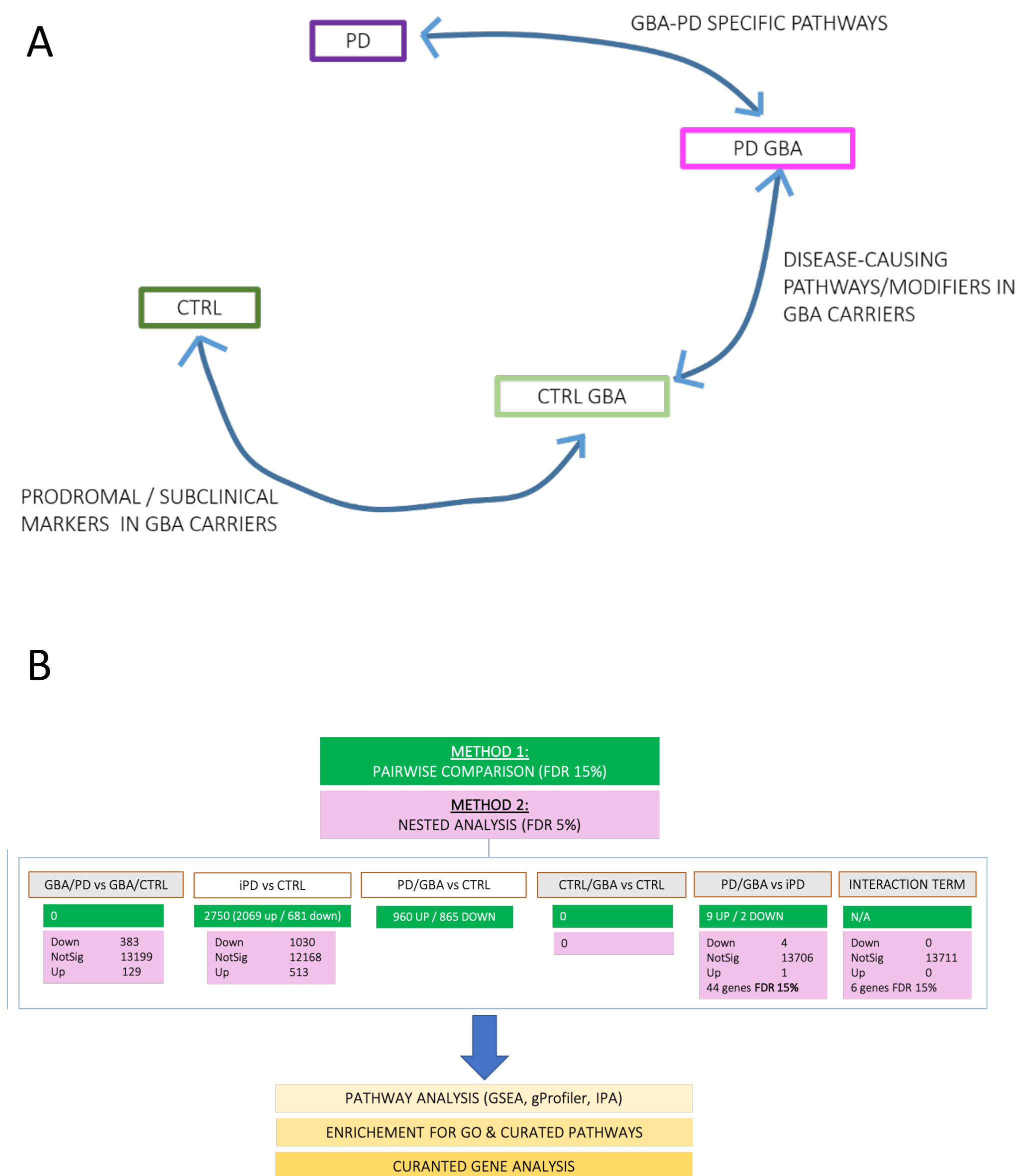


Figure 5. Differential expression analysis of transcriptomic data from purified CD14+ monocytes.
A) Schematic representation of group comparison and expected outcomes.
B) Schematic representation of the pipeline utilized for gene expression analysis (Method 1: pairwise comparison upon limma/voom normalization

methods, considering FDR < 0.15 for significance; Method 2: nested interaction analysis, considering FDR < 0.05 for significance). Numbers of significantly up- and down-regulated genes are reported in the box below each comparison (green box: results from analyses with Method 1, pink box: results from analyses with Method 2). Differentially expressed genes were then analyzed to study pathway enrichment analysis with the listed tools (GSEA, g-profiles, IPA), as well as for enrichment of curated pathway analysis and curated gene analysis (as reported in the yellow boxes).

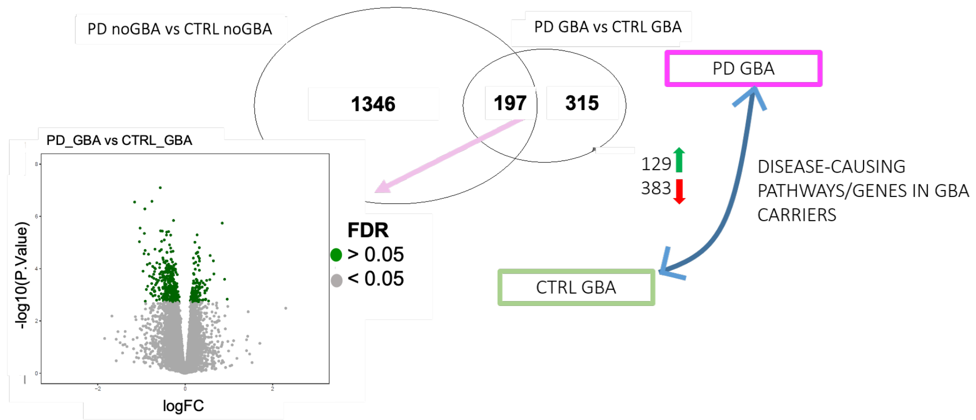
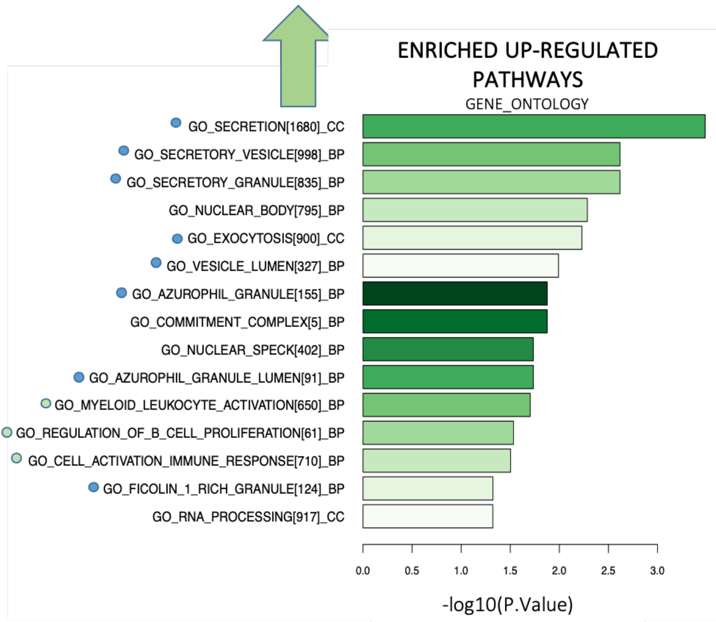
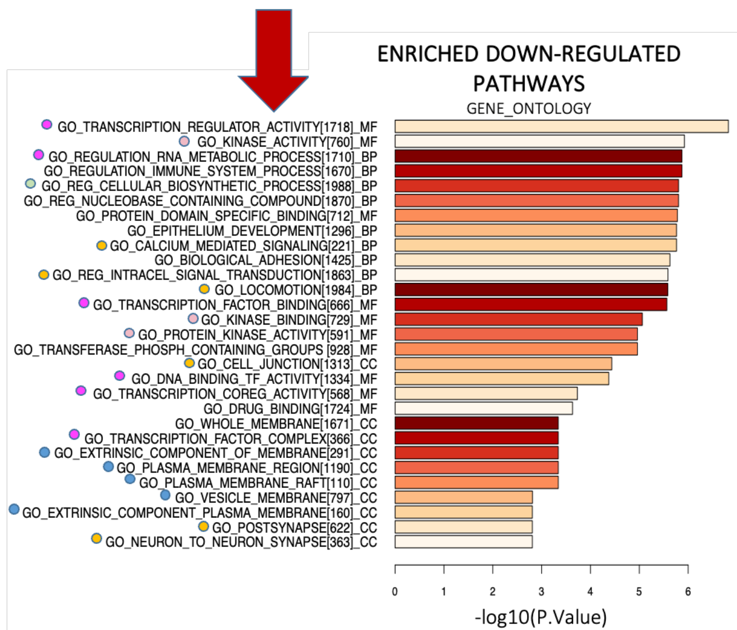


Figure 6. Differential expression profiles between PD and CTRL in *GBA* carriers.

Schematic representation of differentially expressed genes (129 upregulates and 383 downregulated) between PD and CTRL subjects with mutations on the *GBA* gene. Venn-diagram represents the overlap across differentially expressed genes between PD and CTRL subjects with no mutations of the *GBA* genes and differentially expressed genes between PD/GBA+ and CTRL/GBA- subjects (197 overlapping genes). Volcano plot represents logFC (x-axis) and -log10 of P-values (y-axis) of the differentially expressed genes between PD/GBA+ and CTRL/GBA- groups. Green dots represent genes with FDR < 0.05.





VESCICLE/SECRETION
IMMUNE SYSTEM
TRANSCRIPTION
KINASE ACTIVITY
SIGNAL TRANSDUCTION

Figure 7. Pathway enrichment analysis of differentially expressed genes between PD/GBA+ vs CTRL/GBA+ subjects.

Pathway enrichment of genes differentially expressed between PD/GBA+ and CTRL/GBA+ subjects with FDR < 0.05 for GO terms are reported. Go terms are classified according to the following categories: Molecular Function (MF), Biological Processes (BP), Cellular Component (CC), and they are listed in the two bar plots (number of genes for each GO term is listed into parenthesis next to the corresponding GO term). Bar plots represent FDR. Pathways enriched across up-regulated genes in PD/GBA+ vs CTRL/GBA+ (not overlapping with the ones differentially expressed between the PD/GBA- vs CTRL/GBA- cohorts) are listed in the top part of the figures (green barplot), while pathways enriched across down-regulated genes in PD/GBA+ vs CTRL/GBA+ (not overlapping with the ones differentially expressed between the PD/GBA- vs CTRL/GBA- cohorts) are listed in the bottom part of the figures (red barplot). Each GO term is then classified according to functional categories, as indicated by colored dots next to each term (blue: vesicle/secretion pathways, green: immune system, pink: transcription pathways, light pick: kinase activities, orange: cellular signal transduction).

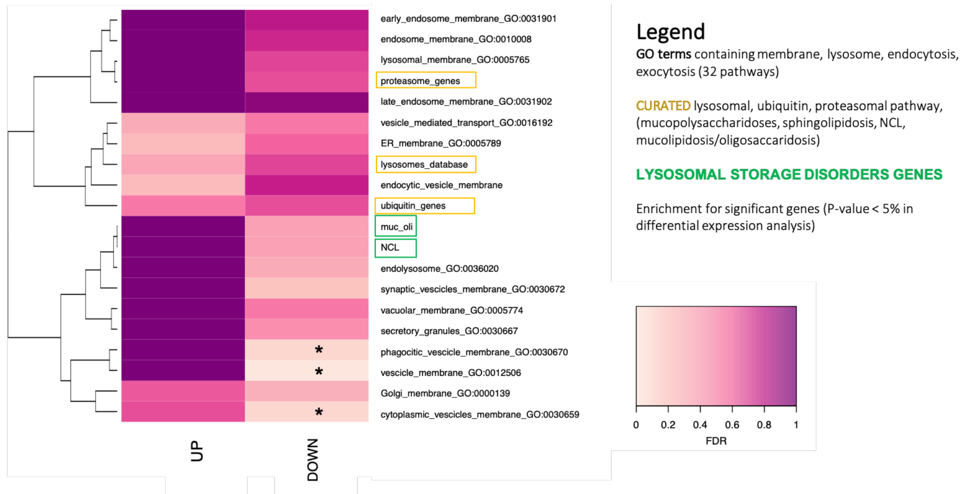


Figure 8. Targeted pathway enrichment analysis in PD/GBA+ vs CTRL/GBA+.

Enrichment analysis of differentially expressed genes at FDR < 0.05 between PD/GBA+ vs CTRL/GBA+ subjects for pathway related to vesicle and membrane trafficking (GO terms) as well as curated pathways (as described in the text, Material and Methods section) related to lysosomal, ubiquitin, proteasomal pathway (highlighted with gold boxes), and lysosomal storage disorders genes (green boxes). Heatmap only shows pathways with enriched genes, divided between up- and down-regulated genes. * indicates pathways with enrichment according to Fisher-exact test, P-value < 0.15.

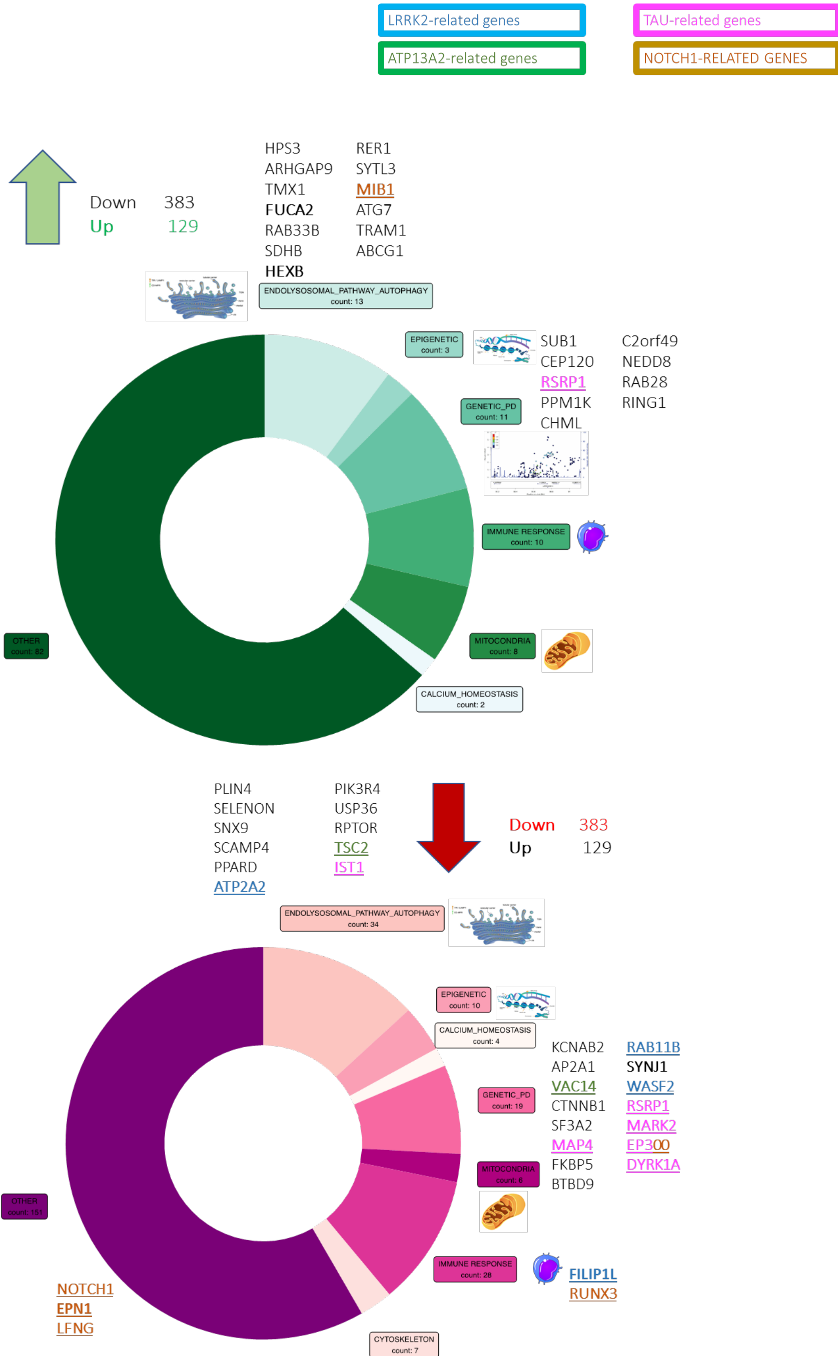


Figure 9. Curated pathway enriched analysis of expression profiles in CD14+ isolated monocytes in PD/GBA+ vs CTRL/GBA+.

Donuts graphs represent the percent number of genes among the up- (right) and down- (left) regulated genes between PD/GBA+ and CTRL/GBA+ subjects. All the up-regulated genes (129) and an equivalent number of down-regulated genes (124) were manually classified based on data reported in the literature according to the following pathways: endo-lysosomal pathway/autophagy, epigenetic, immune response, mitochondria, calcium homeostasis, cytoskeleton, genetic of PD (genes previously associated with PD as monogenic forms of the disease or genetic risk factor for the disease, or genes directly interacting with known PD-genes), and others. Number of genes per each category are reported in the box ("count").
Main genes of some of the categories are listed next to their section. Genes related to selected genes (*LRRK2*, *ATP13A2*, *TAU*, *NOTCH1*) identified across deregulated genes are color-coded according to the boxes reported in the top-right (LRRK2-related genes: blue, ATP13A2-related genes: green, TAU-related genes: pink, NOTCH1-related genes: gold).

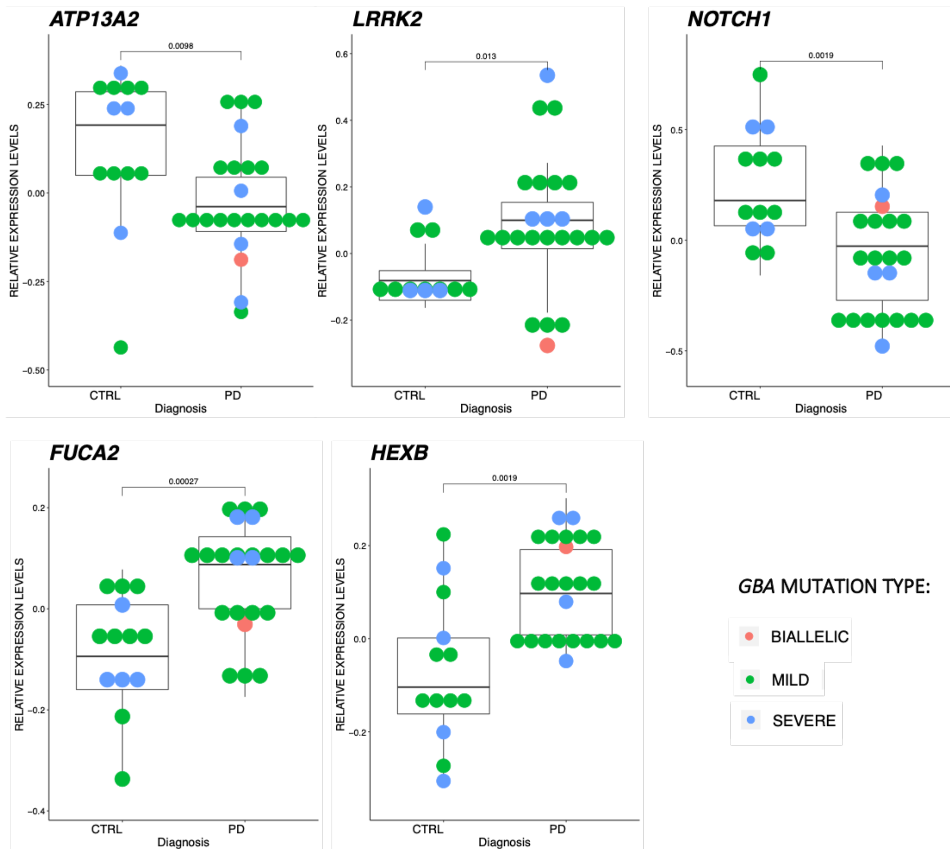
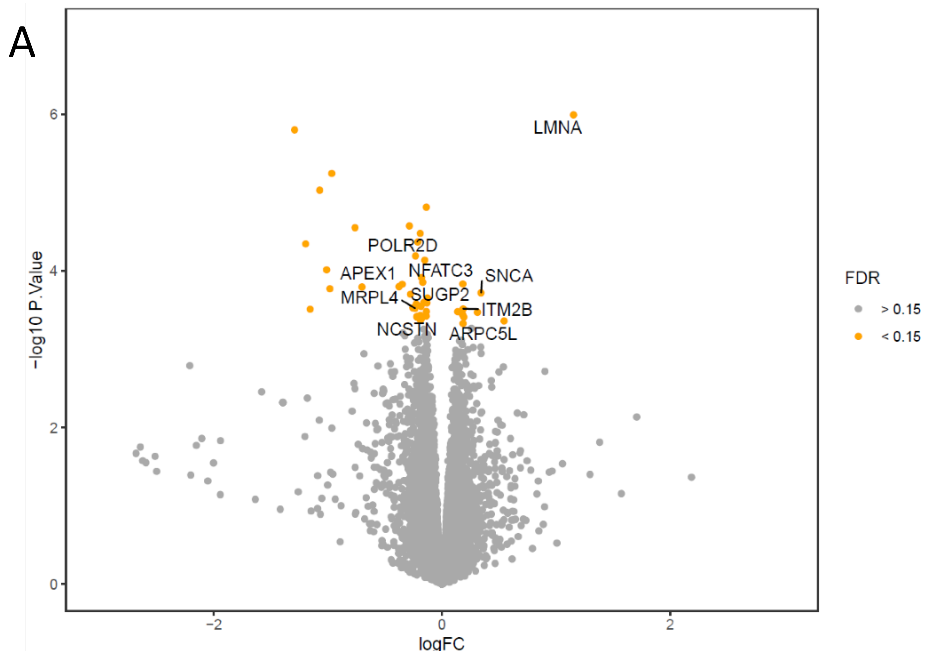
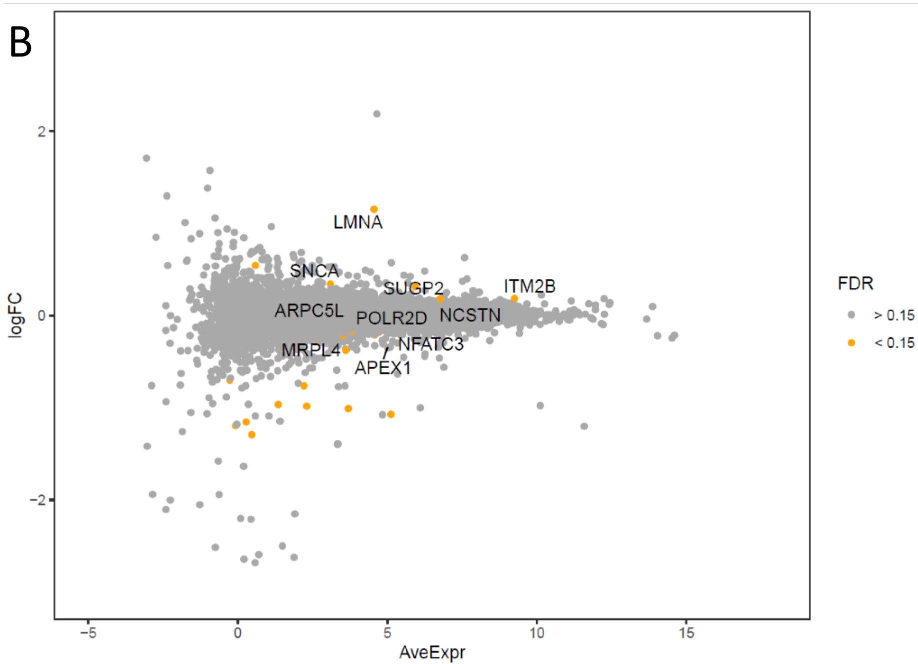


Figure 10. Differential expression of target genes in CD14+ isolated monocytes from PD/GBA+ vs CTRL/GBA+.
Box plot representing differential expression levels (normalized expression count, after regression of SVs) of targeted genes identified in the previous analysis and relevant for PD-related pathogenic mechanisms. Comparisons are between carriers of *GBA* mutations and disease status is labeled in the x-axes. Each dot represents a subject. Dots are colored based on *GBA* and *LRRK2* (G2019S) mutations, as reported in the legend (*GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of different expression levels is reported on top (statistics: Mann-Whitney U test).


A
LMNA
POLR2D
APEX1
NFATC3
SNCA
MRPL4
SUGP2
ITM2B
NCSTN
ARPC5L
−log10 P.Value
logFC
FDR
> 0.15
< 0.15


B
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SNCA
SUGP2
ITM2B
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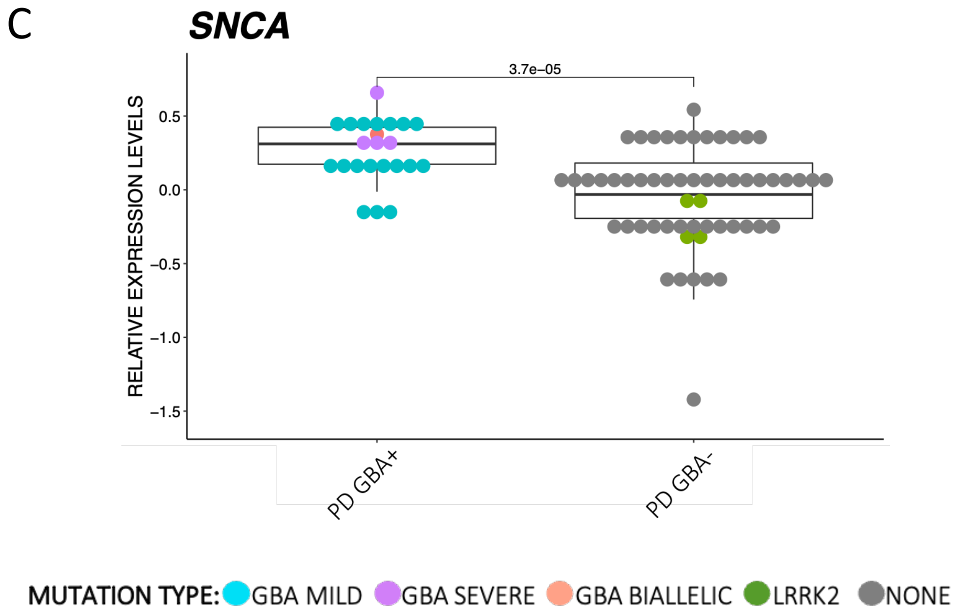


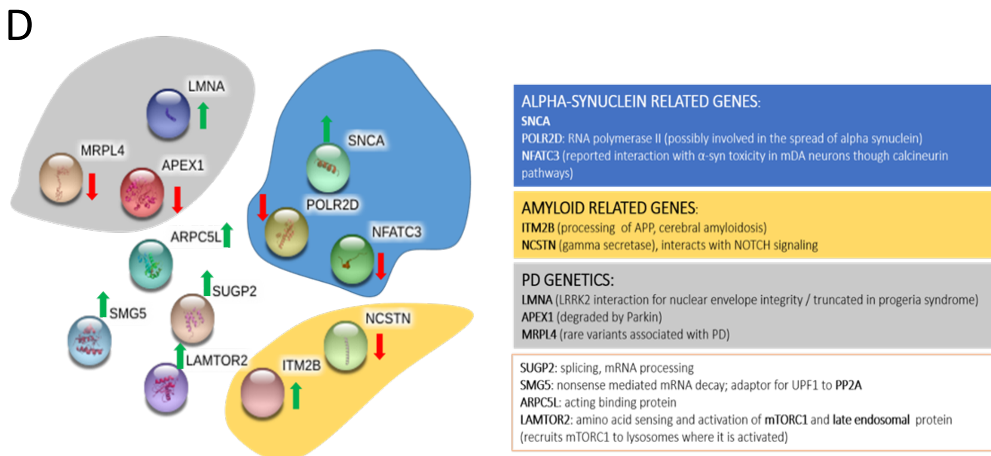
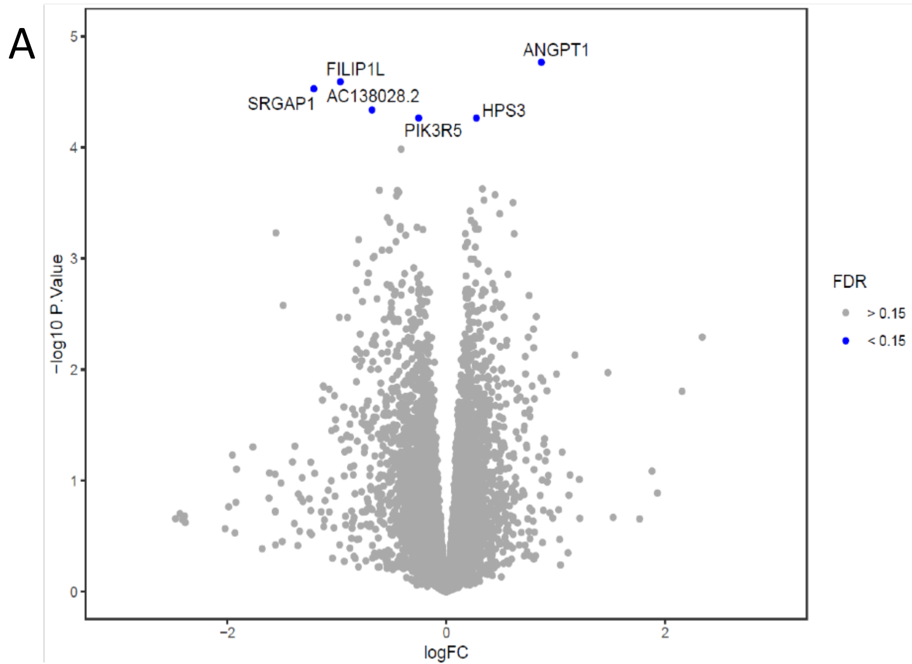


Figure 11. Differential expression profiles between subject with PD in *GBA* carriers vs non carriers.
A) Volcano-plot representing logFC (x-axes) and P-Value (y-axes, -$\log_{10}$ P-Value) of differential expressed genes between PD/GBA+ and PD/GBA- as per nested interaction model. Highlighted in yellow are genes with FDR < 0.15 (44 total genes). ID labels of functionally relevant genes are reported in the plot.
B) MA-plot of differential expressed genes between PD/GBA+ and PD/GBA-. logFC of gene expression is reported in the y-axes while

Average expression is reported in the x-axes. ID of relevant genes are labeled in the plot.
C) Differential normalized expression count (after regression of SVs) of SNCA between PD/GBA+ and PD/GBA-. Disease and genetic status are reported on the x-axes. Each dot represents a subject. Dots are colored based on *GBA* and *LRRK2* (G2019S) mutations and disease and genetic status, as reported in the legend (*GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of pair-wise comparison between the two groups is reported on top of the two boxes (statistics: Mann-Whitney U test).
D) Schematic representation (STRING, [282]) of functionally relevant genes differentially expressed between PD/GBA+ and PD/GBA- cohorts. Genes are grouped in colored circles based on shared functional pathways. Arrows indicate whether genes are up (green) or down (red) regulated in the PD/GBA+ vs the PD/GBA- cohort. On the right a brief description of functional role of each gene is provided.



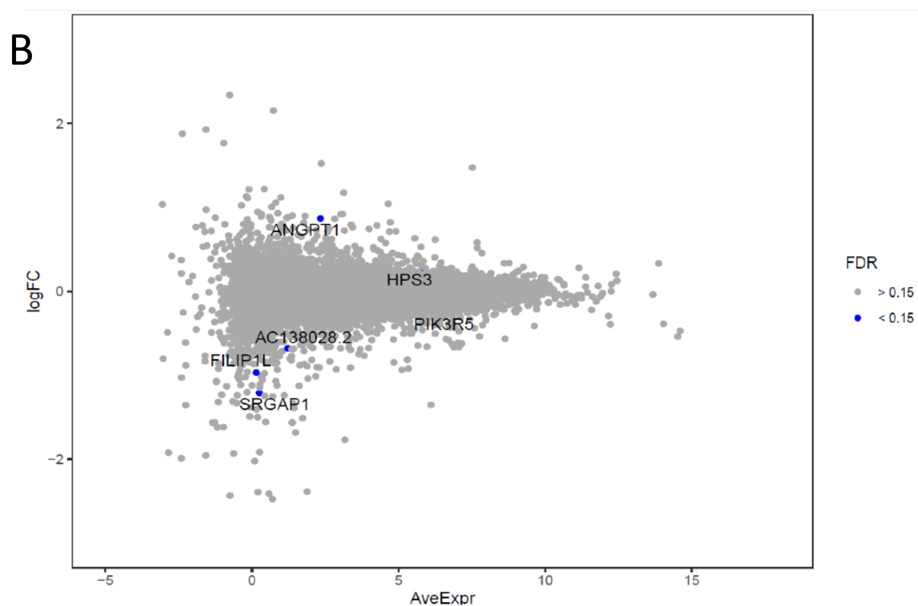
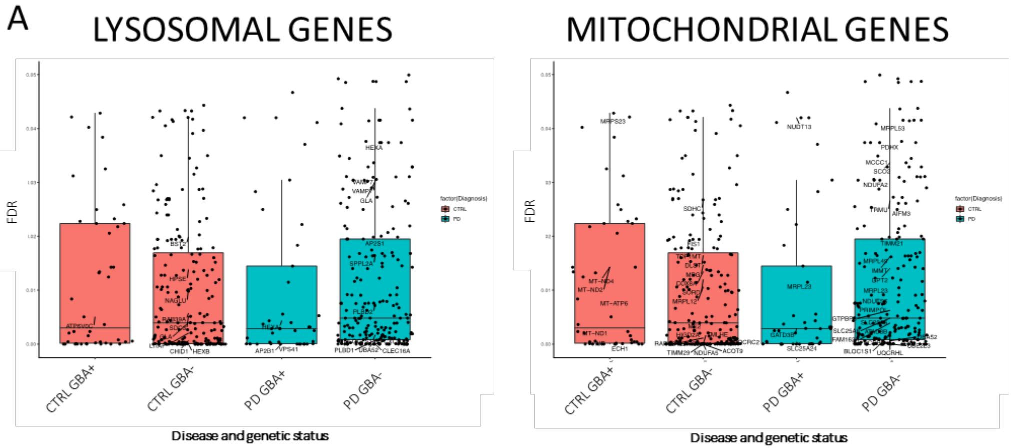
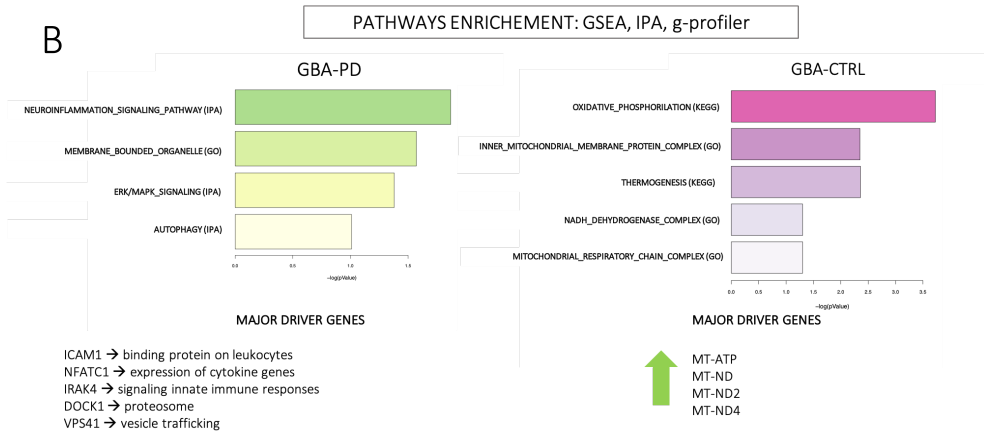


Figure 12. Differential expression profiles across the four cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-) based on diagnosis and genetic status interaction.

A) Volcano-plot representing logFC (x-axes) and P-Value (y-axes, -log10 P-Value) of differential expressed genes based on diagnosis and genetics interaction between the four cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-). Genes with FDR < 0.05 are highlighted in blue and labeled.
B) MA-plot of differential expressed genes based on diagnosis and genetics interaction between the four cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-). logFC of gene expression is reported in the y-axes while Average expression is reported in the x-axes. Genes with FDR < 0.05 are labeled in the plot.





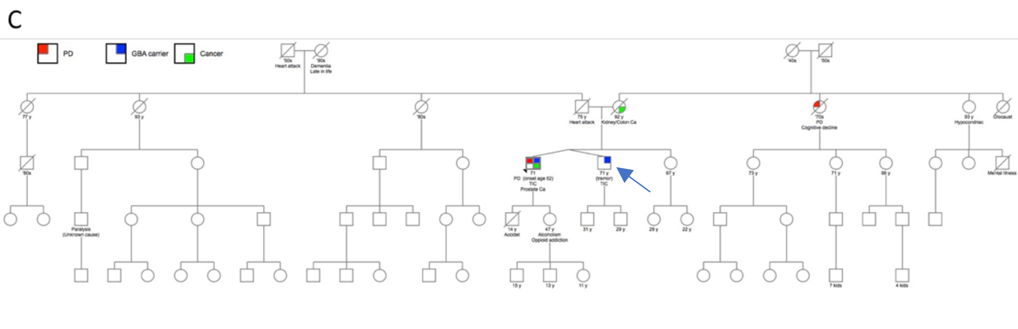
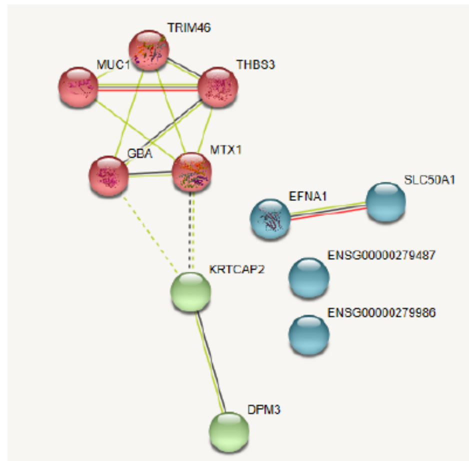


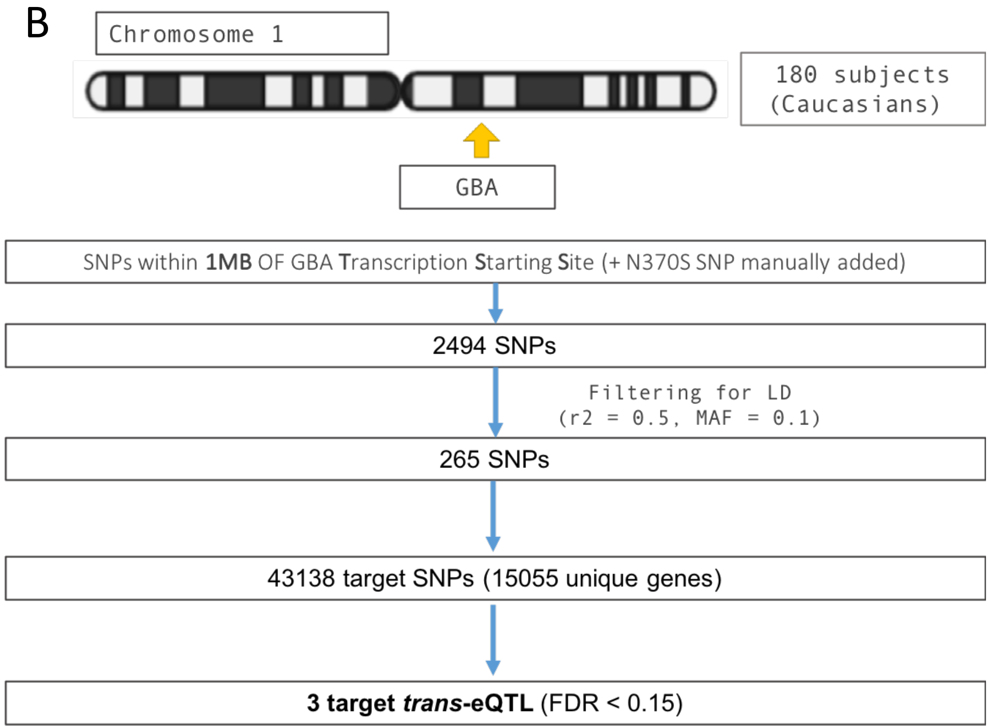
Figure 13. Enrichment analysis of outlier genes in the four cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-).

A) Box plots representing outliers genes in each cohort (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-) where lysosomal genes (left) and mitochondrial genes (right) are labeled. Each dot represent a gene. FDR value per each gene is reported in the y-axes.
B) Pathway enrichment analysis of outlier genes in the PD/GBA+ and CTRL/GBA+ cohort (based on GSEA, IPA and g-profiler tools). Bars represent P-value ($-\log_{10}$(P-value)). Major driver genes of pathway enrichment per each cohort are reported below the bar-plot together with brief description of their function. Green arrow next to outlier genes in the CTRL/GBA+ group indicates that these genes are upregulated in this cohort.
C) Pedigree of the CTRL/GBA+ subjects who presented upregulation of the mitochondrial genes reported in B). CTRL/GBA+ subject is indicated by light blue arrow. Symbol legend for the conditions reported in the family are reported in the top left of the pedigree.

A



B



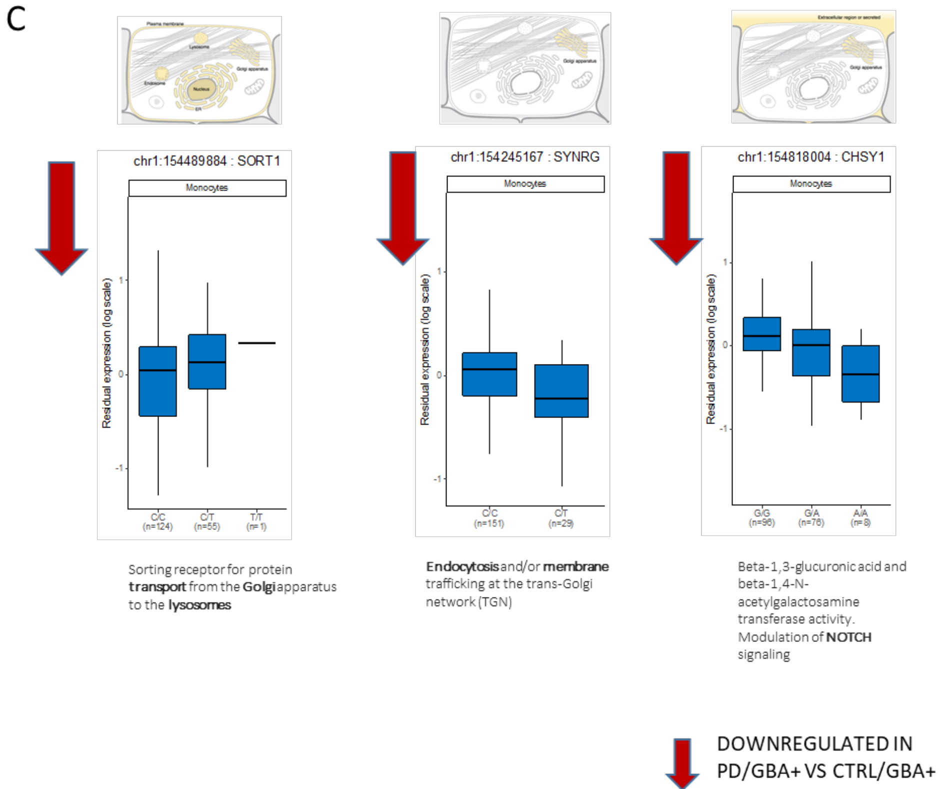


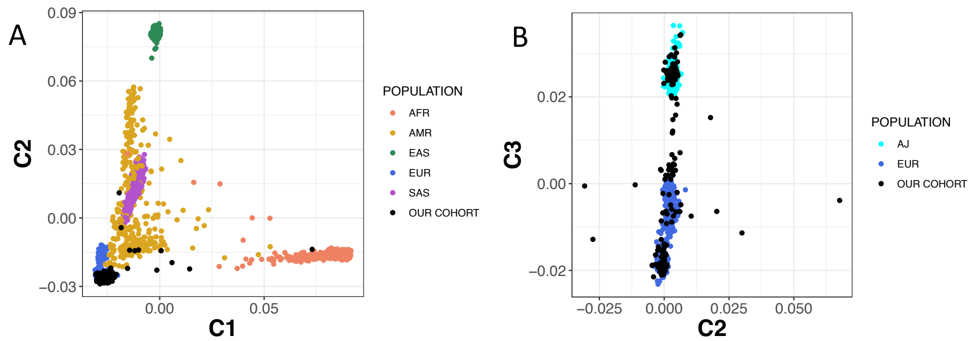
Figure 14. Trans-eQTLs analysis.

A) Connectome between proteins within 1Mb from TSS of *GBA* (STRING, [282]).

B) Schematic representation of pipeline for the filtering of data for trans-eQTL analysis.

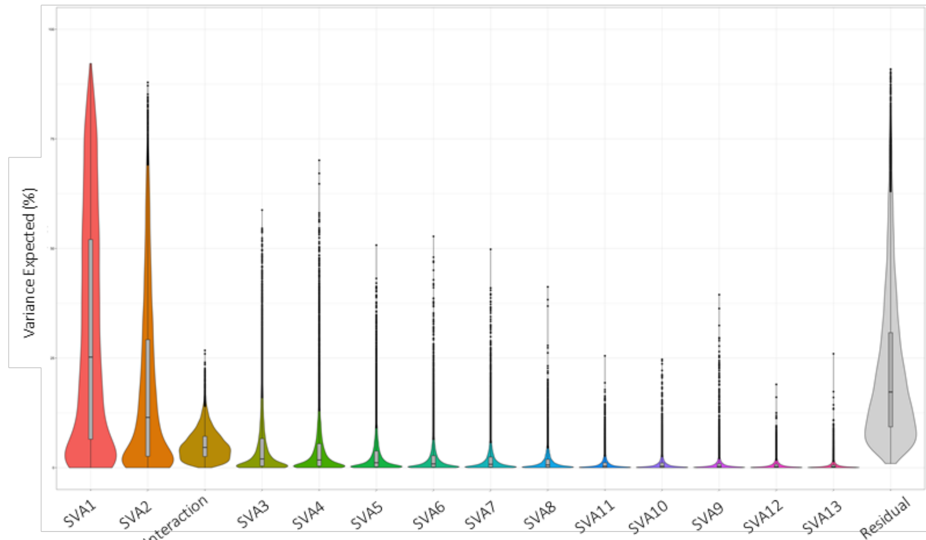
C) Box-plot representing allele frequency across our cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-) of the three trans-eQTL genes with FDR < 0.15 (*SORT1*, *SYNRG*, *CHSY1*). SNP location on *GBA* gene with trans-eQTL effect and targeted gene are reported above each box plot. Red arrow next to each genes indicates that these genes are downregulated in the PD/GBA+ cohort compared to the CTRL/GBA+ cohort according to nested interaction analysis. Molecular functions of significant eQTLs target is reported below the boxplots while their cellular localization is reported above boxplots according to UniProt [224].

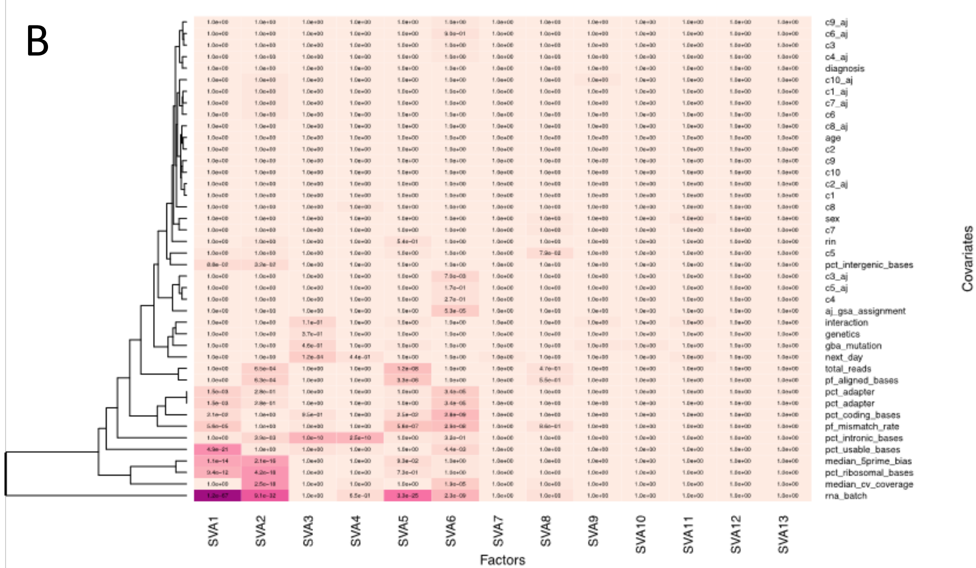
10.2 Supplementary Figures

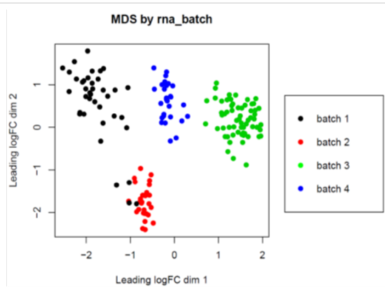


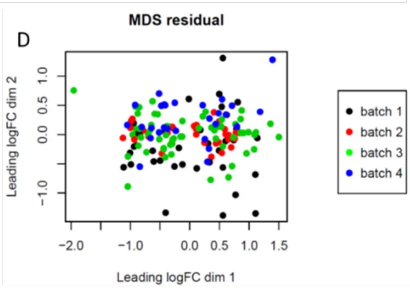
Supplementary Figure 1. Characterization of genetic background of donor population.

A) Representation of PCA analysis of ancestry of MDS values from the cohort of 158 subjects (PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA-) compared to 1000 Genome Project samples (Phase 3). The different ancestry are represented in different colors (Orange: African; Gold: Americans; Green: East Asian; Blue: European; Purple: South Asian; Black: study cohort). B) PCA considering only overlap of MDS values of donor cohort (black) with European ancestry (blue) and AJ ancestry (light blue).

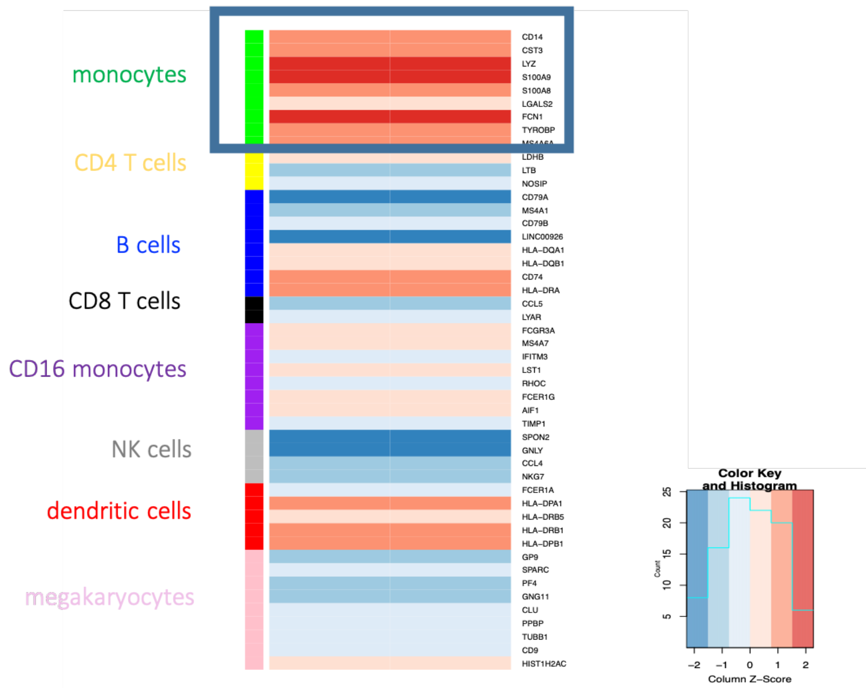

A
Variance Expected (%)
SVA1
SVA2
Interaction
SVA3
SVA4
SVA5
SVA6
SVA7
SVA8
SVA11
SVA10
SVA9
SVA12
SVA13
Residual


B
c9_aj
c6_aj
c3
c4_aj
diagnosis
c10_aj
c1_aj
c7_aj
c6
c8_aj
age
c2
c9
c10
c2_aj
c1
c8
sex
c7
rin
c5
pct_intergenic_bases
c3_aj
c5_aj
c4
aj_gsa_assignment
interaction
genetics
gba_mutation
next_day
total_reads
pf_aligned_bases
pct_adapter
pct_adapter
pct_coding_bases
pf_mismatch_rate
pct_intronic_bases
pct_usable_bases
median_5prime_bias
pct_ribosomal_bases
median_cv_coverage
rna_batch
Covariates
SVA1
SVA2
SVA3
SVA4
SVA5
SVA6
SVA7
SVA8
SVA9
SVA10
SVA11
SVA12
SVA13
Factors


C
MDS by rna_batch
Leading logFC dim 2
Leading logFC dim 1
batch 1
batch 2
batch 3
batch 4

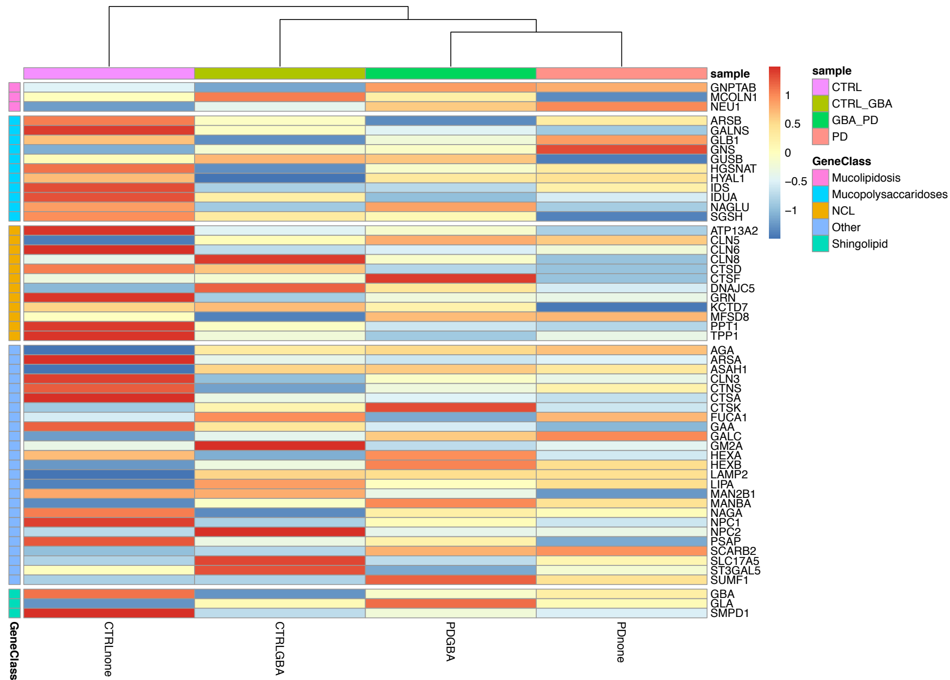

D
MDS residual
Leading logFC dim 2
Leading logFC dim 1
batch 1
batch 2
batch 3
batch 4

Supplementary Figure 2. RNA-seq normalization and quality control.
A) Violin plot representing the contribution of each of the surrogate variable (as explained in the text) to the variability of expression data of the study cohort and residual (158 subjects).
B) Heatmap representing the results of linear regression between the surrogate variables utilized for data normalization and technical variables (from RNA-seq analysis) and metadata. Coefficient of linear regression is reported in the heatmap for each correlation pair.
C) Distribution of MDS values of study cohort identified a clear clustering based on batches used for RNA-seq analysis (batches 1 to 4). D) After regression of SVs, variability of MDS values is significantly reduced, with no significant outliers and no clustering based on experimental batches.



Supplementary Figure 3. Enrichment of CD14+ isolated monocytes expression profiles for markers of immune cells.

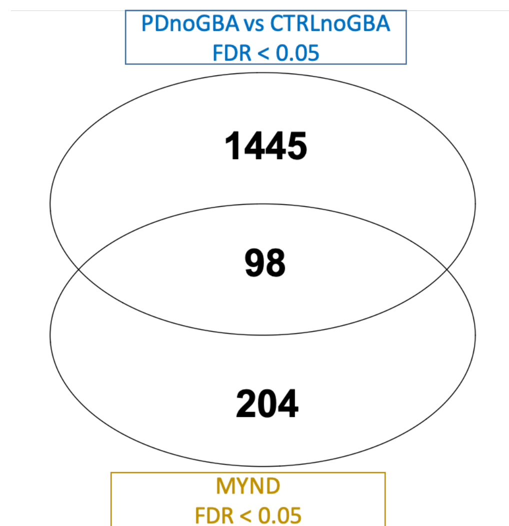
Expression levels of a list of curated markers for the different sub-types of immune cells (monocytes, CD4 T cells, CD8 T cells, B cells, CD16 monocytes, NK cells, dendritic cells, megakaryocytes) were assessed across all samples (158) of the donor cohort. Homogeneous overexpression patterns can be observed for monocytes cells, as expected after processing of PBMC for the purification of this specific cell type. Color legend is reported in the bottom right.



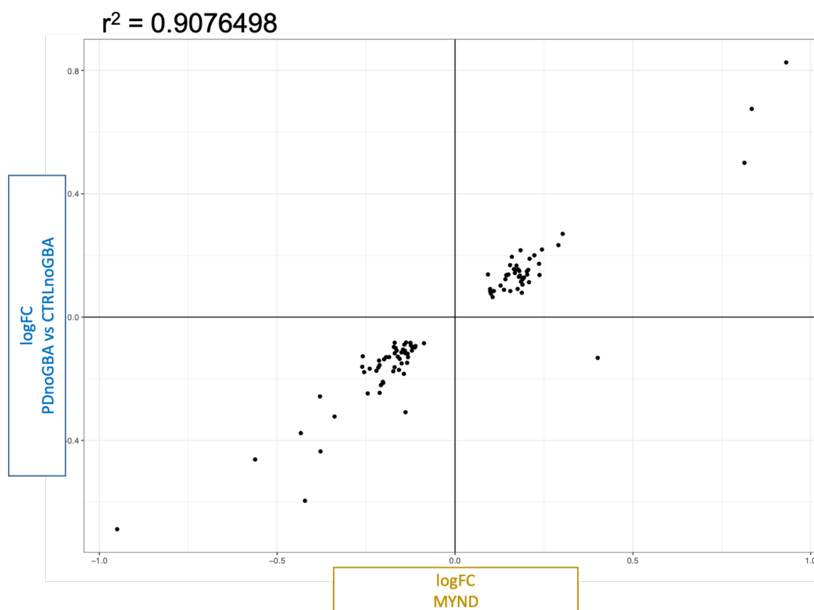
Supplementary Figure 4. Targeted analysis of isolated CD14+ monocytes for LSD genes grouped by disease category.

Normalized expression count of genes associated with Lysosomal Storage Disorders (LSD) in PD/GBA+, PD/GBA-, CTRL/GBA+, CTRL/GBA- (columns). Genes are grouped according to LSD subtypes (Mucolipidosis, Mucopolysaccharidosis, Neuronal Ceroid lipofuscinosis (NCL), Sphingolipidoses, Others) to assess expression pattern of these disease categories across phenotypes.

A



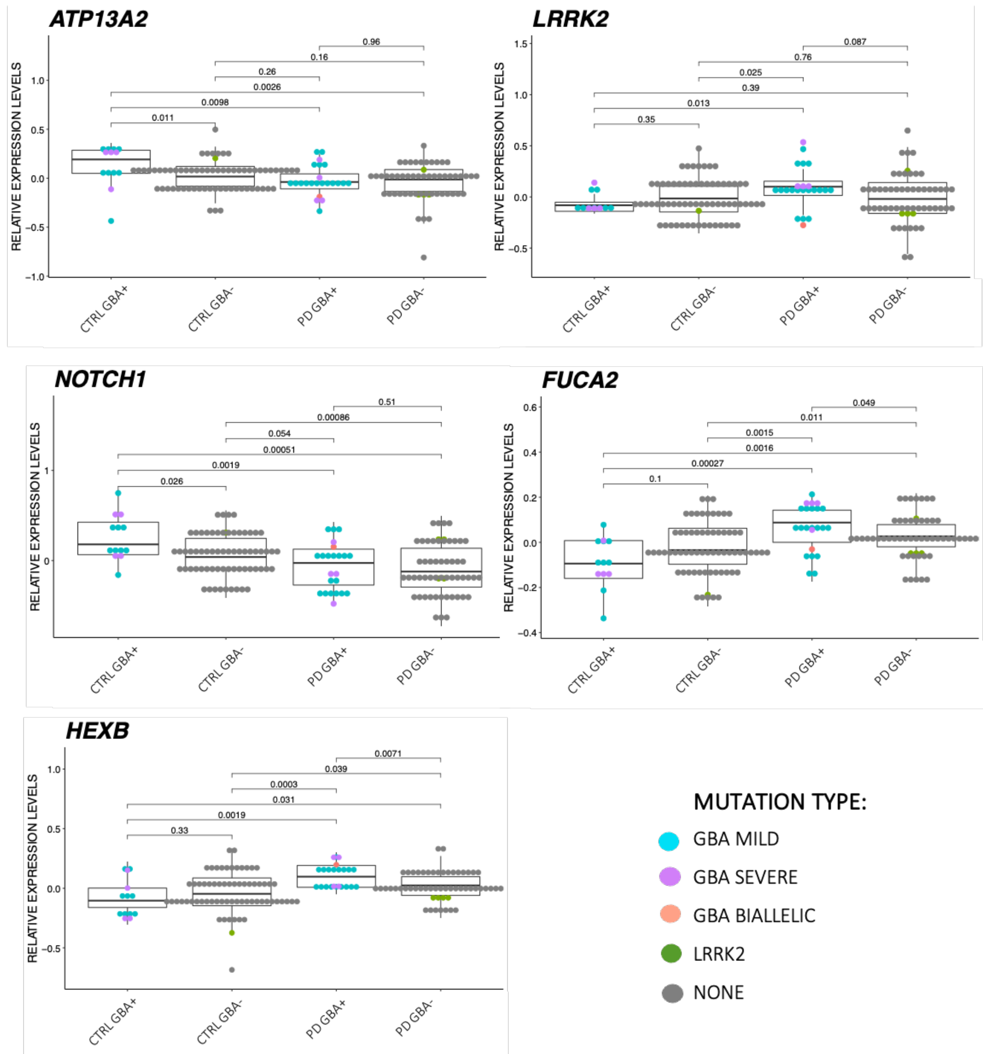
B



Supplementary Figure 5. Differential expression profiles between PD and CTRL subjects with no GBA mutations.

A) Overlap between differentially expressed genes of CD14+ isolated monocytes at FDR < 5% between PD/GBA- and CTRL/GBA- in this work and differentially expressed genes at FDR < 5% between PD and CTRL in our previous cohort (Myeloid cells in Neurodegenerative Disease (MyND)

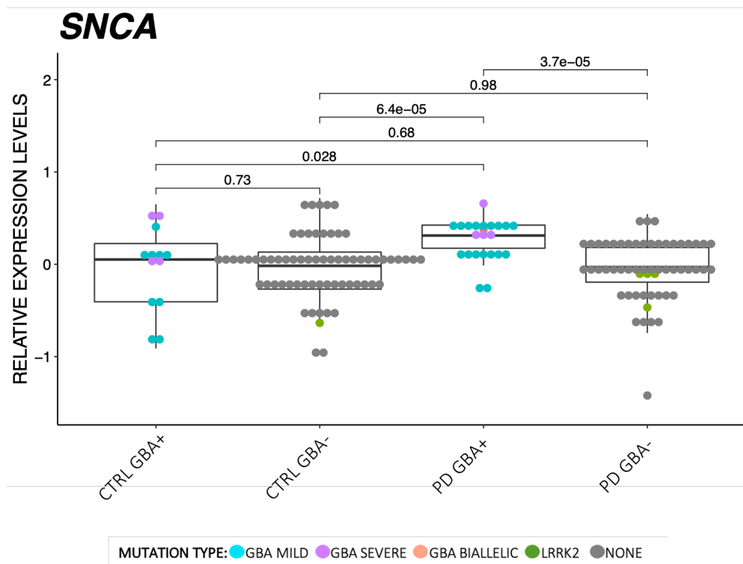
project [211]). A total of 98 genes were overlapping between the two analyses.
B) Linear regression between overlapping differentially expressed genes at FDR < 5% from the same comparison reported in (A) showing concordance of directionality of log FC of the selected genes ($r2 = 0.9076498$).



Supplementary Figure 6. Differential expression of target genes in CD14+ isolated monocytes from the PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA- cohorts.

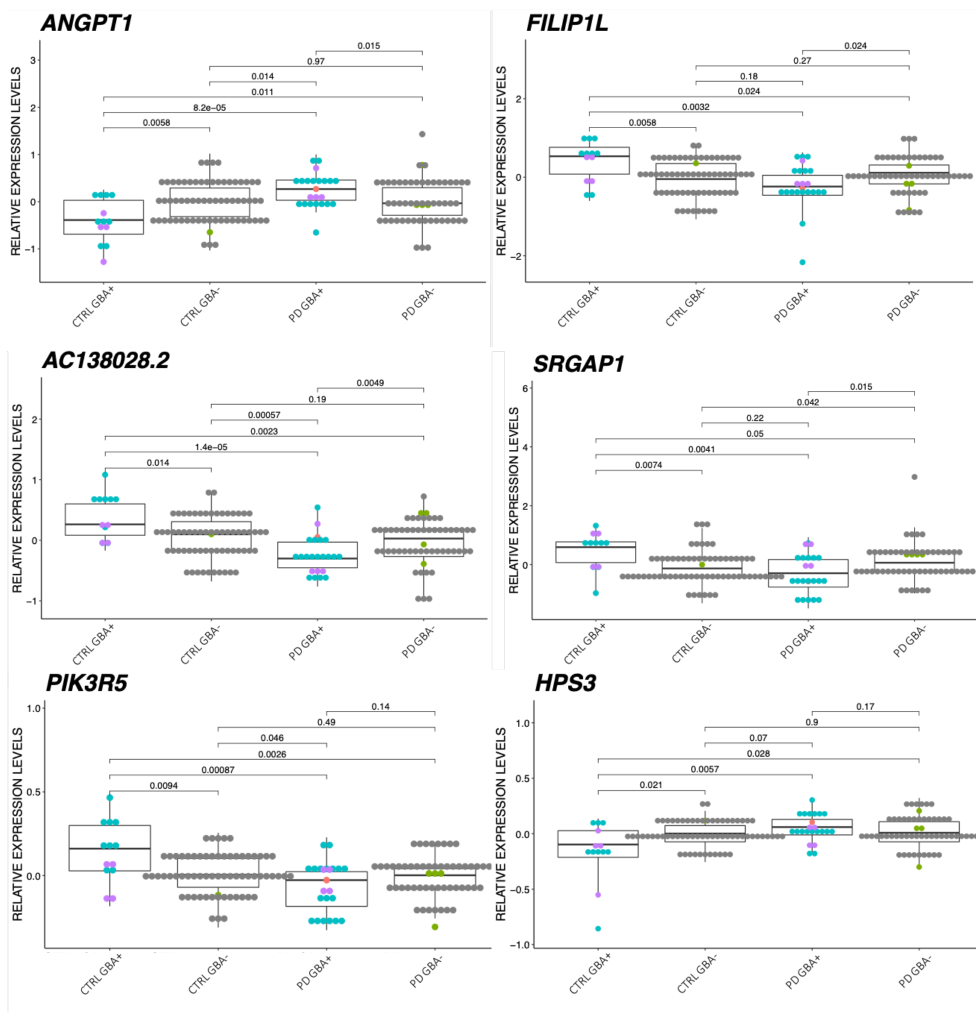
Box plot representing differential expression levels (normalized expression count, after regression of SVs) of targeted genes identified in the previous analysis and relevant for PD-related pathogenic mechanisms. Disease and genetic status are labeled on the x-axes. Each dot represents a subject. Dots are colored based on *GBA* and *LRRK2* (G2019S) mutations, as reported in the legend (*GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M,

N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of pair-wise comparison between different expression levels is reported on top (statistics: Mann-Whitney U test).



Supplementary Figure 7. Differential expression of *SNCA* in CD14+ isolated monocytes from the PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA- cohorts.

Box-plot representing expression levels (expressed as normalized expression count after SVs regression, as detailed in the manuscript) of *SNCA*. Disease and genetic status are labeled on the x-axes. Each dot represents a subject. Dots are colored based on *GBA* and *LRRK2* (G2019S) mutations, as reported in the legend (*GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of pair-wise comparison between different expression levels is reported on top (statistics: Mann-Whitney U test).



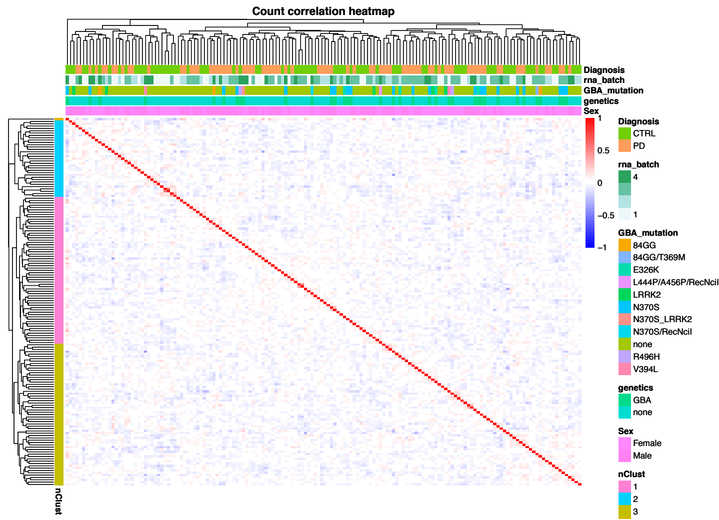
MUTATION TYPE: GBA MILD GBA SEVERE GBA BIALLELIC LRRK2 NONE

Supplementary Figure 8. Differentially expressed genes according to interaction term (diagnosis and genetics interaction) in CD14+ isolated monocytes from the PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA- cohorts.

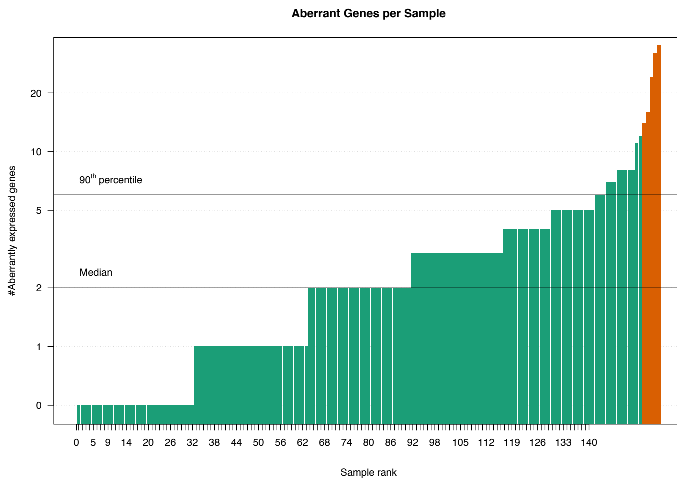
Box plot representing expression levels (expressed as normalized expression count after SVs regression, as detailed in the manuscript) of differentially expressed genes according to interaction term (diagnosis and genetics interaction). Disease and genetic status are labeled on the x-axes. *GBA* and *LRRK2* (G2019S) mutations are labeled, when present (*GBA* mild

mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of pair-wise comparison between different expression levels is reported on top (statistics: Mann-Whitney U test).

A



B



C

493 pairs (subject-gene)

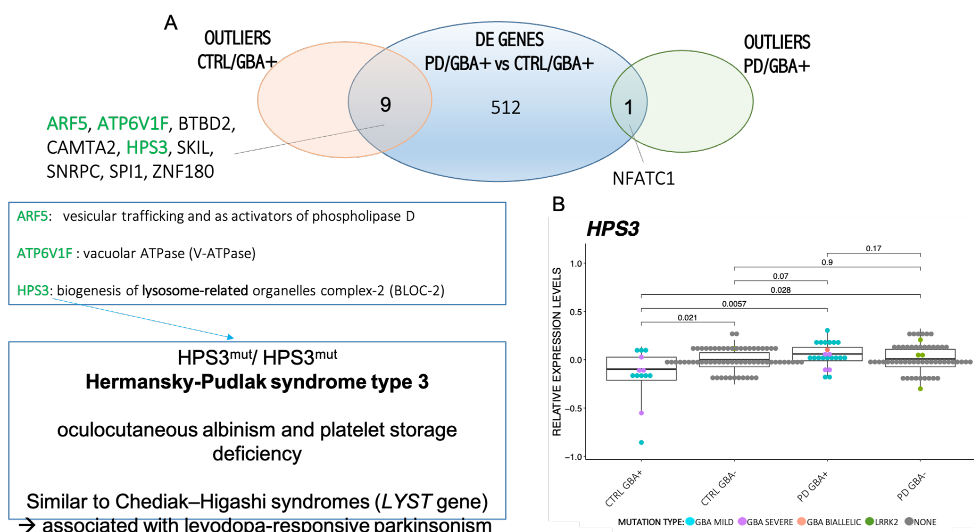
	GBA	none
CTRL	44	200
PD	41	208

125 unique subjects

	GBA	none
CTRL	8	53
PD	19	45

Supplementary Figure 9. QC analysis for analysis of outliers data using OUTRIDER tool.

A) Normalization based on surrogate variables, as provided by the OUTRIDER script, of a total of 13711 genes (considering only genes with > 30% expression). Discrete relevant variables (Diagnosis, batches of RNAseq analysis (rna_batch), GBA mutations, genetic status (GBA+ and GBA-), gender (Sex: male (M) and female (F)) are labeled at the top of the heatmap per each subject.
B) Bar-plot reporting number of outlier genes per each subject (out of 158 subjects).
C) summary tables summarizing the number of outliers genes per cohort (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-) (493 pairs) and the number of subjects per each cohort with at least one outlier gene (125 unique subjects total with at least one outlier gene).



Supplementary Figure 10. Genetic outliers and expression profiles in PD and CTRL/GBA-mutation carriers.

A) Venn diagram reporting number of overlapping genes between outlier genes in the CTRL/GBA+ group (on the left) and the PD/GBA+ group (on the right) with the differentially expressed genes between PD/GBA+ and CTRL/GBA+ cohort (as detected by nested interaction model). Number of overlapping genes is reported in the diagram. Names and function of shared genes is reported in the boxes below the Venn diagram.

B) Box plot of differentially expressed levels of *HPS3* gene between the four cohorts (PD/GBA+, CTRL/GBA+, PD/GBA-, CTRL/GBA-). *GBA* and *LRRK2* (G2019S) mutations are labeled, when present (*GBA* mild mutations (N370, E326K, R496H), *GBA* severe (L444P/A456P/RecNciI, V394L, 84GG, 84GG/T369M, N370S/RecNciI), *GBA* biallelic (N370S/N370S)). P-value of pair-wise comparison between different expression levels is reported on top (statistics: Mann-Whitney U test).

10.3 Tables and Supplementary Tables

	PD	PD_GBA	CTRL	CTRL_GBA
Number of subjects	56	23	66	13
Gender (% of females)	30%	61%	67%	54%
Race (% of caucasian)	100%	100%	100%	100%
Age (mean, min-max)	68.7 (47-88)	60.3 (28-77)	67 (37-86)	58.3 (38-81)
% of N370S *GBA* mutations	0	74%	0	46%
% of *LRRK2* mutations (#)	7% (4)	4.3% (1)	1.5%(1)	0

Table 1. Clinical characterization of study cohort.
Summary of demographic, clinical and genetic features of the cohort of subjects (PD and CTRL) whose purified CD14+ monocytes were used for integrated genomic analysis.

	UP_pVal	genes UP	DOWN_pVal	genes DOWN
NCL	1		0.3809873	TPP1
endolysosome_GO:0036020	1		0.3471233	AP2A1
mucolipidosis_oligosaccaridosis	1		0.3809873	SLC17A5
vacuolar_membrane_GO:0005774	1		0.52566	VAC14
vescicle_membrane_GO:0012506	1		0.03327179	ATP2A2, RIPOR1, SYNJ1
proteasome_genes	1		0.6731331	PSMB5
endocytic_vesicle_membrane_GO:0030666	0.2526243	CAMK2G	0.7863309	AP2A1
phagocitic_vescicle_membrane_GO:0030670	1		0.1067676	RAB11B, PIK3R4, TLR1, RAPGEF1
synaptic_vescicles_membrane_GO:0030672	1		0.2200207	RAB11B, SLC17A5
secretory_granules_GO:0030667	1		0.4707626	COPB1, C5AR1, B4GALT1
late_endosome_membrane_GO:0031902	1		0.9298442	VAC14
early_endosome_membrane_GO:0031901	1		0.8240028	VAC14, LLGL1
cytoplasmic_vescicles_membrane_GO:0030659	0.6527217	GDE1	0.1029702	MYOF, AP1G1, ANKRD27, IFNGR2, SNX9
endosome_membrane_GO:0010008	1		0.7561683	CLCN6, ARHGAP1, VAC14, PLEKHM1, PIP5K1C
vesicle_mediated_transport_GO:0016192	0.3286001	CHML, HSPA1L, ARF5	0.5465935	SEC24C, COPB1, TSC2, AP1G1, CYTH1, AP2A1, SEC16A
lysosomal_membrane_GO:0005765	1		0.6965521	CLCN6, SZT2, ATP11A, AP1G1, PLEKHM1, RPTOR, SLC17A5
ubiquitin_genes	0.5310891	ATG7, MIB1, RING1	0.6525329	HERC2, KMT2D, MGRN1, RNF144B, RNF185, RNF26, RNF44, SYVN1
lysosomes_database	0.3546838	ATP6V1F, FUCA2, HEXB, RNASE6	0.6785794	ADA, ANKRD27, ANXA11, AP1G1, ATP11A, CLCN6, RPTOR, SLC17A5, TPP1
Golgi_membrane_GO:0000139	0.6442496	RER1, ENTPD6, ABCG1, RAB33B	0.3109093	SEC24C, COPB1, TNFRSF1A, PKD1, VAC14, AP1G1, LLGL1, CYTH1, SCAMP4, B4GALT5, SLC9A8, IFNGR2, ZDHHC8, LFNG, B4GALT1, RGP1, SEC16A, NOTCH1
ER_membrane_GO:0005789	0.2552859	FMO5, CYB5R1, DAD1, TMX1, ABCC6, ALDH3A2, RETSAT, ABCG1, TRAM1	0.6093013	SELENON, SEC24C, ERLIN1, COPB1, SYVN1, RNF26, ATP2A2, CTDNEP1, KSR1, PNPLA6, DMPK, TMEM189, IFNGR2, RNF185, CYB5R3, POM121, POM121C, SEC16A, NOTCH1

Supplementary Table 1. Targeted pathway enrichment in PD/GBA+ vs CTRL/GBA+.
List of endolysosomal pathways (from GO terms and curated pathways, i.e. ubiquitin pathway) reported in Figure 6. P-value of enrichment as per Fisher exact test of each pathway within the set of up-regulated (UP) and down-regulated (DOWN) genes. Significant enriched pathways (at P-value < 0.15) are highlighted in red. The list of genes per each pathway which are overlapping with the up- and down-regulated genes in monocytes from the PD/GBA+ compared to PD/GBA- groups.

11. Appendix

Complete list of differentially expressed genes and pathway analysis are available at:
https://www.dropbox.com/scl/fi/ntlqcqgyl4g5l39nwvyut/Appendix.xlsx?dl=0&rlkey=67qrz7uny4i4rbppf5g1uiq9l

Tab1 - DE_ PD-GBA+vsCTR-GBA+. Summary statistic of differentially expression data between the PD/GBA+ and CTRL/GBA+ cohorts after analysis with the nested interaction model.
Significant genes (adj.P.value < 0.05) are labelled in RED and GREEN based on whether they were found to be up- or down-regulated respectively, ("UP/DOWN regulated" column). The functions of the genes relative to the classes of interest ("CLASS" column) were manually annotated based on available data. References from the literature are reported in the "Reference" column.

Tab2 - Pathway_PD_GBA+vsCTR_GBA+. Pathway enrichment analysis of significantly (adj.P.value < 0.05) differentially expressed genes between PD/GBA+ and CTRL/GBA+ cohorts after analysis with the nester interaction model. GSEA and g-profiler were utilized for the analysis. For both up and down-regulated genes analysis were performed considering the GO categories (CC, BP, MF) together as well as separately.

Tab3 - DE_(PD_GBA+vsCTR_GBA+)-overlap. Summary statistic of differentially expression data between the PD/GBA+ and CTRL/GBA+ cohorts not shared with the PD/GBA- vs CTRL/GBA- comparison, after analysis with the nested interaction model.

Tab4 - Pathway_(PD_GBA+vsCTR_GBA+)-overlap. TOP SECTION: Pathway enrichment analysis of significantly (adj.P.value < 0.05) differentially expressed genes between PD/GBA+ and CTRL/GBA+ cohorts

not shared with the PD/GBA- vs CTRL/GBA- comparison, after analysis with the nested interaction model. GSEA was utilized for the analysis. Data were analyzed considering the GO categories (CC, BP, MF) separately as well as merged, ranked by FDR. Pathways with maximum 2000 genes and with enrichment (Fisher exact test) FDR < 0.05 were considered. **LOWER SECTION:** Pathway enrichment analysis of significantly (adj.P.value < 0.05) differentially expressed genes between PD/GBA+ and CTRL/GBA+ cohorts shared with the PD/GBA- and CTRL/GBA- comparison. Pathways with maximum 2000 genes and with enrichment (Fisher exact test) FDR < 0.05 were considered. Pathways with maximum 2000 genes and with enrichment (Fisher exact test) FDR < 0.05 were considered.

Tab5 - DE_PD_GBA-vsCTRL_GBA-. Summary statistic of differentially expression data between the PD/GBA- and CTRL/GBA- cohorts after analysis with the nested interaction model.

Tab6- overlap_GBA_PDCTRL_noGBA_PDCTRL. Summary statistic of overlapping differentially expression data between the comparison of PD/GBA- vs CTRL/GBA- and PD/GBA+ vs CTRL/GBA+ cohorts after analysis with the nested interaction model.

Tab7 - DE_PD_GBA+vsPD_GBA-. Summary statistic of differentially expression data between the PD/GBA+ and PD/GBA- cohorts after analysis with the nested interaction model.
Significant genes (adj.P.value < 0.15) are labelled in RED and GREEN based on whether they were found to be up- or down-regulated respectively, ("UP/DOWN regulated" column). The functions of the significant genes relative were manually annotated based on available data.

Tab8 - DE_Interaction_term. Summary statistic of differentially expression data after analysis with the nested interaction model, interaction term for the following interaction contrast design: [(InteractionPDGBA – InterationCTRLGBA) – (InteractioPDnone – InteractionCTRLnone) = PDCTRL_inGBAcarrier – PDCTRL_inGBANoncarrier]. Genes at FDR < 0.15 are highlighted in yellow.

12. Scientific works and grants

List of scientific products from January 2018 to September 2020

Peer Reviewed Manuscripts:

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Review

GBA, Gaucher Disease, and Parkinson's Disease: From Genetic to Clinic to New Therapeutic Approaches

Giulietta M. Riboldi [1,2,*] and Alessio B. Di Fonzo [2,3]

1 The Marlene and Paolo Fresco Institute for Parkinson's and Movement Disorders, NYU Langone Health, New York, NY 10017, USA

2 Dino Ferrari Center, Neuroscience Section, Department of Pathophysiology and Transplantation, University of Milan, 20122 Milan, Italy; alessio.difonzo@policlinico.mi.it

3 Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Neurology Unit, 20122 Milan, Italy * Correspondence: giulietta.riboldi@nyulangone.org or giulietta.riboldi@unimi.it; Tel.: +1-929-455-5652

1. Introduction

GBA is a gene located on chromosome 1 (1q21) encoding for the glucocerebrosidase (GCase), a lysosomal enzyme involved in the metabolism of glucosylceramide. The mutation of this gene has been classically associated with Gaucher's disease, a systemic disorder with a variable degree of involvement of the central nervous system. Surprisingly, about 14 years ago it was observed that mutations in this same gene were associated with an increased incidence of Parkinson's disease (PD), in both Gaucher's patients as well as asymptomatic carriers [1–4]. PD is the second most common neurodegenerative disorder, affecting 2–3% of the world population over the age of 65 [5]. It is caused by the progressive loss of dopaminergic neurons in the substantia nigra. Classically it presents with a combination of bradykinesia, rigidity, resting tremor, and postural instability. However, a list of non-motor features, such as hyposmia, constipation, urinary symptoms, orthostatic hypotension, anxiety, depression, impaired sleep, and cognitive impairment can present as well in various degrees [5]. Since the first observations of *GBA* and PD, their association has been extensively explored. Different hypotheses have been formulated to explain the causative role of this mutation in PD [6]. First of all, GCase is part of the endolysosomal pathway, which seems to be particularly crucial in the pathogenesis

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Abstract: Parkinson's disease (PD) is the second most common degenerative disorder. Although the disease was described more than 200 years ago, its pathogenetic mechanisms have not yet been fully described. In recent years, the discovery of the association between mutations of the *GBA* gene (encoding for the lysosomal enzyme glucocerebrosidase) and PD facilitated a better understating of this disorder. *GBA* mutations are the most common genetic risk factor of the disease. However, mutations of this gene can be found in different phenotypes, such as Gaucher's disease (GD), PD, dementia with Lewy bodies (DLB) and rapid eye movements (REM) sleep behavior disorders
(RBDs). Understanding the pathogenic role of this mutation and its different manifestations is crucial for geneticists and scientists to guide their research and to select proper cohorts of patients. Moreover, knowing the implications of the *GBA* mutation in the context of PD and the other associated phenotypes is also important for clinicians to properly counsel their patients and to implement their care. With the present review we aim to describe the genetic, clinical, and therapeutic features related to the mutation of the *GBA* gene.

Keywords: glucocerebrosidase; Parkinson's disease; Gaucher's disease; Lewy Body Dementia; REM sleep behavior disorders of PD. Indeed, many different monogenic familial forms of PD are caused by genes involved in this pathway [7]. Moreover, mutated GCase is not able to fold properly and thus can accumulate in different cellular compartments of the dopaminergic neurons, causing a cell stress response that can be deleterious of the cells. In addition, impaired GCase activity seems to cause an accumulation of alpha-synuclein (for a comprehensive review see [8]). Today we know that *GBA* mutations are the major genetic risk factor for PD. Impaired GCase activity has been identified also in idiopathic cases of PD patients who did not carry a mutation in the gene, suggesting a central role of this enzyme in the pathogenesis of the disease [9,10].
In the present review, we aim to summarize the genetic changes and the characteristic features associated with the mutations of this gene, spanning from Gaucher's disease to PD and the other described phenotypes. This will aid in a better understanding of the pathogenic role of this mutation. The identification of these phenotypes will allow for clinicians to offer more appropriate counseling to the patients and their families.

2. Pathogenetic Mutations of the *GBA* Gene

2.1. GBA Mutation and Gaucher's Disease (GD)

Gaucher's disease (GD) is a systemic disorder that can present with a various degree of systemic and neurological manifestations. According to the severity of the disease and the neurological involvement, three different types of GD have been identified. GD type 1 has been classically considered only a systemic disorder, with no neurological involvement whatsoever. Anemia, leukopenia, thrombocytopenia with frequent bleeding, osteopenia with bone pain, easy fractures, Erlenmeyer flask deformity, as well as hepatosplenomegaly, failure to grow, and puberty delay can be presenting features of this disease [11–14]. Monoclonal gammopathy has been reported as well [15]. The disease can manifest early in childhood but it may remain undiagnosed until adulthood when the phenotype is mild. The pathological hallmarks of the disease are the so-called Gaucher cells, macrophages engorged with aberrant lysosomes as a consequence of the GCase-impaired activity. Symptoms are caused by the infiltration of these cells in the reticuloendothelial system of the affected organs [16]. In recent years, the natural history of GD type 1 has dramatically changed since the introduction of target treatments, such as enzyme replacement therapy (ERT) (human recombinant enzyme to be administered intravenously every other week) and oral substrate reduction therapy (SRT) [17]. Treatments with these two approaches are able to address the majority of the systemic symptoms associated with GD type 1 and those in GD type 3. So far, SRT has been approved only for subjects over the age of 18 years. However, in the adult population it represents an important alternative first line treatment. Unfortunately, these therapies are not able to cross the blood-brain barrier and therefore they are not suitable for the treatment of the neurological complications associated with GD type 2 and 3. The two latest forms are also referred to as the acute (type 2) and chronic (type 3) neuronopathic form. Patients affected with GD type 2 start manifesting severe symptoms very early, usually within the first six months of life. They usually present a combination of severe neurological manifestations, with brainstem involvement (i.e., eye movement abnormalities, spasticity, hypotonia) and seizure, as well as life-threatening systemic symptoms, such as respiratory distress and aspiration pneumonia [18,19]. Skin manifestations, like ichthyosis or collodion abnormalities, as well as hydrops fetalis, can be present. Prognosis is very poor and death usually occurs before the age of four [20]. GD type 3 (chronic neuronopathic form) has been further classified as GD type 3a,b,c. GD type 3a presents a milder

visceral phenotype, but can be associated with severe and life-threatening myoclonic seizures. GD type 3b, instead, is characterized by a more prominent visceral involvement [21]. Interestingly, one of the features that have been used to try to discriminate between patients with GD type 1 and the milder neuropathic form GD type 3 is the assessment of the eye movements. Indeed, patients with GD type 3, especially type b, present with characteristic eye movement abnormalities. In particular they show loss of horizontal before vertical gaze palsy and slowing of the saccades, suggesting involvement of the brainstem. GD type 3c, instead, is the only subtype of the disease presenting with cardiac mitral and aortic calcification and poor prognosis [21]. A particular cluster of patients with GD type 3 has been identified among the Swedish population. This is also referred as Norrbottnian form, because of its geographical distribution. It is associated with the c.1448T > G mutation and it presents with an early and severe splenomegaly and a combination in the first or second decade of ataxia, spastic paresis, horizontal supranuclear gaze palsy, kyphoscoliosis and other orthopedic abnormalities, cognitive impairment, and seizures [22].

Those different phenotypes are associated with discrete genetic mutations, as detailed below.

Different Pathogenic Mutations of *GBA* Associated with Gaucher's Disease (GD) Subtypes

More than 300 variants of the *GBA* gene have been associated with Gaucher's disease [23]. GD is an autosomal recessive disorder. In order for the disease to manifest, patients need to carry a pathogenic mutation on both alleles of the GBA gene, either in a homozygous or compound heterozygous fashion. Point mutations, insertion, deletion, missense mutations, splice junctions, and concomitant multiple mutations have been reported [24]. The different variants can be more represented in particular ethnic groups as well as in particular phenotypes. The c.1226A < G (N370S; or N409S according to the new nomenclature) mutation is the most common one among Ashkenazi Jew (AJ) patients, followed by the
c.84dupG (84GG) mutation, which is more rare. The c.115 + 1G > A (IVS2 + 1), c.1504C > T (R463C), and c.1604G > A (R496H) are commonly found in AJ patients with GD type 1 [24]. On the contrary, the N370S mutation is rarely found among Chinese and Japanese patients [24] (Hruska et al., 2008). Among Asian ethnic groups, the c.1448T > C (L444P, or L483P according to the new nomenclature) and the c.754T > A (F252I), usually associated with GD type 2 and 3, are more prevalent, also explaining why among these populations the neuropathic forms of GD are

more frequent [20]. c.1448T > C (L444P) is also the most frequent mutation among Caucasians with a non-Ashkenazi Jew ancestry [25] (Figure 1).

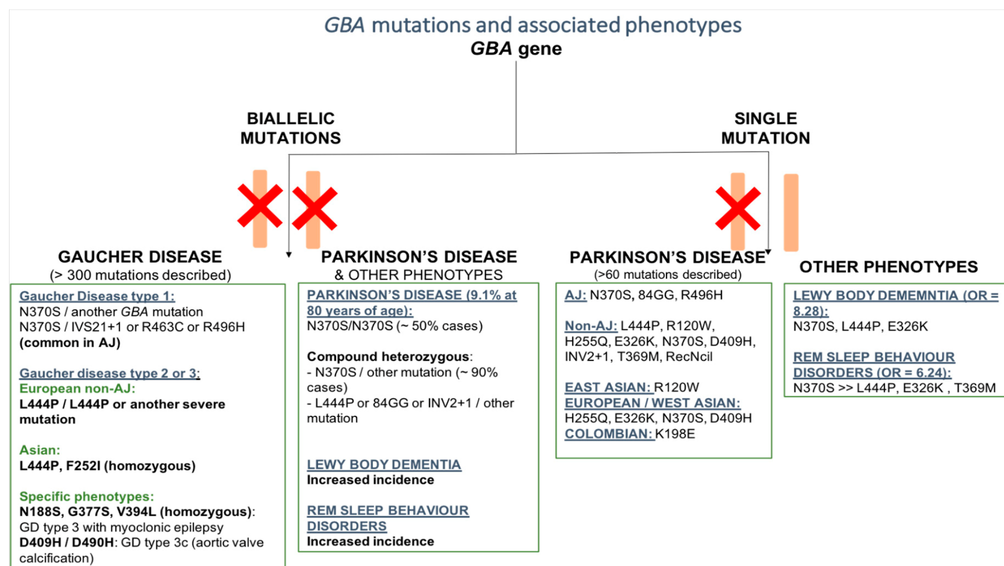

Figure 1. Schematic representation of the most common pathogenic mutations of the *GBA* genes and associated phenotypes. Phenotypes were grouped based on homozygous and heterozygous mutations, ancestry, and specific associated features.

Different mutations can lead to different phenotypes of GD. The c.1226A > G (N370S) mutation is associated only with Gaucher's disease type 1 and it seems to be protective for the development of the neurological involvement characteristic of GD type 2 and 3. Indeed, patients who present the c.1226A > G (N370S) mutation on at least one allele of the *GBA* gene will manifest only GD type 1 [24]. Interestingly, subjects who are homozygous for the N370S variant can also remain asymptomatic for the disease. On the other hand, the c.1448T > C (L444P) mutation is usually associated with GD type 2 or 3, even when presenting in a compound heterozygous state [19]. Homozygous c.1448T > C (L444P) mutation [c.1448T > C]1[c.1448T > C] (L444P/L444P) with no recombinant alleles can be associated with very severe but also milder phenotypes [26]. The c.1342G > C (D409H) variant is responsible for GD type 3c which presents with characteristic cardiac valve calcifications [27]. c.680A > G (N188S), c.1246G > A (G377S), and c.1297G > T (V394L) are more likely to be associated with myoclonic epilepsy [28–30]. Despite previously reported observations, it is commonly found that members of the same family report variability in the manifestation of symptoms even with an identical genotype, suggesting that

a genotype/phenotype correlation is tentative still. Other reported mutations are uniquely rare and oftentimes private among specific families. [12]. Hence, it is difficult to make generalizations about these mutations specific phenotypical profiles.
Another interesting mutation is the c.1093G > A (E326K), which caused a lot of debate in the literature [31]. Indeed, it is not clear whether this mutation is really pathogenic for GD, since it was found also in a significant number of asymptomatic carriers in homozygosity [32,33]. However, when associated with other *GBA* mutations on the same allele, it can cause severe impairment of the GCase activity [34,35]. Interestingly enough, the same mutation seems to be significantly associated with an increased risk of PD [33].

2.2. GBA Mutation and Parkinson's Disease (PD)

2.2.1. Pathogenic Mutations of *GBA* Associated with PD

More than a decade ago, the association between an increased risk of developing PD and the presence of *GBA* mutations was initially noticed in large Gaucher's disease clinics. The incidence of PD among GD patients and their relatives, which were supposedly carriers for the mutation, seemed to be higher than the general population. Initially, only single case reports were suggesting this association. Interestingly, PD was noticed in patients with GD type 1, which has always been considered the non-neuropathic form of the disease [36–41]. It was only when larger populations of PD patients were screened for mutations of this gene that the important role of *GBA* in the pathogenesis of PD was assessed worldwide.
So far, more than 50 population studies have screened the *GBA* gene among PD patients, covering a large number of ancestries (reviewed in [42,43]). Overall, these studies demonstrated that the incidence of *GBA* mutations is significantly higher among PD patients, compared to non-affected subjects. Compared to GD, a smaller number of *GBA* mutations have been reported in patients with PD (about 130 *GBA* mutations) [42]. However, in many of these studies, only the mutations that are most commonly associated with PD were screened. Therefore, less frequent variants still associated with the disease could go undetected. Among all, the c.1226A > G (N370S) and the c.1448T > C (L444P) mutations are the two most common mutations worldwide. Indeed, in some populations they account for the 70–80% of the total number of variants of *GBA* associated with PD [44]. Among subjects from eastern Europe with an AJ ancestry, the c.1226A > G (N370S) mutation is definitely the most frequent one among PD patients, as already reported for GD (Figure 1). Among the non-AJ European descendants, the

c.1448T > C (L444P) mutation is more common. Interestingly, it has been reported that some mutations are able to increase the risk of PD only in the context of specific ancestry [42]. This is the case of the c.84dupG (84GG) and c.1604G > A (R496H) for AJ subjects, the c. 475 C > T (R120W) for East Asian populations, and the c.882T > G (H255Q), c.1093G > A (E326K), c.1342G > C (D409H), and c.1226A < G (N370S), which are only found in subjects of European or West Asian ancestry [42] (Figure 1). A recent study identified an increased incidence of the K198E variant (previously described in GD1 and GD2 patients) in a population of PD patients from Columbia compared to controls [43].
It seems that severe *GBA* mutations (as classified according to the subtype of GD that they are associated with), such as c.84dupG (84GG), c.115 + 1G >A (IVS2 + 1), c.1297G > T (V394L), c.1342G > C (D409H), c.1448T > C (L444P), and c.1263del + RecTL, are associated with a higher risk of causing PD compared to milder mutations, such as the N370S and c.84dupG (84GG) [45]. Moreover, severe mutations are associated with an earlier age of onset, as well as a more rapid progression and increased involvement of cognitive functions [45–47]. In one study, the motor and some of the non-motor symptoms (such as depression, REM sleep behavior disorders, and hyposmia) were significantly worse in PD patients carrying severe *GBA* mutations compared to subjects carrying mild mutations or with idiopathic PD [48].
Interestingly, *GBA* represents only a risk factor for PD. This means that not every carrier will develop the disease. The reason for the reduced penetrance of these mutations has not yet been fully elucidated. Based on large population studies, today we know that, among *GBA* carriers, about 9.1% will develop PD. Some reports suggest that the penetrance of PD in GD patient is 30% at 80 years, but this data needs to be confirmed by further studies [49]. Patients with a homozygous mutation of *GBA*, thus affected with Gaucher's disease, have a higher risk of developing PD and usually with an earlier age of onset of symptoms [48]. Having said that, it is worth noticing that the majority of subjects with GD will never develop PD, even in the case of severe mutations. It is still controversial whether PD in patients with GD presents with a more severe phenotype compared to *GBA* carriers. Carriers of the *GBA* mutation harbor an increased risk of developing PD by five times in heterozygous carriers and 10–20 times in homozygous carriers [50–53]. *GBA* mutations are present in about 2–30% of PD patients [54]. Carrier frequency can be very different across different ancestry. Among AJs, it goes from 10 to 31%, while in Norwegian's it is only 2.3% [54]. In patients of European non-AJ ancestry, it ranges from 2.9 to 12% [54].

In the last few years, there has been a great effort to try to clarify the pathogenic role of the *GBA* mutations in PD and many different hypotheses have been formulated, as reported above (for review see [8]). It is important to note that a growing amount of data is suggesting a failure of the endolysosomal and of the autophagic pathways in PD [55]. These scavenger systems are crucial for the degradation of alpha-synuclein, whose accumulation in the dopaminergic neurons is one of the hallmarks of PD. In the lysosome, GCase plays an important contribution for these processes and, in particular, in the interplay with alpha-synuclein [56]. Therefore, it is not totally surprising that a dysfunction of this enzyme is related to PD. How the different mutations of *GBA* that have been described in PD patients are able to affect the activity of the GCase has not been fully understood. We know that the GCase has three active domains. PD-associated mutations are found in distinctive domains of the protein. The c.1342G > C (D409H) and c.1297G > T (V394L) variants are located in domain I. The c.84dupG (84GG) mutation causes a frameshift that can induce aberrantly shorter or longer proteins that are non-functioning [23]. Other mutations, instead, are not found in the functional domains but do interfere with the final structure of the enzyme, thus making it more unstable or affecting its interaction with other proteins. The c.1226A > G (N370S) and c.1448T > C (L444P) mutations are, for example, located in the proximity of the binding site of the Saposin C, an activator of GCase [57]. More importantly, SapC competes with the binding of alpha-synuclein to GCase, which would cause the inhibition of the enzyme [58,59]. Interestingly, the c.1226A > G (N370S) mutation also seems to affect the ability of the GCase to modify the conformation of one of its loops, loop 3, according to changes in pH [60,61]. Conformational changes in response to the changes of the cellular environment are critical for the proper function of the protein. Despite our knowledge about the structural effects of the different mutations, the exact correlation between the localizations of pathogenetic variants of the gene and the degree of expression of PD has not yet been fully described.

It is also worth noting that *GBA* presents a pseudogene (*GBAP1*) that shares a very high degree of homology—96% sequence identity–located in the proximity of the original gene [62,63]. Therefore, genetic analysis will have to take this into account and should be performed in a specialized laboratory in order to obtain reliable results. New technologies, such as the long-read sequencer, are on the horizon for even more in-depth identification of possible *GBA* mutations [64].

2.2.2. *GBA* Mutations and Parkinson's Disease Phenotype

PD patients carrying *GBA* mutations are not easily recognizable in most cases because they do not present exclusive features that would clearly distinguish them from patients with idiopathic PD (iPD). However, large population studies comparing carriers vs. non-carriers, mild vs. severe mutations, as well as heterozygous manifesting carriers vs. PD–GD patients, allowed the ability to define common traits in these subgroups of patients (for a comprehensive review see [8]). In particular, *GBA*–PD patients present an overall earlier age of onset compared to non-carriers. Disease manifests about 3–6 years earlier in heterozygous carriers, irrespectively of the severity of the mutation, and about 6–11 years earlier in subjects with homozygous mutations [45,46,48,54,65–70]. There are limited reports of *GBA* mutation carriers having an age of onset in the 20^0s. [31,54,71–73].

The progression of the disease has been characterized in many different studies by a more pronounced cognitive deficit in a significant percentage of these patients, with a risk of developing dementia up to three times higher compared to iPD, which is even more increased in patients with severe mutations [46,48,74]. Hallucinations and REM sleep behavior disorders (RBD) also are more common among *GBA* patients in a dose-dependent fashion, being more frequent in subjects with homozygous mutations and in patients carrying severe vs. milder mutations. However, other non-motor symptoms, such as depression and anxiety, constipation, urinary symptoms, orthostatic hypotension, and sexual dysfunctions are over-represented as well in *GBA* carriers compared to iPD, especially in the presence of severe mutations, but with no increased severity in GD patients [46,48,75,76]. An increased incidence of dysautonomic features has been suggested to be the main driver of the slightly reduced survival reported in these patients [77]. Motor complications, such as dysphagia, dysarthria, and freezing of gait, are more frequent as well in *GBA* carriers [46,67].

In patients with *GBA* mutations and PD, the rigid akinetic phenotype seems to be more common. Usually, these patients present a very good response to levodopa, although the progression of the motor symptoms can be slightly faster compared to iPD but without higher rates of motor fluctuations or dyskinesia. Therefore, no specific treatment approaches need to be considered for this subgroup of patients. Interestingly, a recent study evaluated the outcomes of treatment with deep brain stimulation (DBS) in a cohort of PD patients carrying *GBA* mutations [78]. After a follow up of 7.5 years on average, it was noticed that the het-*GBA* cohort presented similar outcomes compared to iPD in terms of motor symptoms, while cognitive

impairment and non-motor symptoms were definitely more represented among carriers [78]. However, because of the beneficial effect on the motor symptoms, DBS should be considered as a suitable option for these patients.

2.2.3. *GBA* Mutations and Other Phenotypes

GBA mutations were identified also in cases of REM sleep behavior disorders (RBD) and in cases of dementia with Lewy bodies (DLB) [79].

GBA and Dementia with Lewy Bodies

A relatively low number of studies have been conducted to explore the incidence of the *GBA* mutation among patients affected with dementia with Lewy bodies (DLB), which was found to be even higher compared to the one in PD patients. In a cohort study of DLB patients, the frequency of *GBA* mutations was 7.49% with an odd ratio of 8.28 [79]. In another study in Spanish subjects, and in a number of autoptic brain tissues from pathologically proven DLB patients, a *GBA* mutation was identified in 12–13% of the cases [80]. Recent genome-wide association studies (GWAS) also confirmed the significant association between *GBA* mutations and DLB (particularly the rs35749011 variant) [81]. Among *GBA* carriers, the risk of developing DLB is about three times greater than developing PD [82].

As well as in PD patients, *GBA* mutations are associated with an earlier age of onset in DLB cases compared to non-carriers (of approximately five years) and a higher disease severity score [79,80]. The association between *GBA* mutations and DLB was found to be higher among male subjects compared to female [80]. These observations were confirmed also in a following study in a cohort of patients with DLB and AJ ancestry [83]. *GBA* mutation carriers (about 11% of the entire cohort) presented more severe symptoms, particularly in terms of increased hallucinations, worse RBD symptoms, and overall cognitive and motor features [83].

A number of different mutations of the *GBA* gene have been reported in DLB patients. Other than the two mutations most frequently associated with PD (c.1226A > G (N370S) and c.1448T > C (L444P)), the E326K variant is over-represented in this cohort of patients compared to controls [79,80]. Interestingly, the c.1093G > A (E326K) mutation also is frequently found in patients with PD dementia (PDD) [84].

Neuropathological data does not significantly differ between DLB patients with and without a *GBA* mutation [79]. However, *GBA* carriers present a reduced GCase activity as well as a more pronounced alteration of lipid profiles in the brain [85]. *GBA* expression profiles have been shown to be reduced in DLB and PDD cases in both specific brain regions (temporal

cortex and caudate nucleus respectively) and in the peripheral blood [86]. *GBA* mutations are more significantly associated with Lewy bodies (LB) pathology (especially with a cortical localization) than with Alzheimer's disease (AD) pathology (i.e., beta-amyloid and neurofibrillary tangles inclusions) [87].

GBA and REM Sleep Behavior Disorders

REM sleep behavior disorders (RBDs) are considered one of the prodromal symptoms of PD and patients affected by this disorder may present with alpha-synuclein accumulation in the brain [88]. According to a recent metanalysis, patients affected with RBDs present an estimated risk of developing a neurodegenerative disorder up to 97% after more than 14 years of follow up [89]. The majority of the cases who present a phenoconversion will develop an alpha-synucleinopathy, represented by PD in the majority of the cases, but also Multiple System Atrophy (MSA), Dementia with Lewy Bodies (DLB), and PD with dementia [90]. In fact, subjects with RBD may present clinical symptoms fulfilling the criteria for prodromal PD in up to 74% of the cases, manifesting worse performances in both motor and non-motor assessments compared to non-affected subjects [91,92]. Notably, many of the studies in this field did not take into consideration the significance of a family history of a neurodegenerative disorder, therefore, it is probable that the percentage of patients that reported a neurodegenerative disease is misrepresented. It would be worth exploring this aspect in future studies.

RBD seems to be more frequent in PD patients with *GBA* mutations compared with patients without this mutation (OR 3.13) [48,65,67,76]. RBDs are also more frequent in PD patients with concomitant GD than in heterozygous carriers [48]. Based on these observations, a few studies explored the incidence of the *GBA* mutation among patients affected with RBD [65,91–93]. These studies reported that among patients with idiopathic RBDs there is an increased frequency of *GBA* mutations (2.6–11.6% of RBD patients vs. 0.4–1.8% of the controls) [65,91,93]. A number of different *GBA* mutations were identified in RBD patients [65,93]. Some of these mutations have already been reported in PD patients, while others still do not have a clear pathogenic role. Among all the reported mutations, the two more commonly found in PD (i.e., c.1226A > G (N370S) and c.1448T > C (L444P), with N370S >> L444P), together with the c.1093G > A (E326K) and the c.1223C > T (T369M), were the most frequently represented in subjects with RBD [65,91–93].

Subjects with homozygous *GBA* mutations, thus affected with GD, and heterozygous carriers with no PD, presented significant worsening of rapid eye movement sleep behavior disorder scores over a period of time of two years compared with non-carrier subjects [92]. Among *GBA* carriers, the odds ratio (OR) for RBD was 6.24 (95% CI 3.76–10.35, $P < 0.0001$) [65]. The presence of *GBA* mutations does not seem to increase the risk among RBD patients of phenoconverting into PD [93]. These observations all together suggest that *GBA* may play a role in the development of RBDs, but not necessarily in determining more severe phenotypes.

Interestingly, no mutations of the *LRRK2* gene, the other common genetic risk factor for PD, have been identified so far in patients with RBDs [91,94].

3. New Targeted Treatments for *GBA*–PD Patients

Despite the very successful treatments that are now available to address the systemic manifestations of Gaucher's disease, unfortunately these approaches (i.e., enzyme replacement therapy and substrate reduction therapy) are not able to reach the central nervous system and thus fail to address the neurological symptoms caused by the disease. Different companies have been working for years to try to address this issue, producing very promising results in cellular and animal models. We are now in a very exciting era where some of these experimental approaches are starting to reach the clinical scene. The treatments available so far in clinical trials try to address two main mechanisms that are thought to be detrimental in linking *GBA* mutations to PD. The first hypothesis is that mutated forms of *GBA* are not able to fold properly in the endoplasmic reticulum (ER) in the cells, causing the protein to accumulate in this cellular compartment [95]. This would trigger a stress response in the dopaminergic neurons leading to their damage and death [95]. Also, the entrapment of the beta GCase in the ER causes reduced levels of the enzyme in the cells, triggering alpha-synuclein accumulation [95]. In order to target this pathogenic mechanism, different chaperones, which are proteins able to facilitate the refolding of their substrates, were tested [95–99]. In 2016, a clinical study assessing the efficacy of ambroxol, one of these chaperones that showed very exciting preliminary results, was started (NCT02914366 study:
https://www.clinicaltrials.gov/ct2/show/NCT02914366?cond=gba+parkinson&rank=7). This is a phase 2 clinical trial to assess the safety and the efficacy of this drug to improve motor and cognitive features of PD patients with a *GBA* mutation. The study is currently ongoing. Another similar

approach has been tested in a phase 1 study by Allergan with LTI-291, a chaperone molecule able to increase the activity of GCase (https://lti-staging.squarespace.com/our-science/#lti-291). Isofagomine is another chaperone protein that has been tested in vitro and in vivo to assess its ability to modulate the phenotype induced by mutations of *GBA* [97]. This molecule is an inhibitory chaperone whose role would be the stabilization of the GCase. Clinical trials with this molecule are not available at the moment. It is also worth considering that small molecules, such as chaperones, can present different therapeutic profiles in carriers of the different mutations of *GBA* according to the effect of these variants on the protein [100].

The second mechanism that has been explored to treat *GBA*–PD patients is the accumulation in the dopaminergic neurons of glucosylceramide (the substrate normally degraded by the GCase) because of the mutation of *GBA* [101–103]. Genzyme recently started a multicenter, randomized, double-blind, placebo-controlled phase 2 study to assess the safety, pharmacokinetics, and pharmacodynamics of an oral compound, ibiglustat (GZ/SAR402671), which is able to reduce the levels of beta-glucocerebrosidase in *GBA* carriers with early-stage PD (MOVES-PD study: https://www.clinicaltrials.gov/ct2/show/NCT02906020?cond=gba+parkinson&rank=2). It is still a long road for the establishment of an effective treatment, but many paths have been established, giving hope for patients with PD.

Mutated GCase is more unstable compared to the wild-type form. Therefore, modulation of the degradation of GCase could be another suitable strategy to increase the activity of the enzyme and thus tackle alpha-synuclein accumulation and neurodegeneration. Hsp90β, together with other heat shock proteins (HSP), such as Hsp27, parkin, and the endoplasmic reticulum-associated pathway, are responsible for the degradation of misfolded GCase. In particular, histone deacetylase inhibitors (HDACis) and direct inhibitors of specific HSP are able to increase the GCase activity, reducing its degradation [104]. Indeed, HDACis prevent the interaction between Hdp90β and GCase through the hyperactivation of one of its domains [105].

GCase plays an important role in the autophagy-lysosomal pathway (ALP), where other genes that have been associated with PD, such as *ATP13A2,* scavenger receptor class B member 2 (*SCARB2*), sphingomyelin phosphodiesterase 1 (*SMPD1*), and others, are also involved (Moors et al., 2016). Failure of the ALP seems to be responsible for the accumulation of alpha-synuclein in neurons. Therefore, a number of pharmacological

approaches directed to the ALP have been attempted in cellular and animal models of PD (for a comprehensive review see [106]). However, the autophagic pathway is broadly represented and active in different cell types and tissues in the organism. Therefore, the identification of approaches with a high selectivity for certain tissues (such as the dopaminergic neurons) or for specific mechanisms within ALP (such as GCase failure) is detrimental for the achievement of effective but also safe treatments for patients.

In order to restore GCase activity, whose failure seems to be responsible for its neuronal pathogenicity, gene therapy approaches are also in the pipeline. Preclinical studies showed that delivery of *GBA* using adeno-associated virus 1 (AAV1) in A53T–alpha-synuclein mice is able to reduce alpha-synuclein accumulation in the brain [107,108]. The field of gene therapy is now continuously growing in the context of the neurodegenerative disorders [109]. Clinical trials to assess the efficacy of this type of approach may soon be a reality in the context of PD and *GBA* mutations.

4. Conclusions

The discovery of the association between mutations of the *GBA* gene and PD allowed important considerations and discoveries that are contributing to a better understating of the pathogenesis of PD. Indeed, after this initial observation, the role of lysosomal impairment has been extensively explored in PD. A growing amount of emerging evidence supports the idea that the endolysosomal trafficking is involved in alpha-synuclein accumulation and dopaminergic neuron degeneration. A number of genes involved in monogenic forms of PD or genetic risk factors for the disease (such as *SNCA*, *ATP13A2*, *VPS35*, *DNAJC6*, *SYNJ1*, *LRRK2*, *RAB39B*) are part of this pathway (for review see [110]). Mutations of genes involved in the endolysosomal pathways are responsible for a group of disorders designated as Lysosomal Storage Disorders (LSD). These are typically rare autosomal recessive diseases which cause systemic involvements with variable degrees of severity and neurological involvement, usually presenting during childhood (reviewed in [111]). It is interesting to note that an increased burden of LSD-associated mutations has been identified in the screening of large PD populations compared to controls [112]. At the same time, among the 39 new gene loci associated with PD reported in the largest genome wide association study (GWAS) performed in PD patients so far, a number of these variants
were found in LSD-associated genes (i.e., *NAGLU*, *GUSB*, *NEU1*, and *GRN*) [113].

The case of autosomal recessive conditions causing severe and rare disorders during childhood, which in turn present as genetic risk factors for common adult neurodegenerative disorders when in a heterozygous state, appears to be more and more frequent, usually presenting an incomplete penetrance. This is the case for a number of LSD in the context of PD or of a parkinsonian degeneration, such as *SMPD1* (sphingomyelin phosphodiesterase, Niemann–Pick disease), *ATP13A2* (P5-type ATPase, Kufor–Rakeb disease), *GALC* (galactosylceramidase, Krabbe disease), *NPC1* (Niemann–Pick type C),
NAGLU (α-N-acetylglucosminidase, Sanfilippo syndrome B, or mucopolysaccharidosis III disease B (MPS-IIIB)), *HEXB* (β-hexosaminidase B, Sandhoff disease (GM2 gangliosidosis)) (summarized in [114]). The association between *GBA* mutations, GD, and PD must be just the tip of the iceberg of a larger phenomenon, where the association between genes initially considered responsible only for autosomal recessive disorders turned out to be risk factors for common neurodegenerative conditions. This association may have been recognized first in GD patients because of the higher frequency of this disease compared with other LSD.
Interestingly, this is also the case for the *TREM2* gene (encoding for Triggering Receptor Expressed on Myeloid cells 2), which seems to be the most frequent genetic risk factor of another common neurodegenerative disorder, Alzheimer's disease (AD) [115]. Autosomal recessive mutations of *TREM2* are responsible for the rare, juvenile condition known as Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy. Of note, *TREM2* plays a crucial role in microglia cells as part of the phagocytic scavenger pathway [116].

The phenomena of one gene presenting with different phenotypes is becoming more common in the context of neurological disorders and in respect to common diseases, such as PD and AD. It is important for clinicians to be familiar with these concepts in order to be able to properly counsel their patients and their family members. Also, the identification of such patients will hopefully offer more effective treatments, once available.
These new insights into the understanding of neurodegenerative diseases and, in particular, PD open new scenarios that only a few years ago were still totally obscure. Hopefully, these discoveries will be important for a real discernment of these severe conditions and for the discovery of more effective therapeutic approaches.

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