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CLINICAL AND BIOCHEMICAL SIGNATURES OF GBA-RELATED PARKINSON DISEASE

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PhD dissertation of
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PREFACE

This research project consists of three studies conducted during my appointment as a research fellow at the Department of Brain and Behavioral Sciences and at the Movement Disorder Center of IRCCS Mondino Foundation.

Part of the third study was undertaken at the "Department of Clinical Neurosciences, UCL, Royal Free Campus" of London (UK), where I worked as a research fellow during my training period abroad from September 2017 to April 2018 before the beginning of the PhD course. This activity is part of the GBA-PARK Project, a multinational collaborative effort supported by The Joint Program for Neurodegenerative Diseases (JPND) that involves the IRCCS Mondino Foundation as an official partner.

CONTENTS

ABSTRACT

Background: Parkinson's disease (PD) is a common neurodegenerative disorder mainly characterized by dopaminergic neuronal loss in the substantia nigra and α-synuclein protein aggregation. Genetic factors are well known to contribute to PD susceptibility. Mutations in the glucocerebrosidase (GBA) gene are the commonest genetic risk factor for PD and also impact on disease development and progression. A better clinical and genetic classification of patients, as well as the identification of clinical and biochemical markers are therefore of utmost importance for multifold reasons:

- to improve the characterization of patient's clinical phenotype of different forms of PD;
- to identify reliable biomarkers for genetic subtypes of PD in order to obtain and early diagnosis and monitor disease progression;
- to include patients with specific mutations in ad hoc clinical trials aiming to tailor medical treatment.

Objective: The present project aims at exploring the bases of PD phenotype through the correlation of specific genetic and biochemical findings with the clinical picture, in subjects with GBA-related PD (GBA-PD) and subjects affected by idiopathic PD (non-mutated PD – NM-PD). We divided the investigation in three studies: i) definition of a clinical and biochemical profile which could distinguish GBA-PD from non-mutated PD (NM-PD) (study I); ii) longitudinal evaluation of the disease course of GBA-PD compared to NM-PD along 2-year follow-up, with a focus on clinical and biochemical parameters (study II); iii) definition of a biochemical prodomal profile in the asymptomatic GBA carriers that could differentiate subjects more at risk to develop PD.

Methods: a comprehensive clinical assessment of motor and non-motor symptoms alongside the analysis of α-synuclein levels, glucocerebrosidase (GCase) enzymatic activity and main GCase-related lysosomal proteins in peripheral blood mononuclear cells (PBMCs) were performed in all the three studies.

Results: At baseline, GBA-PD showed a worse clinical outcome both on motor and non-motor features compared to NM-PD, as well a distinctive biochemical profile in PBMCs showing

significantly higher α –synuclein levels, lower GCase activity, higher LIMP-2 and lower Saposin C levels.

Over time, both the GBA-PD and the NM-PD groups separately displayed a significant deterioration in dysautonomic functions, motor performance, cognitive functions and mood disorder compared to baseline, while GBA-PD had a more severe motor progression with a higher disease severity compared to NM-PD. At 2-year follow-up, the level of α-synuclein in PBMCs was able to differentiate GBA-PD from NM-PD and HC.

Finally, a unique biochemical profile was observed also in the asymptomatic GBA mutation carriers, in which the combination of higher level of α-synuclein with lower Gcase activity was able to define a malignant prodromal profile.

Conclusion: These studies contribute to our current understanding of the role of GBA mutations in the development and progression of PD. We confirm the biological effect of GBA mutations in determining clinical and biochemical distinctive profile of PD. We propose essays in PBMCs as an easily accessible and manageable model to provide a characteristic biochemical profile of GBA carriers, potentially useful for patient stratification or selection in clinical trials.

1. INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder mainly characterized by dopaminergic neuronal loss in the substantia nigra and α-synucleinprotein aggregation.

Genetic factors are well-known to contribute to PD susceptibility. In a subset of patients, the disease recognizes a strong genetic component, caused by dominant mutations (SNCA, LRRK2 genes) or recessive mutations (Parkin, PINK1, DJ-1 genes)[1]. In particular, a single mutation (G2019S) in the LRRK2 gene has been identified as responsible of about 2% of cases of sporadic PD and up to 8% of familial cases in the Caucasian population[2]. More recently, heterozygous mutations in the glucocerebrosidase (GBA) gene, encoding for lysosomal enzyme beta-glucocerebrosidase (GCase), represent the commonest genetic risk factor for PD, occurring in 7-15% PD patients and conferring a 5-25% increased risk of developing the disease[3–5].

Given the relevance and frequency of GBA-related PD (GBA-PD) and the intensive research efforts towards the development of targeted therapeutic strategies, the identification of reliable biomarkers for this genetic PD subtype represents a still unmet need.

Here in this short introduction, we briefly summarize the role of GBA mutations in PD.

1.1 GLUCOCEREBROSIDASE MUTATION IN PARKINSON DISEASE

Interest in GBA as a causative factor for PD followed the clinical observation in the 1990s that among Gaucher Disease (GD) patients and GBA mutation carriers a higher proportion developed Parkinsonian symptoms[6,7].

A confirmation of the association between PD and GBA came from a large multicenter study conducted on 5691 patients and 4898 controls. The study found that GBA mutations were significantly prevalent among a heterogeneous population of PD with an odds ratio of 5.43[3].

Subsequently, several genetic studies have demonstrated a strong association between GBA mutation and an increased risk of PD with aging. Although the proportion of PD patients with GBA mutation varies by ethnicity and sequencing methods used, recent studies suggested that the heterozygous status confers a cumulative risk of developing PD of 5% at age 60, rising to 15-30% at age 80[8,9]. GBA is now considered as one of the most common genetic risk factor for PD, with a mutation prevalence estimated between 2.35 to 9.4% among PD population, arising 31.3% in the PD Ashkenazi Jewish population[10]. The frequency of GBA-related PD in Italy is among the highest worldwide among non–Ashkenazi Jewish populations, rising the 14.3% of

PD subjects, also contributing to a significant proportion of early-onset PD cases[11]. The penetrance of GBA variants in PD is low, age-specific, and controversial across studies, with an estimated 9.1% of carriers developing PD over their lifetime[12]. GD patients and asymptomatic heterozygous GBA mutation carriers are at equal risk of developing PD[13]. Furthermore, not all GBA mutant carriers will develop PD; different GBA mutations have been suggested to result in different risks of developing PD[14,15].

The mechanism by which GBA mutations are linked to PD is still poorly understood. However, several hypotheses have been proposed over the past years on the possible ways by which GBA mutations induce a-synuclein accumulation and PD development[16]. Accumulation evidence support the hypothesis that multiple mechanisms, such as alpha-synuclein accumulation, neuroinflammation, mitochondrial deficiency, autophagic dysfunction, and oxidative stress[5,17] have a role in PD pathogenesis. Moreover, the failure in PD of the autophagic and lysosomal pathway, essential for a-synuclein clearance, lead to aggregation determining dopaminergic neuronal death[18,19].

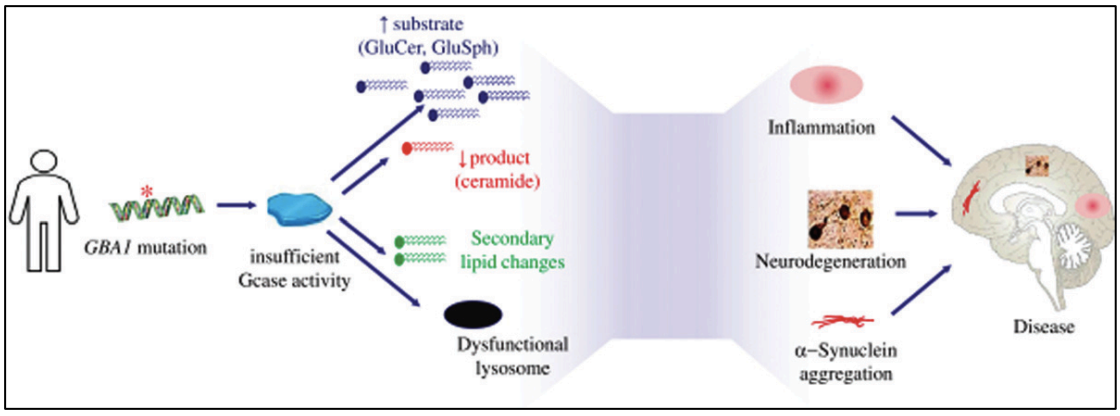

Figure 1 – Pathophysiology of Parkinson disease associated which GBA mutations[20].

1.2 THE LINKAGE BETWEEN GLUCOCEREBROSIDASE AND ALPHA-SYNUCLEIN

One of the key pathological hallmarks of PD is the accumulation of α-synuclein aggregates in the cells of patients. Although the mechanisms linking GBA mutations to PD still remain unclear, a vicious circle between GCase and α-synuclein has been elucidated, with GCase reduction leading to α-synuclein accumulation and in turn, increased α-synuclein inhibiting residual GCase function[21,22]. Experimental findings and clinical observations led to three main hypotheses that may explain this relationship. These hypotheses may not be mutually exclusive and multiple mechanisms might be in place.

The first proposes that a gain-of-function by the misfolded GCase results in its direct interaction with α-synuclein, which then leads to increase α-synuclein accumulation and aggregation[14]. This would require that GCase is able to interact directly with α-synuclein, and that GCase is present in abnormal protein aggregates containing α-synuclein, such as Lewy bodies. Presence of GCase in the brain tissues samples from PD-GBA patients was detected in 32–90% of Lewy bodies and neurites, showing that mutant GCase and α-synuclein co-localize in vivo[4]. One piece of evidence supporting a gain-of-function by the misfolded GCase was provided by a cell model study, where over-expression of mutant GCase in neural cells led to increase in α-synuclein levels [23] (Fig. 4). The relationship between misfolded GCase and SNCA was further strengthened by animal model studies. A progressive increase in α-synuclein levels was shown in the hippocampus of a homozygous GBA mouse model, and a significant increase in α-synuclein levels was detected in the forebrain and cerebellum of hypomorphic prosaposin mice carrying the homozygous V394L GBA mutation [23,24].

The loss-of-function hypothesis proposes that reduced GCase activity and GluCer substrate accumulation leads to perturb lipid homeostasis and subsequently affects α-synuclein trafficking, processing, and clearance. This promotes a-synuclein aggregation and facilitates a-synuclein oligomer formation[14,22,25].

The third hypothesis proposes the existence of a bidirectional feedback loop in which GCase deficiency facilitates formation of α-synuclein oligomers, leading to decrease in normal GCase activity, which in turn promotes formation of additional α-synuclein oligomers [22].

Gegg et al.[26] determined that in cell models increased α-synuclein causes a decrease in GCase activity and protein levels, as overexpression of exogenous SNCA in SH-SY5Y cell lines resulted

in about 44–70% decrease in GCase activity, and about 33–87% decrease in GCase protein levels. Interestingly, a recent study highlighted the importance of the lysosomal protein cathepsin D in mediating the increase of monomeric α-synuclein levels associated with GBA1 mutations, indicating a new player in the relationship between GCase and α-synuclein [27.]

Finally, despite the large body of evidence in favor of the link between GCase and α-synuclein via loss-, gain-of function, or bidirectional feedback loop, neither of this mechanism explains why only a proportion of individuals with GBA mutations develop PD suggesting that other factors must be playing a role. One plausible explanation might be that in order to develop PD, in addition to the GBA mutation, other genetic alternations are involved. Moreover, the mutated GCase on its own is not sufficient to induce α-synuclein pathology and PD can develop only when other changes occur.

1.3 PATHOGENIC MUTATIONS OF GBA-ASSOCIATED PD

More than 300 mutations in the GBA gene, such as insertions, deletions and point mutations, have been discovered so far[9,28]. The N370S (c.1226A > G) and the L444P (c.1448T > C) mutations are the most common mutations worldwide[29].

A recent meta-analysis described other GBA variants, such as R120W, IVS2 + 1G＞A, H255Q, D409H, RecNciI, E326K, and T369M related to PD risk. Ethnic heterogeneity of GBA mutations in PD was also reported. R496H and 84insGG increase PD risks exclusively in AJ populations, while L444P, E326K, T369M, R120W, IVS2+1G>A, H255Q, D409H and RecNciI were found more frequently in non-AJ subjects. N370S is correlated to increased PD risk in all populations[30].

1.4 GENOTYPE-PHENOTYPE CORRELATION IN GBA-PD SUBJECTS

GBA mutations may have differential effects on PD risks, depending on the specific variant, as well as on the clinical profile and disease progression rate. For mild GBA mutation carriers, the odds ratios for developing PD ranged between 2.84 and 4.94, while for severe GBA mutation carriers the odds ratios were between 9.92 and 21.29[31]. The risk for dementia in GBA-PD subjects bearing severe mutations (L444P, splicing mutation IVS10+1G>T) is 5.6 times greater than in PD patients and 2.9 folds greater than in patients carrying mild GBA mutations (N370S)[32]. The risk of hallucinations development is not affected by the severity of GBA mutations[33].

In a more recent study, motor and some non-motor features (depression, RBD, and olfactory loss) were significantly worse in GBA-PD patients with severe mutations than in those bearing mild mutations[34].

E326K is the most prevalent PD-associated GBA mutation and has not been described in GD[35]. PD patients bearing E236K mutations show a faster progression of motor symptoms, with gait disorders and postural instability. They also have a higher risk of cognitive decline, but a lesser risk for motor complications[36–38].

Data on genotype-phenotype correlations between other GBA polymorphisms, clinical features and risk rate of progression are lacking. Further research is therefore mandatory, in

order to better clarify the role of GBA variants in specific clinical manifestations and disease progression.

1.4.1 Prodromal features in GBA mutation carriers

A substantial body of evidence suggests the existence of a prodromal phase of idiopathic PD, of variable length, preceding the onset of the classical signs of the disease[39–41]. The existence of such premotor period has been proposed based on imaging, pathology, clinical and epidemiological studies, suggesting that the nigrostriatal lesion might evolve in 7-10 years before becoming clinically manifest[39,42]. Although stiffness, tremor or imbalance may be present as prodromal features[43], the vast majority of early symptoms and signs in PD are of the non-motor type, including hyposmia, REM behavior disorder (RBD), constipation, depression, and color discrimination [40,42,44].

A prodromal phase was also described in both GD and heterozygous GBA carriers without a clinical diagnosis of PD[45,46]. Cognitive and olfactory functions were significantly impaired, and motor testing was abnormal, in GD patients and GBA mutation carriers without PD, compared to healthy subjects. No differences were found in terms of sleep abnormalities or autonomic function[47,48].

2. AIMS OF THE STUDY

Despite several studies have attempted to assess the different phenotypic features of patients with PD, the impact of genetic variants on the risk of developing specific phenotypes is still poorly defined. A better clinical and genetic classification of patients, as well as the identification of clinical and biochemical markers are therefore of utmost importance to:

- improve the characterization of patient's clinical phenotype of different forms of PD;
- identify reliable biomarkers for genetic subtypes of PD in order to obtain and early diagnosis and monitor disease progression;
- include patients with specific mutations in ad hoc clinical trials aiming to tailor medical treatment.

The present project aims at exploring the bases of the PD phenotype through the correlation of specific genetic and biochemical findings with the clinical picture, in subjects with GBA-related PD (GBA-PD) and subjects affected by idiopathic PD (non-mutated PD – NM-PD).

The main objectives of this thesis were:

1- the identification of a clinical profile of GBA-PD by comparing motor and non-motor features with a group of NM-PD (Study I);

2- the identification of a biochemical profile of GBA-PD by comparing α-synuclein levels in plasma, exosomes and in peripheral blood mononuclear cells (PBMCs), as well as GCase-related lysosomal proteins (GCase, lysosomal integral membrane protein-2 (LIMP-2), Saposin C, Cathepsin D and lysosome-associated membrane glycoprotein 1 (LAMP-1) in PBMCs obtained from PD patients with and without GBA mutations (Study I);

3- the evaluation of the clinical and biochemical profile of GBA-PD over a 2-year follow-up compared with the NM-PD group (Study II);

4- the investigation of changes in the GCase activity and α-synuclein levels in PBMCs in a cohort of asymptomatic subjects carrying both heterozygous and homozygous GBA mutations and whether these parameters are associated with a more severe prodromal PD profile.

3. MATERIALS AND METHODS

3.1. PARTICIPANTS

Study I-II

These studies involved patients with PD with and without GBA mutations and genetically unrelated healthy individuals (HC) that were recruited at Mondino Foundation - IRCCS from 2018 to 2020. All patients provided written informed consent to participate in the study. The study was approved by the local Ethics Committee.

Inclusion criteria were: i) a diagnosis of PD according to the MDS-PD criteria[49]; ii) duration of motor symptoms ≥3 years; iii) H&Y stage ≤3. Exclusion criteria were: i) diagnosis of dementia; ii) any other neurological or systemic diseases; iii) presence of a pathogenic mutation in another major PD-related gene. Controls had no neurological disease or systemic disease that could impair motor function.

At baseline, we recruited 66 PD (29 GBA-PD and 37 NM-PD) and 40 HC as control group (Study I).

All the participants from baseline were invited to take part in the 2-year follow-up assessment (Study II). We used a rolling recruitment model to enroll new participants throughout the duration of the study. At 2-year follow-up, 43 subjects (17 GBA-PD, 13 NM-PD, 13 HC) completed the 2-year assessment. Some subjects were excluded because they no longer met the inclusion criteria or were too frail to undergo assessment. The main reasons of dropout were failure to contact or withdrawal of consent.

Study III

A total of 98 subjects were considered for this study. This sample included 17 heterozygous GBA mutation carriers (Het GBA), 13 Homozygous GBA mutation carriers (Hom GBA), 29 patients with GBA-PD and 39 HC. The subjects were enrolled at IRCCS Mondino Foundation and at Department of Clinical and Movement Neurosciences at UCL Queen Square Institute of Neurology (London) while the Hom GBA subjects were enrolled at Lysosomal Storage Disorder Unit at the Royal Free London NHS Foundation Trust. Inclusion criteria for the asymptomatic GBA carriers were the presence of GBA mutations and absence of any neurological or systemic conditions neither PD nor dementia. Inclusion and exclusion criteria for the GBA-PD and HC were the same used in the studies I and II.

3.2. GENOTYPING

All participants were genotyped in order to confirm their GBA mutation status using Sanger sequencing of the GBA gene50. Identified variants were confirmed by repeating amplification and sequencing with alternative primers. In all subjects, pathogenic variants and rearrangements in the other major PD-related genes (SNCA, LRRK2, PARK2, PINK1 and DJ-1) were previously excluded.

3.3. CLINICAL EVALUTATION

In all three studies, all subjects underwent a complete neurological assessment, including the Unified Parkinson's Disease Rating Scale motor subscale (MDS-UPDRS part III), the University of Pennsylvania Smell Identification Test (UPSIT), the Montreal Cognitive assessment (MoCA), the REM behavior Disorder Questionnaire (RBDq) with the Parkinson's Disease Sleep Scale (PDSS), the Beck's Depression Inventory (BDI), and the Scale for Outcomes in Parkinson Disease – Autonomic (SCOPA-AUT).

Study II. The above described clinical work-out was performed at 2-year follow-up evaluation.

3.4. BIOCHEMICAL EVALUATION

Details in the Supplementary Materials.

Study I. A 35-ml blood sample was obtained from all subjects for isolation of whole plasma, exosomes and PBMCs. α-synuclein levels in plasma and exosomes were tested by ELISA [51]. In PBMCs, expression levels of α-synuclein, GCase, LIMP-2, Saposin C, Cathepsin D and LAMP-1 were assessed by Western blotting, while GCase activity was measured fluorometrically.

Study II. At the 2-year follow-up, all subjects underwent a blood test to measure the GCase enzymatic activity, α-synuclein levels in PBMCs and the expression of the main GCase-related lysosomal proteins (GCase, LAMP1, LIMP2, Saposin C).

Study III. A 35-ml blood sample was obtained from all subjects for isolation of PBMCs. GCase enzymatic activity and α-synuclein levels were measured fluorometrically and by Western blotting, respectively.

3.5. STATISTICAL ANALYSIS

Statistical analysis was performed using "Stata" version v.13.0 (StataCorp, Texas). We set statistical significance at $p<0.05$ for all statistical tests performed.

Study I and II. Groups were homogenous for gender, age and years of education. For categorical variables, differences between the groups were tested with the Fisher's exact test. The following analysis were performed both at baseline (T0) and at 2-year follow-up assessment (T1). Clinical data comparison among PD groups (GBA-PD and NM-PD) was performed with Wilcoxon signed-rank test. Biochemical data comparison among the three groups (GBA-PD, NM-PD, HC) was performed by Kruskal-Wallis test followed by Dunn's Pairwise test (Bonferroni adjustment). Percentage of fold changes (relative to controls) for each biochemical parameter was next calculated in GBA-PD and NM-PD groups; differences were analysed using Student's t-test. Correlations between biochemical and clinical parameters were assessed by Spearman test.

The intra-group analysis for both the clinical and biochemical data ("T0" vs "T1") in each group separately, was performed with the Mann–Whitney test.

At T0, GBA-PD were further subdivided into sub-groups according to mutation severity; groups comparison for both clinical and biochemical data was performed using Kruskal-Wallis test followed by Dunn's Pairwise test (Bonferroni adjustment). The unknown variants were excluded from the comparison analysis. At T1, due to the small sample size of each GBA-PD subgroups a descriptive analysis was performed.

Study III. First, we performed a correlation analysis (Pearson correlation coefficient) between biological parameters –GCase activity and α-synuclein levels– and the single clinical variables, to assess the presence of a relationship between biological and clinical profiles. Moreover, motor and non-motor features (UPDRS-III, BDI, SCOPA-AUT, MoCA, RBDsq, PDSS, UPSIT) for prodromal PD of each subjects were merged in a 7-item cumulative clinical index (CI). Specifically, we considered each scale as an item, and according with the references listed below the corresponding cut-off were used (MDS-UPDRS-III >3[49]; MoCA <26[52]; BDI >9[53]; RBDSQ >4[54]; SCOPA-AUT ≥14[55]; UPSIT ≤27[56,57]; PDSS <82[58]) as a criterion to classify subjects' scores in a set of binary variables. We assigned a 0 when subjects' performance/score was within a normal range (0=absence of a deficit), and 1 when their performance/score was below the cut-off (1=presence of a deficit). For each subject, the CI score was then computed by dividing the sum of the deficits presented by the total number of variables measured. The

correlation analysis (Pearson correlation coefficient) between biological parameters and CI score was also performed in the whole sample.

Then, we used a classification procedure to split the whole sample into different clusters on based on their biochemical profile analysing GCase and α –synuclein separately and in combination independently from the GBA mutation status. In particular, we performed a Two Step Cluster Analysis using i) GCase; ii) a-synuclein and ii) combined GCase and α-synuclein as clustering variables. A major advantage of this classification approach is related to the determination of the number of clusters, which is not grounded on an arbitrary choice like more traditional clustering techniques, but rather relies on a statistical measure of fit (e.g., Bayesian information criterion or BIC, like in our case). One-way ANOVA assessed the effect of cluster analysis groupings on the CI. Finally, for each clinical variable, we calculated the positive and negative Post-Test Probability considering Het GBA and Hom GBA subjects polled together in the combined GCase/α-synuclein clusters. Briefly, positive and negative Post-Test Probability indicate the probability of being included or not within the combined GCase/α-synuclein clusters previously identified.

4. RESULTS

Study I

Among GBA-PD subjects, 7 patients carried severe variants (L444P, R296X, R131C), 7 had the mild variant N370S, 10 carried risk alleles (T369M or E326K) and 1 had a complex allele. In 4 patients (carrying E388K, M85V or R272C), the severity of the variants could not be assessed [11] (Table 1).

Table 1. GBA variants identified in GBA-PD group

Cases	Allele name	Aminoacidic change	Nucleotidic change	Exon	Class of mutation	RS (dbSNP)/ CM (HGMD)
5	L444P	p.Leu483Pro	c.1448T>C	10	severe	rs421016
1	R296X	p.Arg296Ter	c.886C>T	8	severe	rs1553217626
7	N370S	p.Asn409Ser	c.1226A>G	9	mild	rs76763715
5	E326K	p.Glu365Lys	c.1093G>A	8	risk	rs2230288
5	T369M	p.Thr408Met	c.1223C>T	8	risk	rs75548401
2	E388K	p.Glu427Lys	c.1279G>A	9	unknown	rs149171124
1	M85V	p.Met124Val	c.370A>G	5	unknown	rs758455177
1	- -	p.Gly234Glu p.Pro189Leu	c.701G>A c.566C>T	7 6	Complex	CA342723758+ rs74462743
1	R131C	p.Arg170Cys	c.508C>T	8	severe	rs398123530
1	R272C	p.Arg209Pro	c.626G>C	7	unknown	CM980831

Groups were comparable for age, although GBA-PD subjects had an earlier disease onset but similar disease duration (Table 2) and showed worse scores at the MoCA, RBDsq, PDSS, UPDRS-III, BDI, UPSIT and SCOPA-AUT scales (Table 3).

Table 2. Demographics data of the study cohort

	HC n= 40	GBA-PD n= 29	NM-PD n= 37	p (between)		
				p1	p2	p3
Male, n (%)	15 (38)	20 (69)	25 (68)	0.25	0.07	1.0
Age (yrs)	60.6 ± 7.1	58.7 ± 9.4	61.2 ± 6.7	0.18	1.0	0.14
Age at onset (yrs)		51.1 ± 10.3	57.2 ± 9.2	-	-	0.01*
Disease duration (yrs)		6.6 ± 4.8	7.7 ± 4.7	-	-	0.36

*Data are presented as mean ± SD. Group comparison performed with one-way ANOVA for age and years of education, Fisher exact test was used for sex variable. p1: HC vs GBA-PD; p2: HC vs NM-PD; p3: GBA-PD vs NM-PD. *Significant difference.*

Table 3. Clinical data of PD groups (GBA-PD and NM-PD)

	GBA-PD n= 29	NM-PD n= 37	p
H&Y	2.1 ± 0.6	1.6 ± 0.6	0.01
UPDRS-III	18.0 ± 10.8	14.6 ± 5.4	0.04
MoCA	21.5 ± 3.4	23.6 ± 2.3	0.002
BDI	7.9 ± 5.3	4.6 ± 2.7	<0.001
UPSIT	17.7 ± 5.8	22.3 ± 3.4	<0.001
RBDsq	4.8 ± 3.3	3.3 ± 1.5	0.01
PDSS	99.8 ± 30.5	112.6 ± 17.1	0.02
SCOPA-AUT	12.7 ± 6.5	9.9 ± 3.5	0.01

Data are reported as means as mean ± SD. p: GBA-PD vs NM-PD. All differences were statistically significant.

Among GBA-PD, no significant difference was observed between groups in the subgroup analysis according mutation severity except for BDI scores that were significantly higher in the mild subgroups compared to risk variant subgroup (Table 4).

On the biochemical side, both GBA-PD and NM-PD groups showed significantly higher levels of exosomal, but not plasma, α-synuclein, compared to controls. When assessing PBMCs, GBA-PD significantly differed from other groups for higher levels of α-synuclein and lower GCase activity, while NM-PD behaved similarly to controls (Table 5).

When comparing relative changes (relative to controls), we did not observe significant differences between GBA-PD and NM-PD groups in both plasma and exosomal α-synuclein levels, the latter being similarly elevated in both groups (Figure 1A-B). Conversely, Biochemical analysis of PBMCs disclosed a GBA-PD specific profile, characterized by significantly higher α-

synuclein levels and significantly lower GCase activity compared to NM-PD (Figure 1C-D). Moreover, measurement of GCase-related lysosomal proteins showed lower Saposin C and higher LIMP-2 levels in GBA-PD compared to NM-PD (Figure 1E-F). No significant differences between groups were found for the other GCase-related lysosomal proteins (Figure 2).

Table 5. Biochemical data of the study cohort

	HC n= 40	**GBA-PD n= 29**	**NM-PD n= 37**	**p (between)**		
				p1	**p2**	**p3**
Plasma total α-synuclein (ng/mL)	14.7 ± 3.1	15.8 ± 3.4	14.1 ± 4.5	0.30	0.61	0.07
Exosomal α -synuclein (pg/mL)	14.2 ± 10.9	22.0 ± 16.1	22.9 ± 10.2	<0.001*	<0.001*	0.41
PBMCs - α-synuclein (% HC)	100.5 ± 19.9	145.3 ± 42.9	103.8 ± 26.7	<0.001*	0.84	<0.001*
PBMCs - GCase activity (nmol/mg protein/h)	10.0 ± 2.9	5.5 ± 1.1	9.3 ± 2.9	<0.001*	0.51	<0.001*
PBMCs - LIMP-2 protein (% HC)	103.3 ± 33.2	107.1 ± 30.5	92.8 ± 25.3	0.44	0.14	0.01*
PBMCs - Saposin C protein (% HC)	100.7 ± 14.6	92.0 ± 17.1	106.8 ± 19.2	0.06	0.30	0.002*
PBMCs - LAMP-1 protein (% HC)	100.9 ± 16.0	99.4 ± 33.5	96.0 ± 22.9	0.50	0.12	0.79
PBMCs - GCase protein (% HC)	101.5 ± 28.9	109.9 ± 67.7	120.4 ± 58.2	1.0	0.33	0.32
PBMCs - Cathepsin D protein (% HC)	100.3 ± 25.1	99.9 ± 34.7	97.3 ± 23.2	0.83	0.70	1.0

*Data are reported as mean ± SD. Group comparison performed with Kruskal-Wallis followed by post-hoc analysis with Dunn's Pairwise Comparison test (Bonferroni correction). p1: HC vs GBA-PD; p2: HC vs NM-PD; p3: GBA-PD vs NM-PD. *Significant difference.*

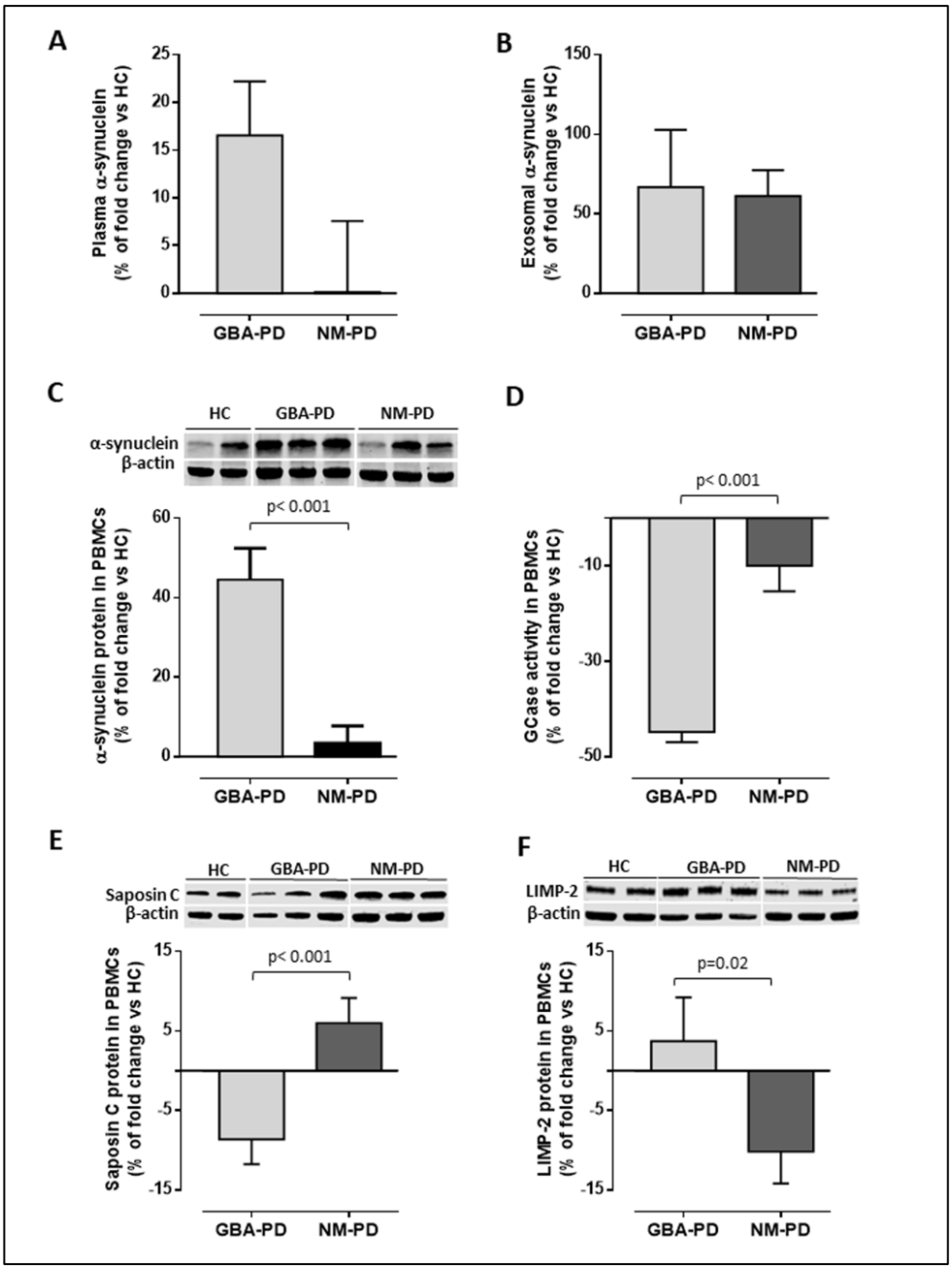

Figure 1 - Bar graph of the percentage fold changes of A) plasma α-synuclein; B) exosomal α-synuclein; C) α-synuclein in PBMCs; D) GCase activity in PBMCs; E) PBMCs levels of LIMP-2 and F) Saposin C in GBA-PD and NM-PD relative to controls.

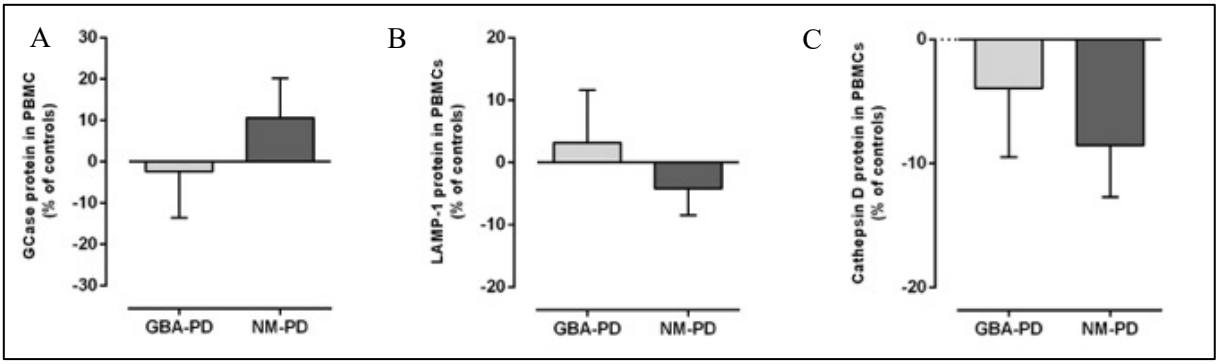

Figure 2. PBMCs protein levels of GCase-related lysosomal proteins in GBA-PD and NM-PD relative to controls. A) GCase; B) LAMP-1; C) Cathepsin D.

When GBA-PD were stratified by mutation type, carriers of severe variants showed higher levels of PBMCs a-synuclein compared to other categories, while GCase activity was significantly higher in carriers of risk variants than in the other subgroups (Table 6).

Investigation of clinical-biochemical links in GBA-PD group showed a negative correlation between PBMCs α-synuclein and MoCA scores (r=-0.44, p=0.01) (Table 7).

Table 4. Clinical parameters of the GBA-PD group according to genotype stratification

	GBA-PD						
	Mild (M) n= 7	Risk (R) n= 10	Severe (S) n= 8	Unknown (U) n= 4	P M vs R	M vs S	R vs S
H&Y	1.3 ± 0.5	1.7 ± 0.6	1.6 ± 0.5	1.3 ± 0.6	NS	NS	NS
UPDRS III	20.2 ± 12.1	22.1 ± 11.3	14.8 ± 12.7	12 ± 5.3	NS	NS	NS
MoCA	23.6 ± 2.6	20.8 ± 3.7	22.7 ± 3.5	21.2 ± 7.1	NS	NS	NS
BDI	10.6 ± 8.8	4.4 ± 3.1	7.1 ± 4.2	7.7 ± 6.0	**0.009***	NS	NS
UPSIT	15.8 ± 4.7	16.3 ± 5.1	15.8 ± 2.6	24.3 ± 3.1	NS	NS	NS
RBDsq	3.2 ± 1.5	5.6 ± 5.9	5.4 ± 3.4	5.6 ± 5.5	NS	NS	NS
PDSS	94.2 ± 25.0	103.6 ± 34.6	104.5 ± 37.6	115.3 ± 39.7	NS	NS	NS
SCOPA-AUT	11.8 ± 8.8	10.7 ± 5.4	11.7 ± 9.2	8.0 ± 6.1	NS	NS	NS

Data are reported as means (± standard deviation). Group comparison performed with Kruskal-Wallis followed by post-hoc analysis with Dunn's Pairwise Comparison test. *statistically significant differences.

Table 6. Biochemical parameters of the GBA-PD group according to genotype stratification

	GBA-PD						
	Mild (M) n= 7	Risk (R) n= 10	Severe (S) n= 8	Unknown (U) n= 4	p M vs R	M vs S	R vs S
Plasma total α -synuclein, (ng/mL)	16.2 ± 3.1	16.9 ± 4.1	16.9 ± 4.1	17.1 ± 1.4	NS	NS	NS
Exosomal α -synuclein (pg/mL)	20.7 ± 7.7	22.2 ± 19.2	22.2 ± 19.2	20.2 ± 2.2	NS	NS	NS
PBMCs α -synuclein (% HC)	139.9 ± 58.2	135.5 ± 32.6	167.1 ± 33.7	135.2 ± 52.8	NS	0.05	**0.03***
PMBC - GCase activity (nmol/mg protein/h)	4.9 ± 0.9	6.6 ± 0.8	4.6 ± 0.5	5.3 ± 1.1	**0.004***	NS	**0.0003***
PMBC - LIMP-2 protein (% HC)	115.5 ± 22.8	109.3 ± 38.7	94.1 ± 22.6	113.1 ± 20.7	NS	NS	NS
PMBC - Saposin C protein (% HC)	103.5 ± 13.5	86.6 ± 10.3	81.7 ± 48.7	106.1 ± 16.3	NS	**0.02***	NS
PMBC - LAMP-1 protein (% HC)	92.1 ± 27.2	107.6 ± 38.8	86.5 ± 30.8	117.9 ± 31.5	NS	NS	NS
PMBC - GCase protein (% HC)	78.3 ± 34.8	121.1 ± 78.7	105.6 ± 54.4	146.1 ± 101.4	NS	NS	NS
PMBC - Cathepsin D protein (% HC)	105.4 ± 22.2	109.9 ± 48.3	90.6 ± 24.9	83.5 ± 28.4	NS	NS	NS

Data are reported as means (± standard deviation). Group comparison performed with Kruskal-Wallis followed by post-hoc analysis with Dunn's Pairwise Comparison test. *statistically significant differences.

Table 7. Correlation analysis between biochemical and clinical parameters in GBA-PD group.

	GBA-PD *n= 29*						
	UPDRS-III	**MoCA**	**BDI**	**UPSIT**	**RBDsq**	**PDSS**	**SCOPA-AUT**
Plasma total α -synuclein, (ng/mL)	**0.4447***	-0.0925	0.3353	-0.1715	0.0197	-0.0242	-0.1676
Exosomal α -synuclein (pg/mL)	-0.0581	-0.1452	0.2500	-0.1904	-0.1994	-0.1864	0.2260
PBMCs α -synuclein (% HC)	-0.2114	**-0.4370***	-0.2550	-0.2449	0.0364	-0.0173	-0.1200
PBMCs GCase activity (nmol/mg protein/h)	-0.2136	-0.1972	**-0.4079***	0.2936	-0.0681	0.1157	0.0352
PBMCs LIMP-2 protein (% HC)	-0.0725	-0.0890	-0.0309	0.2750	-0.0884	-0.1459	0.0303
PBMCs Saposin C protein (% HC)	0.2799	-0.0420	0.1032	0.1445	0.0874	0.2271	-0.3581
PBMCs LAMP-1 protein (% HC)	-0.2176	-0.2081	0.0235	0.0871	-0.1170	-0.1513	0.2147
PBMCs GCase protein (% HC)	0.0071	0.0242	-0.2293	0.0581	-0.2114	**0.4550***	-0.4538
PBMCs Cathepsin D protein (% HC)	0.2844	0.1787	-0.1445	-0.2400	-0.1206	-0.1167	-0.2916

Data are reported as Spearman's Rho coefficient. * Significant difference: p=<0.05 from Spearman correlation analysis.

Study II - ***evolution of clinical and biochemical markers over 2 years***

After 2 years of observation, 43 subjects (17 GBA-PD, 13 NM-PD, 13 HC) completed the follow-up assessment. Subjects' demographic characteristics are shown in Table 8. No significant difference in gender and disease duration was found between groups, while age at follow-up was lower in the GBA-PD group.

Table 8. ***Demographics data of the study cohort at 2-year follow-up.***

	HC	GBA-PD	NM-PD	P (between)		
	n= 13	n= 17	n= 13	P1	p2	p3
Male, n (%)	8 (61.5)	11 (64.7)	8 (61.5)	>0.05	>0.05	>0.05
Age at follow-up (yrs)	65.8 ± 3.4	61.3 ± 7.9	66.2 ± 6.7	0.01*	0.86	0.11
Disease duration (yrs)		8.3 ± 4.3	9.8 ± 5.4	-	-	0.24

*Data are presented as mean ± SD. Group comparison performed with one-way ANOVA for age and years of education, Fisher exact test was used for gender variable. p1: HC vs GBA-PD; p2: HC vs NM-PD; p3: GBA-PD vs NM-PD. *Significant difference.*

Among GBA-PD subjects, 5 patients carried severe variants (L444P, R296X, R131C), 4 had the mild variant N370S, 5 carried risk alleles (T369M or E326K) and 1 had a complex allele. In 2 patients (carrying M85V or R272C), the severity of the variants could not be assessed[11] (Table 9).

Table 9. GBA variants identified in GBA-PD group at 2-year follow-up

Cases	Allele name	Aminoacidic change	Nucleotidic change	Exon	Class of mutation	RS (dbSNP)/ CM (HGMD)
2	L444P	p.Leu483Pro	c.1448T>C	10	severe	rs421016
1	R296X	p.Arg296Ter	c.886C>T	8	severe	rs1553217626
4	N370S	p.Asn409Ser	c.1226A>G	9	mild	rs76763715
3	E326K	p.Glu365Lys	c.1093G>A	8	risk	rs2230288
2	T369M	p.Thr408Met	c.1223C>T	8	risk	rs75548401
1	M85V	p.Met124Val	c.370A>G	5	unknown	rs758455177
1	R272C	p.Arg209Pro	c.626G>C	7	unknown	CM980831
1	- -	p.Gly234Glu p.Pro189Leu	c.701G>A c.566C>T	7 6	Complex	CA342723758+ rs74462743
2	R368C	p.Arg368Cys	c.508C>T	8	severe	rs398123530

At 2 years, GBA-PD group had significantly worse scores than NM-PD in UPDRS-III (p=0.002) and HY (p=0.008) (Table 10).

In the within group analysis (T0 vs T1), the GBA-PD displayed a significant deterioration in the UPDRS-III (p=0.001), HY (p=0.001), BDI (p= 0.002), MoCA (p=0.049) and SCOPA-AUT (p =0.002) compared to baseline. The NM-PD group displayed a significant worsening in UPDRS-III (p=0.002), HY (p=0.03), UPSIT (p=0.01), SCOPA-AUT (p=0.009), BDI (p=0.03) and MoCA (p =0.009) compared to baseline (Table 10 and Figure 3).

On biochemical side at 2-year evaluation, GBA-PD significantly differed from other groups (NM-PD and HC) for higher PBMCs levels of α-synuclein and lower GCase activity, while NM-PD behaved similarly to controls (Table 11). No differences between groups were observed for the other lysosomal proteins measured. In the within group analysis (T0 vs T1), HC showed a significant reduction in GCase activity compared to baseline (p= 0.005); no other differences emerged in the other biochemical parameters in the longitudinal analysis (Table 11).

When comparing relative changes (to controls) at T1, GBA-PD showed the unique profile observed at baseline, characterized by significantly higher α-synuclein levels and significantly lower GCase activity compared to NM-PD. No significant differences between PD groups were found for the other GCase-related lysosomal proteins (Fig. 4).

When GBA-PD were stratified by mutation type, carriers of mild variants displayed a more benign outcome than risk or severe variants in particular regarding motor and cognitive performance, while depression seemed prevalent comparing with the other mutations (Table 12.a). On biochemical side, carriers of severe variants showed higher levels of PBMCs a-synuclein compared to other categories, while GCase activity was higher in carriers of risk variants than in the other subgroups (Table 12.b).

Investigation of clinical-biochemical correlations in NM-PD group showed a negative correlation between PBMCs α-synuclein and MoCA scores (r=-0.87, p=0.02).

Tab.10 – Evolution of clinical markers over 2 years and comparison between groups.

		GBA-PD (n=17)	NM-PD (n=13)	*p (between)*
H&Y	Baseline	1.5 ± 0.6	1.5 ± 0.6	
	Follow-up	2.3 ± 0.5	1.8 ± 0.6	**<0.001**
	p (within)	**<0.001**	0.06	
UPDRS-III	Baseline	15.9 ± 5.5	11.5 ± 3.6	
	Follow-up	25.3 ± 10.4	14.9 ± 3.7	**0.008**
	p (within)	**<0.001**	**0.002**	
MoCA	Baseline	21.9 ± 2.9	25.1 ± 2.2	
	Follow-up	20.4 ± 2.9	21.63 ± 2.7	0.59
	p (within)	**0.049**	**0.009**	
BDI	Baseline	8.6 ± 5.9	5.8 ± 2.9	
	Follow-up	12.2 ± 7.4	9.9 ± 6.9	0.40
	p (within)	**0.002**	**0.03**	
UPSIT	Baseline	15.8 ± 6.3	20.0 ± 3.3	
	Follow-up	15.6 ± 5.2	16.6 ± 2.9	0.22
	p (within)	0.83	**0.01**	
RBDsq	Baseline	4.8 ± 3.0	4.1 ± 3.1	
	Follow-up	5.0 ± 3.0	4.1 ± 3.1	0.55
	p (within)	0.90	0.93	
PDSS	Baseline	99.4 ± 34.4	103.5 ± 23.8	
	Follow-up	95.8 ± 31.8	110.3 ± 27.2	0.13
	p (within)	0.93	0.27	
SCOPA-AUT	Baseline	12.2 ± 7.8	12.1 ± 7.8	
	Follow-up	17.1 ± 8.3	16.5 ± 7.4	0.69
	p (within)	**0.002**	**0.009**	

Data are presented as mean ± SD. Intra-group comparisons performed with the Wilcoxon signed-rank test, between-group comparison with the Wilcoxon–Mann–Whitney test. *Significant difference.

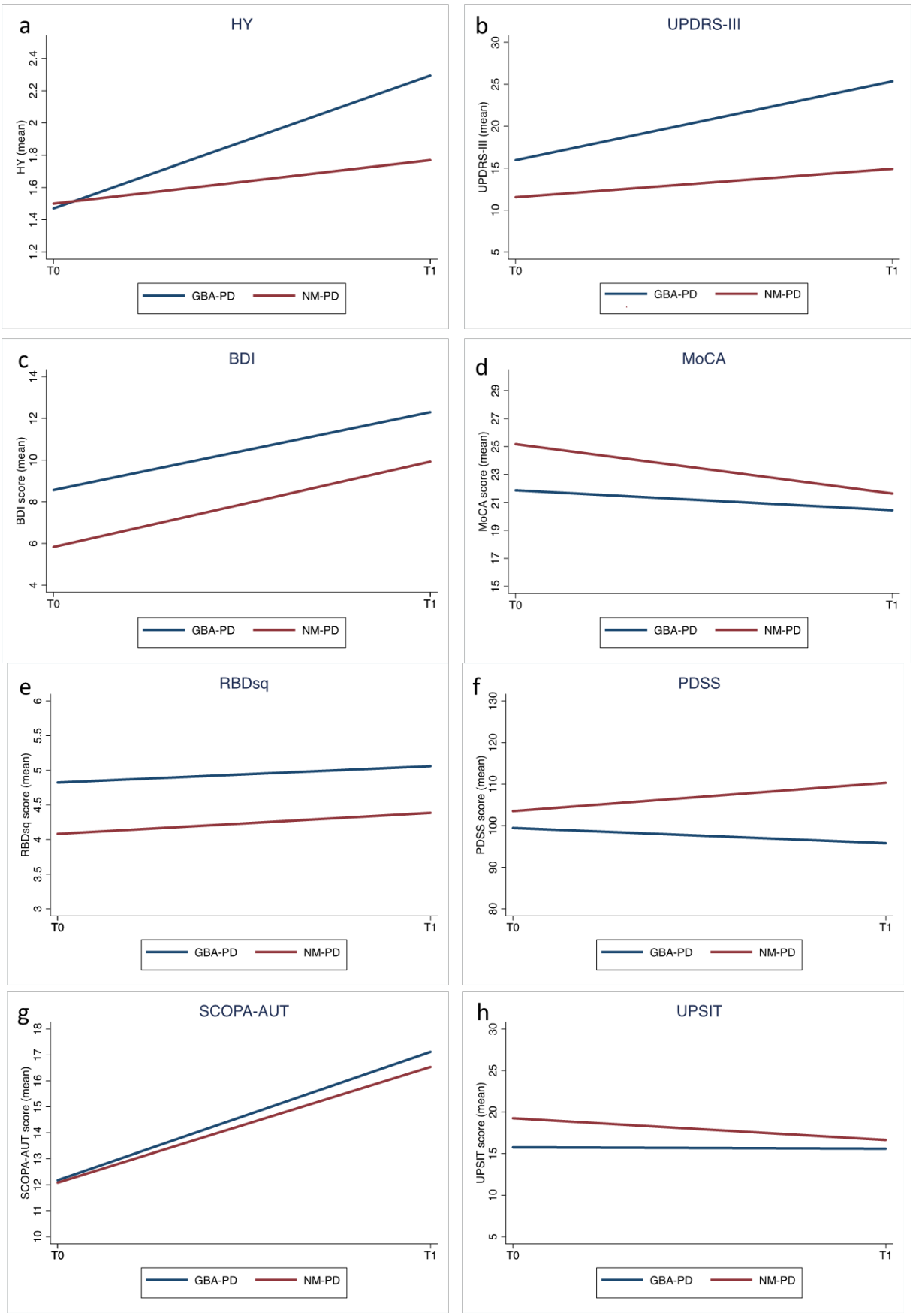

Figure 3 – Evolution of clinical markers over 2-year follow-up. The graphs show the mean scores at basaline and T1 for GBA-PD and NM-PD group. a) HY; b) UPDRS-III; c) BDI; d) MoCA; e) RBDsq; f)PDSS; g) SCOPA-AUT; h) UPSIT.

Tab 11. Evolution of biochemical parameters over 2 years and comparison between groups

		HC n=13	**GBA-PD n=17**	**NM-PD n=13**	***p (between)***		
					p1	p2	p3
PBMCs – α-synuclein (% HC)	Baseline	97.8 ± 12.5	154.4 ± 46.7	111.61± 26.9			
	Follow-up	100.8 ± 14.5	131.6 ± 15.2	105.6 ± 31.4			
	p (within)	0.44	0.06	0.42	**0.001**	0.86	**0.007**
PBMCs – GCase activity (nmol/mg protein/h)	Baseline	10.4 ± 1.8	5.5 ± 0.9	8.7 ± 3.9			
	Follow-up	8.1 ± 1.2	5.4 ± 1.4	8.5 ± 2.2			
	p (within)	**0.005**	0.89	0.86	**<0.001**	1.0	**<0.001**
PBMCs – LIMP-2 protein (% HC)	Baseline	95.3 ± 16.8	105.9 ± 19.4	85.7 ± 14.5			
	Follow-up	100.3 ± 15.5	102.5 ± 26.2	91.7 ± 15.5			
	p (within)	0.37	0.76	0.21	1.0	0.22	0.33
PBMCs - Saposin C) protein(% HC)	Baseline	101.1 ± 19.0	87.8 ± 20.3	102.4 ± 26.1			
	Follow-up	99.8 ±5.7	100.7 ± 33.6	91.9 ± 25.7			
	p (within)	0.86	0.23	0.26	0.15	0.16	1.0
PBMCs - LAMP1 protein (% HC)	Baseline	98.7 ± 10.3	101.1 ± 31.7	100.7 ± 23.6			
	Follow-up	100.5 ± 6.2	104.2 ± 17.6	96.7 ± 13.0			
	p (within)	0.72	0.50	0.60	1.0	0.39	0.26
PBMCs - GCase protein (% HC)	Baseline	105.1 ± 18.1	94.4 ± 44.7	101.1 ± 26.2			
	Follow-up	97.0 ± 13.6	85.5 ± 29.9	102.5 ± 33.9			
	p (within)	0.39	0.51	0.60	0.14	1.0	0.26

Data are presented as mean ± SD. Intra-group comparisons performed with the Wilcoxon signed-rank test; *between-group comparison performed with Kruskal-Wallis followed by post-hoc analysis with Dunn's Pairwise Comparison test (Bonferroni correction). p1: HC vs GBA-PD; p2: HC vs NM-PD; p3: GBA-PD vs NM-PD. *Significant difference.*

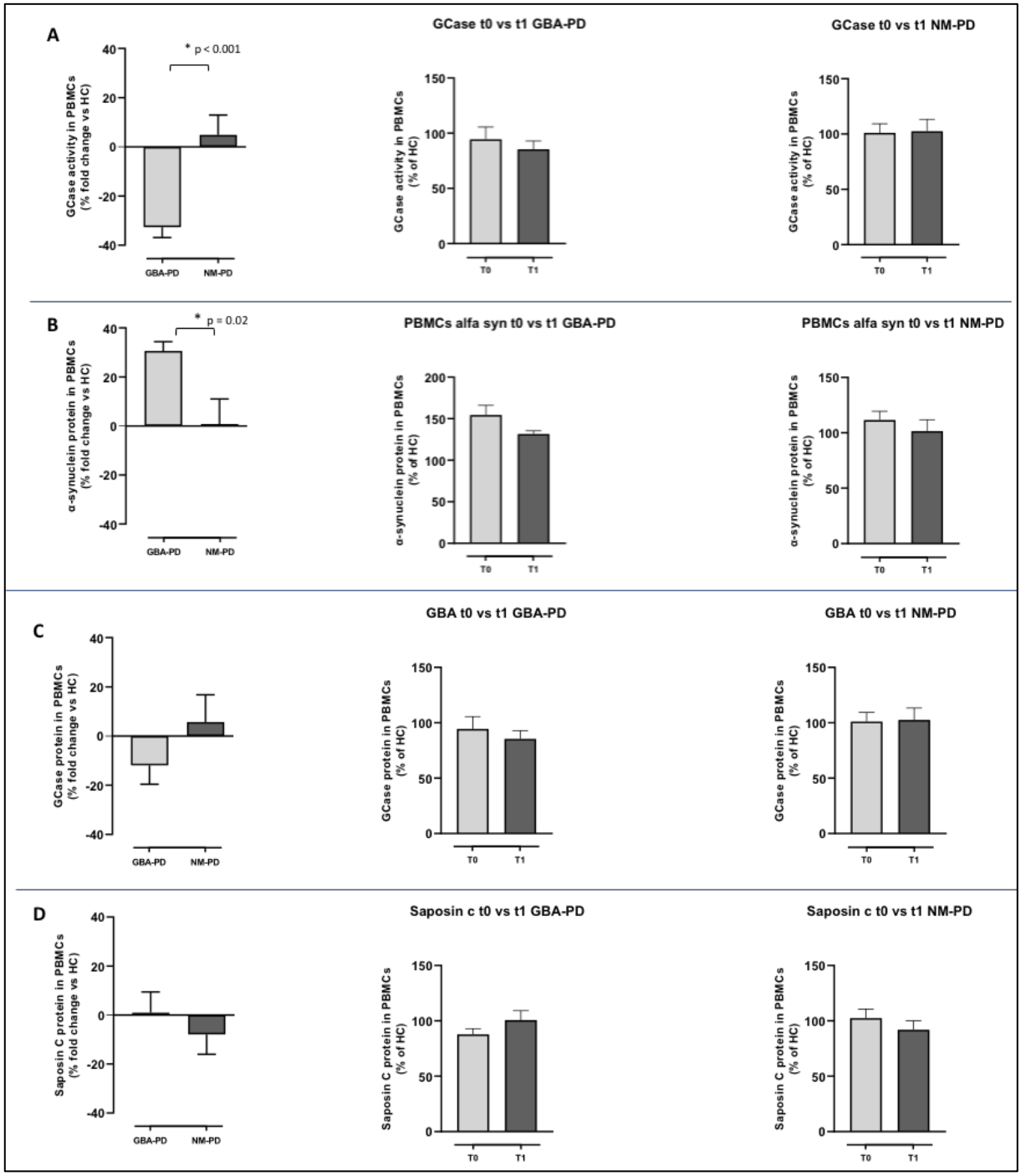

Figure 4 - Bar graph of the percentage fold changes of A) GCase activity in PBMCs; B) α-synuclein in PBMCs; C) Gcase protein levels in PBMCs; D) Saposin C protein levels in PBMCs; E) levels of LIMP-2 and F) LAMP-1 levels in PBMCs in GBA-PD and NM-PD relative to controls.

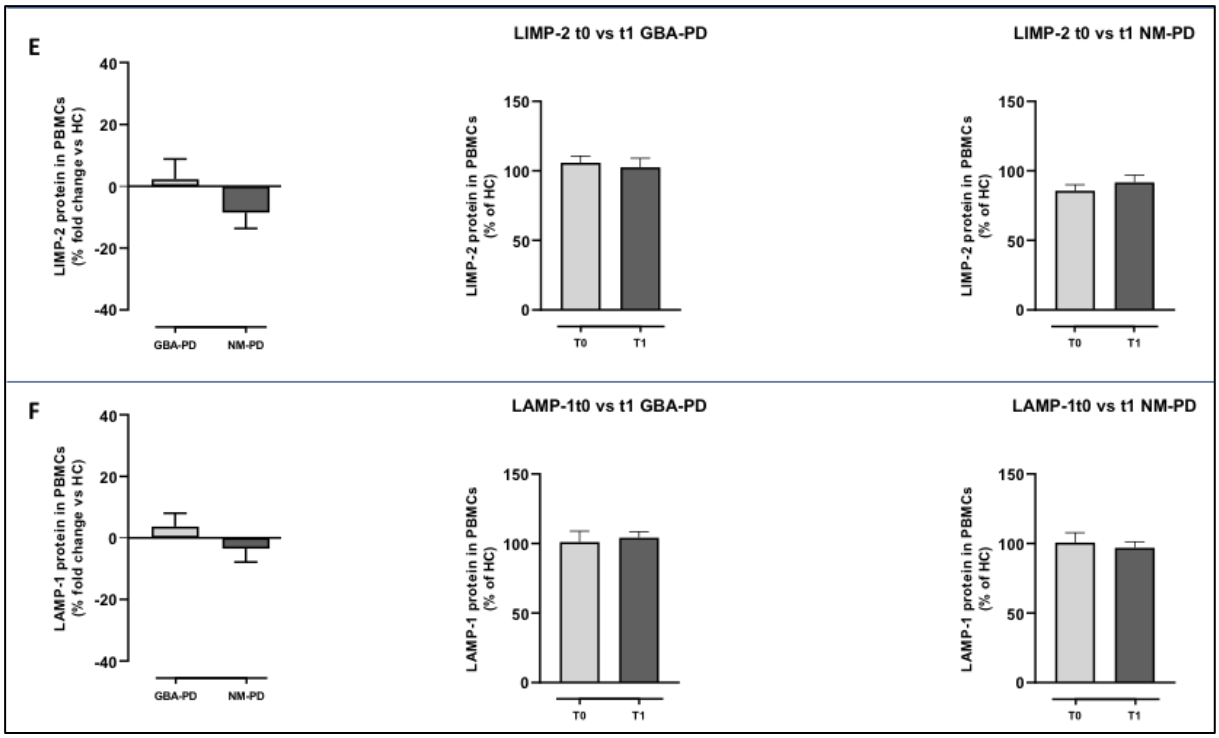

Figure 4 - Bar graph of the percentage fold changes of E) levels of LIMP-2 and F) LAMP-1 levels in PBMCs in GBA-PD and NM-PD relative to controls.

Table 12.a. Clinical parameters of the GBA-PD group according to genotype stratification at 2-year follow-up

	Mild (M) n= 4	Risk (R) n= 5	Severe (S) n= 6	Unknown (U) n= 2
H&Y	2.3 ± 0.5	2.6 ± 0.6	2.2 ± 0.4	2.0 ± 0
UPDRS III	23.0 ± 10.2	29.8 ± 14.4	26.0 ± 7.7	17.0 ± 5.7
MoCA	22.30 ± 2.2	20.0 ± 3.7	19.5 ± 2.7	21.0 ± 0.2
BDI	16.0 ± 8.5	4.4 ± 3.1	10.5 ± 5.5	12.0 ± 11.3
UPSIT	17.0 ± 9.3	14.8 ± 3.9	14.2 ± 3.5	19.0 ± 1.4
RBDsq	4.5 ± 2.9	5.6± 1.7	5.0 ± 3.4	5.0 ± 5.7
PDSS	97.0 ± 25.0	97.8 ± 38.5	90.6 ± 34.4	101.5 ± 33.2
SCOPA-AUT	20.3 ± 9.6	15.2 ± 8.3	16.3 ± 8.8	18 ± 9.9

Data are reported as means (± standard deviation).

Table 12.b. Biochemical parameters of the GBA-PD group according to genotype stratification at 2-year follow-up

	Mild (M) n= 4	Risk (R) n= 5	Severe (S) n= 6	Unknown (U) n= 2
PBMCs α -synuclein (% HC)	128.12 ± 19.19	125.83 ± 7.73	139.35 ± 13.70	133.67 ± 29.40
PMBC - GCase activity (nmol/mg protein/h)	4.91 ± 1.30	5.75 ± 1.55	5.60 ± 1.60	5.43 ± 1.24
PMBC - LIMP-2 protein (% HC)	96.87 ± 24.95	112.27 ± 25.47	106.39 ± 31.14	80.05 ± 15.90
PMBC - Saposin C protein (% HC)	97.01 ± 34.15	93.64 ± 27.96	113.56 ± 47.64	93.85 ± 5.17
PMBC - LAMP-1 protein (% HC)	91.38 ± 3.89	114.27 ± 24.59	100.04 ± 11.70	114.92 ± 11.57
PMBC - GCase protein (% HC)	82.42 ± 45.37	69.17 ± 17.80	103.99 ± 27.26	86.09 ±0.84

Data are reported as means (± standard deviation).

Study III - Prodromal PD Patterns in asymptomatic GBA Carriers

A total of 98 subjects were considered for the study. This sample included asymptomatic heterozygous and homozygous GBA carriers (17 Het GBA, 13 Hom GBA), 29 GBA-PD and 39 healthy controls.

Details of demographic and clinical characteristics of the sample groups, are reported in table 13.

Table 13. Demographic and clinical parameters of the study cohort.

	Het GBA n=17	***Hom GBA n=13***	***GBA-PD n=29***	***HC n=39***	**p (between)**
Gender (male:female)	9:8	9:4	19:10	15:24	0.053
Age *(yrs)*	60 ± 12	56 ± 10	57 ± 10	63 ± 9	0.052
Education *(yrs)*	14 ± 3	15 ± 3	11 ± 3	12 ± 4	>0.05
MDS-UPDRS-III	2.2 ± 2.5	3.4 ± 4.6	18.9 ± 10.2	0.1 ± 0.3	<0.001*
MoCA	27.6 ± 1.7	26.6 ± 2.6	21.4 ± 3.4	28.8 ± 0.8	<0.001*
BDI	4.3 ± 3.3	8.7 ± 10.3	8.0 ± 5.4	1.8 ± 2.1	<0.001*
SCOPA-AUT	5.9 ± 2.8	8.4 ± 7.3	12.8 ± 6.6	4.1 ± 3.3	<0.001*
RBDSQ	2.9 ± 2.7	4.1 ± 3.8	4.8 ± 3.3	1.0 ± 0.9	<0.001*
PDSS	115.8 ± 21.1	109.8 ± 28.8	99.9 ± 31.0	130.8 ± 8.5	<0.001*
UPSIT	31.2 ± 4.3	33.4 ± 3.5	17.5 ± 5.9	32.1 ± 2.6	<0.001*

*Data are presented as mean ± SD. Group comparison performed with one-way ANOVA; Fisher exact test was used for gender variable. *Significant difference.*

On the biochemical side, the Hom GBA group displayed a significantly lower Gcase activity compared with the other groups. Between heterozygous GBA carriers, GCase activity was significantly lower in the affected GBA-PD group (p=0.04). The PBMCs a-synuclein level was significantly higher in the GBA-PD group and in the GBA carriers (both Hom GBA and Het GBA) compared to the HC group (both p< 0.001) (Table 14).

Tab 14. Biochemical parameters of the study cohort.

	Het GBA n=17	*Hom GBA n=13*	*GBA-PD n=29*	*HC n=39*	**P (between)**
PBMCs – GCase activity (nmol/mg protein/h)	7.6 ± 2.2	1.2 ± 0.9	5.5 ± 1.2	10.1 ± 2.9	P^1<0.001* p^2=0.04*
PBMCs – α-synuclein (% HC)	136.4 ± 107.8	136.3 ± 101.2	146.2 ± 43.4	100.7 ± 20.2	P^3<0.001* P^4<0.001* P^5<0.001*

*Data are presented as mean ± SD. Group comparison performed with one-way ANOVA. p1: Hom vs other groups; p2: GBA-PD vs Het GBA; p3: GBA-PD vs HC; p4: Het GBA vs HC; p^5 Hom GBA vs HC. *Significant difference.*

The correlation analysis between the biochemical and clinical variables in the whole sample showed that GCase activity was significantly linked with all clinical variables except for UPSIT (r=0.17, p=0.092). Specifically, while we found a positive relationship linking GCase with MoCA performance (r=0.26, p<0.05) and PDSS score (r=0.31, p<0.01), we observed a negative relationship with MDS-UPDRS-III (r=-0.30, p<0.005), BDI (r=-0.38, p<0.001), RBDsq (r=-0.30, p<0.005), and SCOPA-AUT (r=-0.28, p=0.005).

Concerning PBMCs α-synuclein level, we observed a significant negative relationship with MoCA (r=-0.25, p<0.05) and a positive relationship with RBDsq (r=0.24, p<0.05). Moreover, both GCase activity and a-synuclein level are significantly correlated to the clinical index (CI) score (rGCase=-0.39, p<0.001; rα-syn=0.21, p=0.037) in the whole sample.

Two Step Cluster Analysis based on GCase activity divided the whole sample into three clusters (mean silhouette=0.7), namely low (mean=1.34) medium (mean=6.57) and high (mean=12.34) GCase clusters. The clustering procedure on PBMCs α-synuclein divided the whole sample into two clusters (mean silhouette=0.7), indicating a mid-low α-synuclein cluster (mean=97.09) and a high α-synuclein (mean=209.41) cluster (table 15). However, the combined GCase/ α-synuclein levels provided the best performance splitting the sample in two clusters (average silhouette=0.5), the high GCase/mid-low α-synuclein (mean Gcase=8.28, mean α-syn=94.23) and the low GCase/high α-synuclein cluster (mean Gcase=4.51; mean α-syn=193.34), discriminating the HC group from both GBA carriers and GBA-PD. Details about subjects' membership to clusters a reported in Table 15 and figure 5.

Table 15. Subjects' cluster membership to the different clusters.

	Het GBA (n=17)	Hom GBA (n=13)	GBA-PD (n=29)	HC (n=39)
Gcase cluster				
Low GCase (n=14, 14.3%)	0 (0.0%)	13 (93.3%)	1 (6.7%)	0 (0.0%)
Medium GCase (n=62, 63.3%)	15 (24.6%)	0 (0.0%)	28 (44.3%)	19 (31.1%)
High GCase (n=22, 22.4%)	2 (9.1%)	0 (0.0%)	0 (0.0%)	20 (9.9%)
α-synuclein clusters				
High α-synuclein (n=25, 25.5%)	8 (33.3%)	7 (29.2%)	9 (33.3%)	1 (4.2%)
Mid-low α-synuclein (n=73, 74.5%)	9 (12.3%)	6 (8.2%)	20 (27.4%)	38 (52.1%)
Gcase/α-synuclein cluster				
Low GCase & High α-synuclein (n=40, 40.9%)	8 (20%)	11 (27.5%)	**21(52.5%)**	**0 (0.0%)**
High GCase & Mid-low α-synuclein (n=58, 59.1%)	9 (24.6%)	2 (3.4%)	8 (13.8%)	**39 (58.2%)**

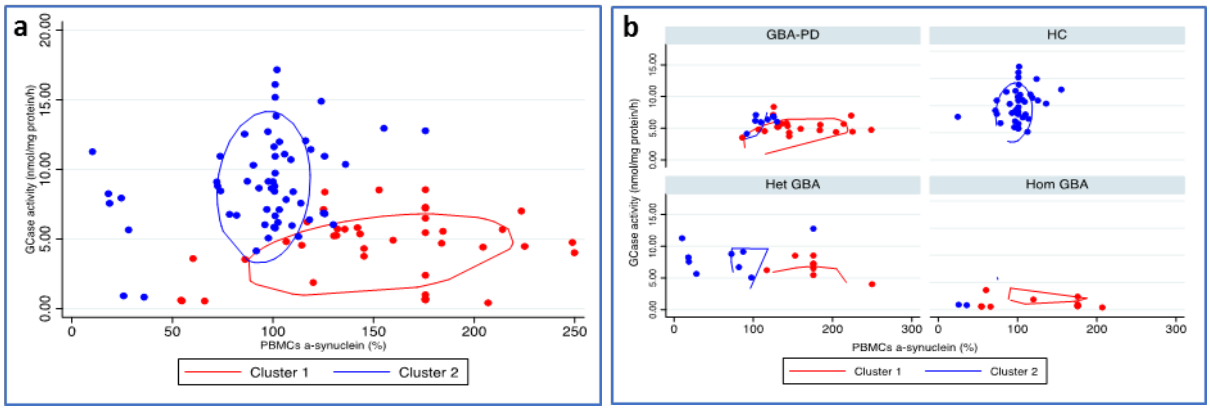

Figure 5. a) combined GCase/ α-synuclein clusters; b) groups' membership to the combined GCase/ α-synuclein clusters according to different groups (Cluster 1: low GCase/high α-synuclein; Cluster 2: high GCase/mid-low α-synuclein).

When we assessed the effect of cluster analysis groupings on the CI, we found a significant effect of combined GCase/α-synuclein clusters ($F(1,95)=15.495$, $p<0.001$) revealing a significant difference between high GCase/mid-low α-synuclein and low GCase/high α-synuclein clusters, discriminating subjects with a benign profiles (high GCase/mid-low α-synuclein) against a malignant profile (low GCase/high α-synuclein). The medium GCase cluster showed a significant main effect on the CI compared to the high GCase cluster

(p=0.001), while we did not find a significant effect of α-synuclein clusters suggesting that α-synuclein levels are not able alone to discriminate subjects with different clinical profiles (Fig. 6).

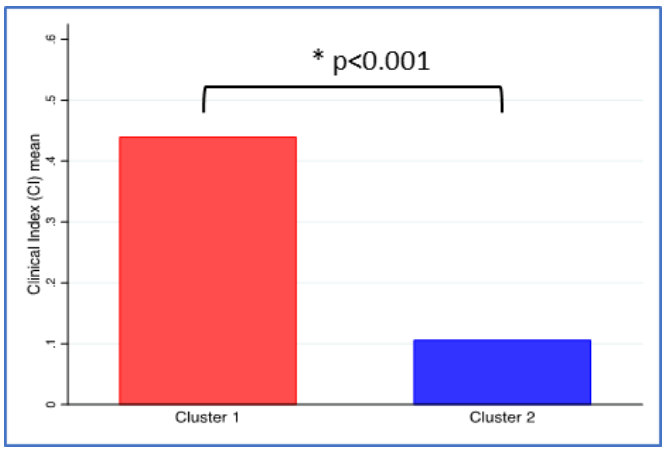

Figure 6. Effect of the conbined GCase/ α-synuclein clusters on the clinical index (CI).
Cluster 1: low GCase/high α-synuclein; Cluster 2: high GCase/mid-low α-synuclein.

Finally, within the GBA carriers (both Het GBAand Hom GBA) the positive post-test probability reveling that dysautonomia, mood and sleep disorders were the most relevant features of the low GCase/high α-synuclein cluster (malign profile) (Table 16).

Table 16. Positive and negative Post-Test Probability in GBA carriers with high low GCase/α-synuclein as a reference condition.

	pPTP	nPTP
MDS-UPDRS-III	20%	***60%***
MoCA	40%	48%
BDI	***80%***	40%
SCOPA-AUT	***67%***	44%
RDBSQ	***57%***	45%
PDSS	33%	48%
UPSIT	33%	48%

The table shows for each clinical variable the positive (pPTP) and negative (nPTP) Post-Test Probability in Het GBA and Hom GBA subjects polled together, with high low GCase/α-synuclein as a reference condition. > 50% are considered significant.

5. DISCUSSION

Profiling the clinical and biochemical signature of GBA-Related PD (Study I)

Given the high frequency of GBA mutations among PD, a deep phenotypic and biochemical characterization of this genetic subgroup is now becoming mandatory[59,60]. Along with a better understanding of the molecular mechanisms predisposing to PD, clinical-biochemical profiles could be crucial to predict PD development in GBA carriers, also paving the way to personalized treatment strategies.

As α-synuclein is the best-known player in PD pathogenesis and progression, most biomarker studies have focused on the relationship between defective GCase activity and α-synuclein levels in biological fluids. Indeed, a lower GCase activity and reduced α-synuclein levels have been reported in the CSF of GBA-PD compared to NM-PD, with differences related to the severity of the mutation[61–63]. Yet, only few studies have explored this relationship in easily accessible body tissues such as blood of GBA mutation carriers, showing a good correlation between GCase activity reduction and increased levels of oligomeric α-synuclein in plasma and dimeric α-synuclein in erythrocytes[64,65].

To our knowledge, this is the first study reporting a thorough biochemical profiling of GBA-PD in blood, compared both with NM-PD patients and HC. Firstly, we assessed α-synuclein levels not only in total plasma but also in plasma exosomes, because of their ability to reflect brain-related pathological changes[66,67]. Then, we performed a complete characterization of PBMCs by measuring α-synuclein levels, GCase activity, as well as the expression levels of several GCase-related lysosomal proteins.

Although small variations in plasma α-synuclein levels were observed in GBA-PD and NM-PD compared with HC, no statistical differences emerged among groups, showing that this parameter is not distinctive for GBA-PD condition and confirming its unreliability as surrogate marker of synucleinopathy [68,69]. Conversely, we found significantly higher levels of exosome-associate α-synuclein in both PD groups compared to HC, in line with previous studies which specifically associated increased exosomal α-synuclein to PD[67,70,71]. The similar increase of exosomal α-synuclein in both PD groups suggests that this parameter is likely unrelated to GCase deficiency, but it might rather reflect the overall neurodegenerative process[72], as well as other PD-associated lysosomal dysfunctions[73].

Interestingly, PBMCs showed a unique biochemical profile that clearly distinguished GBA-PD from NM-PD. In fact, besides the clear reduction in GCase activity, we reported for the first time a significant increase of α-synuclein levels in PBMCs of GBA-PD compared to HC and NM-PD, while these two latter groups showed comparable levels, in line with previous data[74,75]. This difference could result from the synergistic effect of impaired GCase activity and dysregulation of chaperone-mediated autophagy observed in PBMCs of GBA-PD[76,77] , representing a potentially relevant biomarker of GBA-related disease. Of note, these parameters not only were able to differentiate GBA-PD from NM-PD and HC, but they also varied according to mutation severity, suggesting a potential utility as stratification biomarkers.

On the clinical side, as expected, we observed an increased prevalence of non-motor symptoms in GBA-PD compared to NM-PD, and worse scores in terms of motor functions, sleep disorders, cognition, olfactory functions and mood [9,34,78].

Moreover, we observed a negative correlation between PBMCs α-synuclein levels and MoCA scores in GBA-PD, suggesting that increased α-synuclein levels could mirror a more rapid progression of the disease, particularly on the cognitive side.

We did not observe any difference in GCase activity levels between NM-PD and HC. Despite some studies have reported a reduction of GCase activity in the brain of NM-PD patients[26,79], data concerning this activity in peripheral blood are still conflicting, showing small or negligible variations compared with HC[64,80,81].

Besides GCase and α-synuclein, PBMCs from GBA-PD also showed distinctive alterations in Saposin C and LIMP-2 levels compared to NM-PD. Saposin C facilitates GCase activity and protect the enzyme from intracellular proteolysis and α-synuclein-mediated inhibition[82] . The observed reduction of Saposin C in GBA-PD is a potential consequence of a negative feedback loop due to increased α-synuclein levels or GCase unavailability within lysosomes[83,84], and might further affect GCase function, increasing cell susceptibility to α-synuclein accumulation. Conversely, the increased levels of GCase transporter LIMP-2 could represent a compensatory mechanism to sustain GCase trafficking toward the lysosomes and, consequently, functionality. Overall, our findings highlight the key role of PBMCs as potential sources of biomarkers for PD[85,86], and suggest for the first time that lysosomal alterations in these cells may be considered valuable parameters to identify a biochemical profile distinctive of GBA-PD. Importantly, PBMCs can be collected easily and repeatedly throughout minimally invasive

procedures in comparison to CSF or skin biopsy, supporting their usefulness also in clinical trials.

Longitudinal clinical and biochemical changes of GBA-Related PD over 2-years (Study II)

With study II, we aim to explore the longitudinal disease course of GBA-PD patients compared to a group of NM-PD along a 2-year follow-up. For this purpose, we investigated the evolution of the clinical (motor and non-motor) features and the biochemical profile measuring the changes in the alpha-synuclein levels in PBMCs, GCase enzymatic activity and the expression of the main GCase-related lysosomal proteins.

GBA-PD individuals have an increased risk of having a faster disease progression and a more severe clinical phenotype than the NM-PD, developing at an earlier stage a more disability condition and a reduced autonomy in daily life activities[87]. However, is not clear why the rapid progression observed in PD subjects bearing GBA mutations is directly related to the GBA mutation itself or whether it reflects the difficulty in distinguish clinically the subjects form NM-PD. In this view, the identification of valuable biomarkers for PD subjects, may help to identify earlier the subjects most at risk of rapid progression, monitor the response to drug treatment and therefore to intervene in an earlier stage with device-aided therapies, such as deep-brain stimulation.

In our previous study (study I) we demonstrated that GBA-PD subjects in the early phases of the disease was characterized by a distinctive both clinical and biochemical profile able to differentiate them with NM-PD. In this work we combined the longitudinal clinical investigation with biochemical findings, in order to better understand the underlying neurodegeneration process in this specific mutated population and if the PBMCs a-synuclein may still represent a valuable biomarker of GBA-related disease.

At baseline, the two PD groups were matched for age and disease severity but showed worse outcome in all motor and non-motor features compared to the NM-PD. At 2-year follow-up, a significant deterioration in parkinsonian motor and non-motor symptoms from baseline was observed in both in GBA-PD and NM-PD group. However, despite the two PD groups were comparable for the disease duration, the GBA-PD subjects showed a major disease progression with higher disease severity (HY) and motor scores (UPDRS-III) than NM-PD. This finding characterized by a faster progression of the GBA-PD in particular in the motor

symptoms was in line with a previous study reporting, in a large cohort of GBA-PD patients, a greater deterioration in motor and functional impairment over 7 years follow-up from diagnosis[88]. The absence of differences in rate of non-motor symptoms progression over time between the two PD groups could be explain that in the advance stages of the disease we observe a global performances deterioration also in the NM-PD patients in which dysautonomia, cognitive impairment and mood disorders also develop in these subjects. Indeed, the difference in the non-motor features observed at the baseline could reflect a specific condition of the initial phases in which the GBA-PD are from the outset more strongly compromised.

Moreover, in our cohort, both GBA-PD and NM-PD subjects demonstrated a significantly lower MoCA score at 2 years follow-up, although a significant worsening between groups were not found. These findings, seems to be in contrast with some previous reports[78,89] in which the risk of progression toward dementia is increased in GBA-PD subjects. However, two longitudinal prospective studies with a small sample size showed inconsistent results regarding progression to dementia among GBA-PD compared to NM-PD[38,90]. Our finding regarding the cognitive profile could be explained by the presence at baseline of higher MoCA score in the NM-PD groups that in the GBA-PD, within normal values or mild cognitive impairment, while GBA-PD patients already at baseline showed an overt deterioration of cognitive functions. In addition, in the GBA-PD group the most cognitive compromised subjects were lost at the T1 evaluation, and the reduced number of subjects may have affected the statistical findings.

Finally, it is also possible that the different GBA mutations play a different role in the cognitive impairment in GBA-PD patients[32].

Nonetheless, on the biochemical side over time we observed significantly higher levels of PBMCs α-synuclein and lower GCase activity in the GBA-PD than NM-PD. Despite both the PBMCs α-synuclein levels and the GCase activity did not change from baseline within each group, these parameters in PBMCs were able to differentiate GBA-PD from NM-PD and HC, identifying a biochemical profile distinctive of GBA-PD not only in the early stages of the disease but also in the late stage. Although, at follow-up we do not observed specifically correlation of this parameters with the disease severity or the clinical features, we could confirm that they are proper of the GBA condition.

At follow-up, no other distinctive alterations emerged among PD groups for Saposin C and LIMP-2 levels nor for the other lysosomal GCase-related protein, showing that these parameters are not distinctive for the advanced stage of GBA-PD condition and confirming their peculiarity of the early stages. The previously reported (study I) reduction of Saposin C as well as the increase of LIMP2 could be related to a compensatory mechanism that act in the early stages of the disease to contrast the increased α-synuclein accumulation, becoming ineffective when the disease progress.

Although these findings must be taken with caution as they are derived from a small sample size with a limited number of GBA variant carriers, it is worth noting that the study was based on a solid prospective design, included control groups (NM-PD and HC) and combined a complete neurological assessment with the biochemical characterization of subjects. Indeed, other studies that evaluated longitudinal aspects of PD with GBA mutations were based on retrospective data or collected from non-specific clinical evaluations[88,91].

Our longitudinal observation highlights for the first time the role of the PBMCs a-synuclein as a valuable parameter able to distinguish the GBA-PD condition even in the advanced phases of the disease. However, given the prevalence of GBA-PD toward a faster motor progression and worsening of the disease stage, with this study we underlie the importance of the early detection of GBA-PD for defining prompt the disease prognosis and, especially, targeted early intervention.

Combined GCase/alpha-synuclein pattern may identify specific prodromal PD patterns in GBA carriers: a cluster analysis study (Study III)

Although GBA mutations are well recognized genetic risk factors for developing PD, both in the biallelic and in heterozygote carrier states[14,39], the underlying mechanisms that determine penetrance are incompletely understood. Hyposmia, cognitive dysfunction, autonomic dysfunction, RBD and depression are recognized prodromal features of PD and they are prevalent among GBA-positive individuals[48,60]. However, clinical features alone have still poor sensitivity to detect subtle changes which may characterize the evolution toward PD development. Hence, the identification of early diagnostic PD biomarkers able to accurately diagnose the disease years before the clinical major manifestation is mandatory.

With study III, we investigated in asymptomatic subjects carrying GBA mutations (GBA carriers) whether the biochemical parameters (GCase activity and PBMCs a-synuclein), previously investigated in Study I, were also altered in these subjects and how they were associated with a more severe clinical prodromal PD profile.

As expected, GCase enzymatic activity in Hom GBA was significantly lower compared with the other groups, while it was intermediate in Het GBA carriers. Reduced GCase enzymatic activity is reported in patients with PD and Lewy body dementia in cerebrospinal fluid, leucocytes and monocytes, suggesting a lower GCase enzymatic activity in peripheral cells may be a potential early marker of PD.

Of note, in the heterozygous carriers GCase activity was lower in the GBA-PD than the asymptomatic Het GBA and it is associated with higher PBMCs a-synuclein, suggesting that this difference could result from the synergistic effect of impaired GCase activity and dysregulation of chaperone-mediated autophagy observed in PBMCs of GBA-PD[76,77].

Moreover, PBMCs a-synuclein level in the asymptomatic GBA carriers (both Het and Hom GBA) were significantly higher compared to controls. Higher level of α-synuclein were found in serum of asymptomatic GBA carriers and they were correlated with higher combined clinical non motor features, suggesting that monitoring this parameter could be predictive of phenoconvertion[60]. To date, little is known about the total α-synuclein level in PBMCs of asymptomatic GBA carriers. A recent study published by Emekyanov et al.[92] reported increased PBMCs a-synuclein level in asymptomatic Het GBA carriers compared to NM-PD. Our findings are in line with these previous studies and strongly support the influence of GBA mutations in determining the increase a-synuclein level in PBMCs, even in the prodromal phase, years or decades before PD diagnosis.

As exploratory analysis, to specifically investigate the presence of a distinctive biochemical prodromal profile in the asymptomatic GBA carriers, a two-step cluster analysis was performed to split the subjects into different clusters based on their biochemical characteristic analysing GCase and α-synuclein separately and in combination.

Therefore, we found that the combined GCase activity/ α-synuclein levels cluster provided the best performance splitting the sample into a benign (high GCase/mid-low α-synuclein) and malignant (low GCase/high α-synuclein) profile, discriminating HC from both GBA carriers and GBA-PD. When we explore the effect of the biochemical cluster on the clinical index, we did not find a significant effect of the GCase and α-synuclein clusters alone on the clinical index,

suggesting that individually these parameters are not crucial to discriminate subjects with different clinical profiles. Nevertheless, a significant effect of the combined GCase/α-synuclein clusters on clinical index was found, revealing a significant difference between the malignant and the benign profiles, with the first showing significantly higher values in the clinical index with dysautonomia, mood and sleep disorders as the most relevant features.

To the best of our knowledge, this is the first report showing the presence of a distinctive biochemical and clinical profile in the GBA asymptomatic carriers and provides novel information about the relationship between biochemical and phenotypic prodromal PD signatures of GBA carriers.

With this pilot study, we suggest that a combination of GBA genotyping, screening of prodromal PD features alongside with the biochemical analysis may help to identify those subjects more likely to develop PD.

We are aware that, given the incomplete penetrance of GBA mutations in PD, larger cohorts with a longitudinal study design are needed to better detect the phenoconversion of the asymptomatic GBA carriers. However, if our findings will be confirmed, they may allow for even earlier detection of prodromal neurodegeneration and lay the ground for future targeting neuroprotective drugs for those subject bearing GBA mutations without PD.

Limitations

We are aware that these studies have several limitations. The main limitation of studies I and II is the small sample size of the three groups and, within GBA-PD, the limited number of subjects carrying mutations of different severity, which could mask additional significant differences. Thus, our data warrant replication in larger cohorts, especially to strengthen the outcome of stratification analysis by mutation type. Indeed, it is now clearly emerging that GBA mutation severity may influence PD characteristics and disease progression, and this could also potentially impact on the biochemical profile.[32,62,93] Furthermore, the small sample size of the follow-up study, as a consequence of the high drop-out rate, calls for validation of our results in a larger independent cohort. The main limitation of study III is the lack of longitudinal measurement of Gcase enzymatic activity and PBMCs α-synuclein. It would be interesting to assess potential changes in these biochemical parameters over time and correlate to disease progression. Further studies on a large asymptomatic GBA positive

population are needed to identify a precise phenotypic characteristic and to determine a reliable biomarker that could help to identify subjects at higher risk to develop PD.

An additional potential limitation is the use of questionnaires to assess autonomic symptoms (SCOPA-AUT) and sleep disorders (RBDsq and PDSS) instead of objective measures. While these questionnaires are considered good screening tools, we acknowledge that objective assessments with autonomic testing and polysomnography are required to confirm the diagnosis of cardiovascular dysautonomia and RBD, respectively. Studies aiming at exploring autonomic dysfunction in GBA-PD subjects using instrumental and objective measurements are lacking and it is certainly interesting to better characterize this population by means of more detailed and reliable measures.

Finally, we acknowledge a potential technical limitation associated to the semiquantitative nature of Western Blot analysis, albeit this technique is routinely employed for biomarker discovery.

6. CONCLUSION

GBA mutations play a pivotal role in the molecular pathogenesis of PD and are considered one of the most frequent risk factors for the disease.

In these three studies we were able to confirm the biological effect of GBA mutations in determining clinical and biochemical distinctive profile of PD.

On clinical side, GBA-PD showed a worse clinical outcome both on motor and non-motor features compared to NM-PD, as well a distinctive biochemical profile.

Over time, both the GBA-PD and the NM-PD groups separately displayed a significant deterioration in dysautonomic functions, motor performance, cognitive functions and mood disorder compared to baseline, while GBA-PD had a more severe motor progression with a higher disease severity compared to NM-PD.

Moreover, PBMCs α-synuclein could be a potentially relevant biomarker of GBA-related disease even in the late stage, able to differentiate GBA-PD from NM-PD and HC and varied according to mutation severity, suggesting a potential utility as stratification biomarkers. If replicated in larger independent cohorts, this signature may serve as classifier for patient stratification or selection in clinical trials.

Finally, we propose PBMCs as a widely accessible and manageable model providing a unique biochemical profile in both GBA-PD and in the asymptomatic GBA mutation carriers, in which we confirm the presence of a distinctive prodromal signature both on clinical and biochemical side.

In conclusion, albeit preliminary and with some limitations, our findings contribute to the current understanding of the role of GBA mutations in the development and progression of PD. Continued research into its pathophysiology footprint is imperative in order to individuate a panel of biomarkers for characterizing PD subjects with and without GBA mutations that could be used not only for early diagnosis, monitor disease progression and optimize therapeutic intervention, but also for predict the risk for PD, and develop new target treatment options.

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SUPPLEMENTARY MATERIALS

Biochemical Assessment

Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugations and stored with plasma at -80°C. Expression levels of GCase, cathepsin D, LAMP-1, LIMP-2, saposin C and α-synuclein in PBMCs were evaluated by Western blotting. Thirty μg of protein were separated by PAGE (Biorad) and transferred onto 0.2μm nitrocellulose membranes (Invitrogen).

Membranes were incubated with the following primary antibodies: α-synuclein (Abcam, 1:1000), β-glucocerebrosidase (Sigma, 1:500), Saposin C (SantaCruz, 1:1000), LIMP-2 (Abcam, 1:1000), LAMP-1 (NovusBiologicals, 1:2000), Cathepsin D (Abcam, 1:2000), β-actin (SantaCruz, 1:10000). Blots were imaged with the Odyssey System (LiCor, Biosciences) and the target protein signal was normalized with the corresponding β-actin signal. Then, the abundance of target proteins in GBA-PD and NM-PD groups was expressed as percentage relative to mean of the control samples (three) in each independent experiment/gel. Exosomes were isolated from plasma (1 mL) by sequential centrifugations and filtration, as described in [51]. α -synuclein concentration in plasma exosomes and whole plasma was assessed by ELISA assay (Anaspec), according to manufacturer's instructions. Optical density at 450 nm was determined using a microplate reader (Biotek). GCase activity was measured fluorimetrically on a microplate reader (Molecular Devices) following the protocol described by Vaccaro et al.[94].

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APPENDIX - *Article of Study I*

Profiling the Biochemical Signature of GBA-Related Parkinson's Disease in Peripheral Blood Mononuclear Cells

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ABSTRACT: Background: *GBA* mutations are the commonest genetic risk factor for Parkinson's disease (PD) and also impact disease progression.
Objective: The objective of this study was to define a biochemical profile that could distinguish GBA-PD from non-mutated PD.
Methods: 29 GBA-PD, 37 non-mutated PD, and 40 controls were recruited; α-synuclein levels in plasma, exosomes, and peripheral blood mononuclear cells were analyzed, GCase and main GCase-related lysosomal proteins in peripheral blood mononuclear cells were measured.
Results: Assessment of plasma and exosomal α-synuclein levels did not allow differentiation between GBA-PD and non-mutated PD; conversely, measurements in peripheral blood mononuclear cells clearly distinguished GBA-PD from non-mutated PD, with the former group showing significantly higher α-synuclein levels, lower GCase activity, higher LIMP-2, and lower Saposin C levels.
Conclusion: We propose peripheral blood mononuclear cells as an easily accessible and manageable model to provide a distinctive biochemical profile of GBA-PD, potentially useful for patient stratification or selection in clinical trials.

Key Words: α-synuclein; glucocerebrosidase; Parkinson's disease; exosomes; phenotyping

Parkinson's disease (PD) is a common neurodegenerative disorder mainly characterized by dopaminergic neuronal loss in the substantia nigra and α-synuclein protein aggregation.

Genetic factors are well known to contribute to PD susceptibility. In particular, heterozygous mutations in the *GBA* gene, encoding lysosomal enzyme glucocerebrosidase (GCase), represent the commonest genetic risk factor for PD, occurring in 7%–15% of PD patients and conferring a 5%–25% increased risk of developing the disease.[1-3] Given the relevance and frequency of GBA-related PD (GBA-PD) and the intensive research efforts toward the development of targeted therapeutic strategies, the identification of reliable biomarkers for this genetic PD subtype represents a still unmet need.

Although the mechanisms linking GBA mutations to PD still remain unclear, a vicious circle between GCase and α-synuclein has been elucidated, with GCase reduction leading to α-synuclein accumulation and, in turn, increased α-synuclein inhibiting residual GCase function.[4,5] Because lysosomes are known to play a major role in α-synuclein degradation,[6] the levels of GCase and other lysosomal enzymes have been measured in cerebrospinal fluid (CSF) of patients in combination with pathological α-synuclein, suggesting these values could provide a biochemical fingerprint of PD.[7,8] CSF collection, however, requires a relatively invasive procedure, not suitable for mass screenings.

Pathological α-synuclein, as well as other misfolded proteins, can spread from cell to cell through exosomes. These are extracellular vesicles that can be easily isolated from peripheral blood and whose content may reflect disease-specific changes. In the presence of dysfunctional lysosomal activity, the accumulation of cytosolic α-synuclein may result in its increased release through exosomes,[9,10] making them a promising biomarker for PD.[11,12]

Here we attempted to define a biochemical profile of GBA-PD by comparing α-synuclein levels in plasma, exosomes, and peripheral blood mononuclear cells (PBMCs), as well as GCase-related lysosomal proteins (GCase, lysosomal integral membrane protein-2 [LIMP-2], saposin C, cathepsin D, and lysosome-associated membrane glycoprotein 1[LAMP-1]) in PBMCs obtained from PD patients with and without GBA mutations.

Methods

The details are in the Supplementary File.

Subjects

We recruited 66 PD (29 GBA-PD and 37 non-mutated [NM-PD]) and 40 healthy individuals (HC) as a control group. The study was approved by the local ethics committee. PD patients underwent a complete neurological assessment to detect and quantify motor and nonmotor signs.

Biochemical Assessment

A 35-mL blood sample was obtained for isolation of whole plasma, exosomes, and PBMCs. The α-synuclein levels in plasma and exosomes were tested by enzyme-

TABLE 1. Demographics and biochemical data of the study cohort

	HC n = 40	GBA-PD n = 29	NM-PD n = 37	*P* (between) p1	p2	p3
Male, n (%)	15 (38)	20 (69)	25 (68)	0.25	0.07	1.0
Age (y)	60.6 ± 7.1	58.7 ± 9.4	61.2 ± 6.7	0.18	1.0	0.14
Age at onset (y)		51.1 ± 10.3	57.2 ± 9.2	—	—	0.01[a]
Disease duration (y)		6.6 ± 4.8	7.7 ± 4.7	—	—	0.36
Plasma total α-synuclein (ng/mL)	14.7 ± 3.1	15.8 ± 3.4	14.1 ± 4.5	0.30	0.61	0.07
Exosomal α -synuclein (pg/mL)	14.2 ± 10.9	22.0 ± 16.1	22.9 ± 10.2	< 0.001[a]	< 0.001[a]	0.41
PBMCs — α-synuclein (% HC)	100.5 ± 19.9	145.3 ± 42.9	103.8 ± 26.7	< 0.001[a]	0.84	< 0.001[a]
PBMCs — GCase activity (nmol/mg protein/h)	10.0 ± 2.9	5.5 ± 1.1	9.3 ± 2.9	< 0.001[a]	0.51	< 0.001[a]
PBMCs — LIMP-2 protein (% HC)	103.3 ± 33.2	107.1 ± 30.5	92.8 ± 25.3	0.44	0.14	0.01[a]
PBMCs — saposin C protein (% HC)	100.7 ± 14.6	92.0 ± 17.1	106.8 ± 19.2	0.06	0.30	0.002[a]
PBMCs — LAMP-1 protein (% HC)	100.9 ± 16.0	99.4 ± 33.5	96.0 ± 22.9	0.50	0.12	0.79
PBMCs — GCase protein (% HC)	101.5 ± 28.9	109.9 ± 67.7	120.4 ± 58.2	1.0	0.33	0.32
PBMCs — cathepsin D protein (% HC)	100.3 ± 25.1	99.9 ± 34.7	97.3 ± 23.2	0.83	0.70	1.0

Data are reported as mean ± standard deviation. Group comparisons were performed with the Kruskal–Wallis test followed by post hoc analysis with the Dunn's pairwise comparison test (Bonferroni correction).
[a]Significant difference.
p1, HC vs GBA-PD; p2, HC vs NM-PD; p3, GBA-PD vs NM-PD.

linked immunosorbent assay.[11] In PBMCs, expression of α-synuclein, GCase, LIMP-2, Saposin C, cathepsin D, and LAMP-1 was assessed by Western blotting, whereas GCase activity was measured fluorometrically.

Statistics

Statistical analysis was performed using Stata 13.0 (StataCorp, College Station, TX). Biochemical data comparison among the 3 groups was performed by the Kruskal–Wallis test followed by Dunn's pairwise test (Bonferroni adjustment). GBA-PD patients were further divided into subgroups according to mutation severity; group comparison was performed using the analysis described above. The percentage of fold changes (relative to controls) for each biochemical parameter was next calculated in GBA-PD and NM-PD groups; differences were analyzed using Student's *t* test. Correlations between biochemical and clinical parameters were assessed by the Spearman test.

Results

Groups were comparable for age, although GBA-PD subjects had an earlier disease onset but similar disease duration (Table 1), and showed worse scores on the Montreal Cognitive Assessment (MoCA), REM behavior Disorder Questionnaire (RBDsq), Parkinson's Disease Sleep Scale (PDSS), Movement Disorder Society - Unified Parkinson's Disease Rating Scale - motor subscale (MDS-UPDRS part III), Beck's Depression Inventory (BDI), University of Pennsylvania Smell Identification Test (UPSIT) and Scale for Outcomes in Parkinson Disease – Autonomic (SCOPA-AUT) scales (Table S1).

On the biochemical side, both the GBA-PD and NM-PD groups showed significantly higher levels of exosomes, but not plasma or α-synuclein, compared with controls. When assessing PBMCs, GBA-PD significantly differed from other groups for higher α-synuclein and lower GCase activity, whereas the NM-PD group behaved similarly to controls (Table 1).

When comparing relative changes, we did not observe significant differences between GBA-PD and NM-PD groups in both plasma and exosomal α-synuclein levels, the latter being similarly elevated in both groups (Fig. 1A,B). Conversely, biochemical analysis of PBMCs disclosed a GBA-PD-specific profile, characterized by significantly higher α-synuclein and significantly lower GCase activity compared with NM-PD (Fig. 1C,D). Moreover, measurement of GCase-related lysosomal proteins showed lower saposin C and higher LIMP-2 in GBA-PD compared with NM-PD (Fig. 1E,F). No significant differences between groups were found for the other GCase-related lysosomal proteins (Fig. S1).

When GBA-PD participants were stratified by mutation type, carriers of severe variants showed higher PBMC a-synuclein compared with other categories, whereas GCase activity was significantly higher in carriers of risk variants than in the other subgroups (Table S3).

Investigation of clinical-biochemical links in the GBA-PD group showed a negative correlation between PBMC α-synuclein and MoCA scores ($r = -0.44$, $P = 0.01$; Table S4).

Discussion

Given the high frequency of *GBA* mutations among PD patients, a deep phenotypic and biochemical characterization of this genetic subgroup is now becoming mandatory.[13,14] Along with a better understanding of the molecular mechanisms predisposing to PD, clinical-

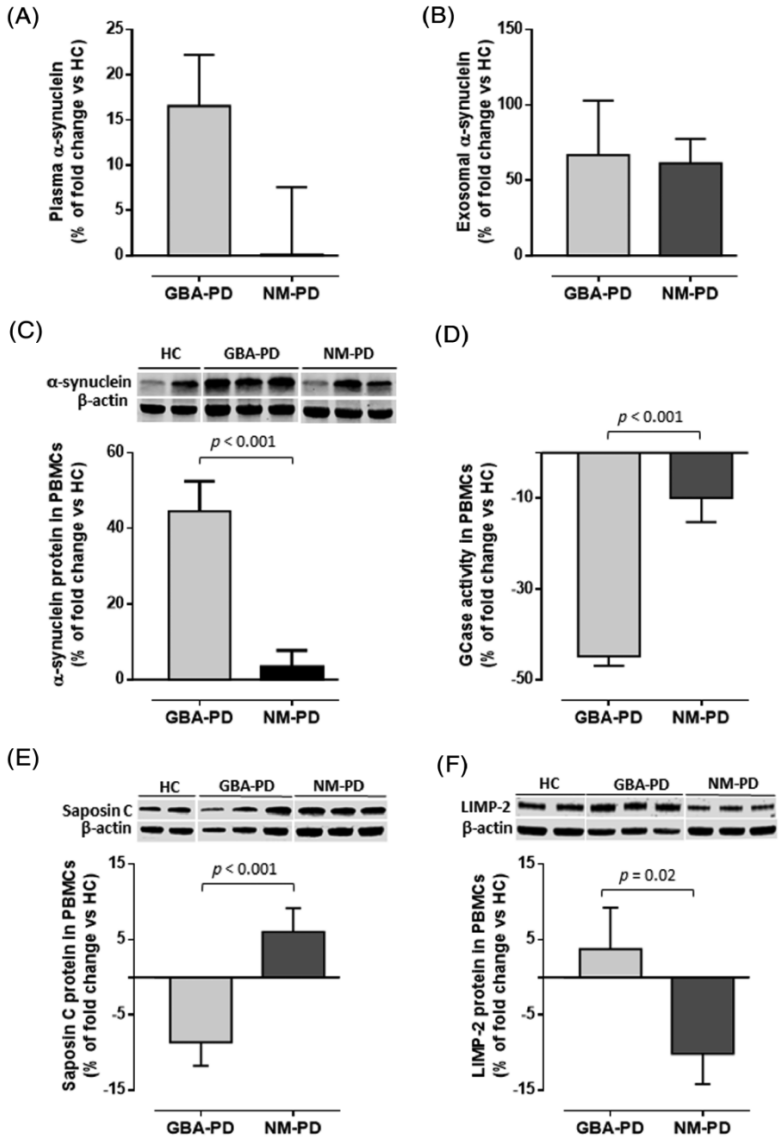

FIG. 1. Bar graph of the percentage fold changes of (**A**) plasma α-synuclein, (**B**) exosomal α-synuclein, (**C**) α-synuclein in PBMCs, (**D**) GCase activity in PBMCs, (**E**) PBMC levels of saposin C, and (**F**) LIMP-2 in GBA-PD and NM-PD relative to controls.

biochemical profiles could be crucial to predicting PD development in *GBA* carriers, also paving the way to personalized treatment strategies.

As α-synuclein is the best-known player in PD pathogenesis and progression, most biomarker studies have focused on the relationship between defective GCase activity and α-synuclein levels in biological fluids. Indeed, lower GCase activity and reduced α-synuclein levels have been reported in the cerebrospinal fluid (CSF) of GBA-PD patients compared with NM-PD, with differences related to the severity of the mutation.[7,8,15] Yet, only a few studies have explored this relationship in easily accessible body tissues such as the blood of *GBA* mutation carriers, showing good correlation between GCase activity reduction and increased oligomeric α-synuclein in plasma and dimeric α-synuclein in erythrocytes.[16,17]

To our knowledge, this is the first study reporting a thorough biochemical profiling of GBA-PD in blood, compared with both NM-PD patients and HC. First, we assessed α-synuclein levels not only in total plasma but also in plasma exosomes, because of their ability to reflect brain-related pathological changes.[12,18] Then we

performed a complete characterization of PBMCs by measuring α-synuclein levels, GCase activity, and the expression of several GCase-related lysosomal proteins.

Although small variations in plasma α-synuclein levels were observed in GBA-PD and NM-PD compared with HC, no statistical differences emerged among groups, showing that this parameter is not distinctive for the GBA-PD condition and confirming its unreliability as a surrogate marker of synucleinopathy.[19,20] Conversely, we found significantly higher exosome-associated α-synuclein in both PD groups compared with HC, in line with previous studies that specifically associated increased exosomal α-synuclein with PD.[12,21,22] It was previously shown that pharmacological modulation of GCase activity in vivo was able to increase exosome-associated α-synuclein levels.[10] However, the similar increase of exosomal α-synuclein in both PD groups suggests that this parameter is likely unrelated to GCase deficiency; but rather might rather reflect the overall neurodegenerative process[23] and other PD-associated lysosomal dysfunctions.[9]

Interestingly, PBMCs showed a unique biochemical profile that clearly distinguished GBA-PD from NM-PD. In fact, in addition to the clear reduction in GCase activity, we reported for the first time a significant increase in α-synuclein in PBMCs of GBA-PD compared with HC and NM-PD, whereas the 2 latter groups showed comparable levels, in line with previous data.[24,25] This difference could result from the synergistic effect of impaired GCase activity and dysregulation of chaperone-mediated autophagy observed in PBMCs of GBA-PD,[26,27] representing a potentially relevant biomarker of GBA-related disease. Of note, these parameters not only were able to differentiate GBA-PD from NM-PD and HC, but they also varied according to mutation severity, suggesting potential utility as stratification biomarkers. Moreover, we observed a negative correlation between PBMC α-synuclein levels and MoCA scores in GBA-PD, suggesting that increased α-synuclein could mirror more rapid progression of the disease, particularly on the cognitive side.

We did not observe any difference in GCase activity levels between NM-PD and HC. Despite some studies having reported a reduction in GCase activity in the brains of NM-PD patients,[28,29] data concerning this activity in peripheral blood are still conflicting, showing small or negligible variations compared with HC.[16,30,31]

In addition to GCase and α-synuclein, PBMCs from GBA-PD patients also showed distinctive alterations in saposin C and LIMP-2 levels compared with NM-PD patients. Saposin C facilitates GCase activity and protects the enzyme from intracellular proteolysis and α-synuclein-mediated inhibition.[32] The observed reduction of saposin C in GBA-PD is a potential consequence of a negative feedback loop from increased α-synuclein or GCase unavailability within lysosomes[33,34] and might further affect GCase function, increasing cell susceptibility to α-synuclein accumulation. Conversely, the increased levels of GCase transporter LIMP-2 could represent a compensatory mechanism to sustain GCase trafficking toward the lysosomes and, consequently, functionality. Accordingly, increased expression of LIMP-2 was observed in induced Pluripotent Stem Cells (iPSCs) derived from PD patients carrying the heterozygous *GBA* N370S mutation, compared with HC.[35] In our study, the lack of significant differences in LIMP-2 levels between GBA-PD and HC could possibly relate to the limited sample size in both groups and the presence of distinct mutations that can affect GCase function differently, as demonstrated by stratified analysis.

Overall, our findings highlight the key role of PBMCs as potential sources of biomarkers for PD[36,37] and suggest for the first time that lysosomal alterations in these cells may be considered valuable parameters to identify a biochemical profile distinctive of GBA-PD. Importantly, PBMCs can be collected easily and repeatedly throughout minimally invasive procedures in comparison with CSF or skin biopsy, supporting their usefulness also in clinical trials.

We acknowledge that a limitation of our study relates to the small sample sizes of the 3 groups and, within GBA-PD, to the limited number of subjects carrying mutations of different severity, which could mask additional significant differences. Thus, our data warrant replication in larger cohorts, especially to strengthen the outcome of stratification analysis by mutation type. Indeed, it is now clearly emerging that *GBA* mutation severity may influence PD characteristics and disease progression, and this could also potentially impact the biochemical profile.[8,38,39] A second limitation may reside in the semiquantitative nature of Western blot analysis, albeit this technique is routinely employed for biomarker discovery.

In conclusion, we propose PBMCs as a widely accessible and manageable model providing a distinctive biochemical profile of GBA-PD. If replicated in larger independent cohorts, this signature may serve as classifier for patient stratification or selection in clinical trials and would also deserve exploitation in prospective longitudinal studies to assess its value in monitoring disease progression and response to treatment or in detecting *GBA* carriers at higher risk of PD conversion, who would benefit from early start of neuroprotective strategies. ■

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Data Availability Statement

The data sets generated during the current study are available in the ZENODO repository (10.5281/zenodo.4300469). ■

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Supporting Data

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