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RESEARCH ARTICLE

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# Comparing *GBA* -Parkinson s disease and idiopathic Parkinson s disease: -Synuclein oligomers and synaptic density as biomarkers in the skin biopsy

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## Abstract

The main genetic risk factors for Parkinson s disease (PD) are presently represented by variants in *GBA1* gene encoding for the -glucocerebrosidase (GCase). Searching for a peripheral biomarker that can be used for selecting and monitoring patients in clinical trials targeting *GBA1*-associated PD (*GBA1*-PD) is a current challenge. We previously demonstrated that α-synuclein oligomers expressed as proximity ligation assay (PLA) score in synaptic terminals of skin biopsy are a reliable biomarker for distinguishing idiopathic PD (iPD) from healthy controls (HC). This cross-sectional study investigates an unexplored cohort of *GBA1*-PD ($n = 27$) compared to 28 HC, and 36 iPD cases to (i) analyze α-synuclein oligomers and quantify them throughout PLA score, (ii) investigate GCase expression in brain and synaptic terminals targeting the sweat gland, (iii) unravel indicators that could differentiate patients with specific *GBA1* mutations. PLA score discriminates *GBA1*-PD from HC with sensitivity = 88.9 (95 CI 70.84–97.65), specificity = 88.5 (95 CI 69.85–97.55), and PPV = 88.9 (95 CI 73.24–95.90), AUC value = 0.927 (95 CI 0.859–0.996). No difference was found between *GBA1*-PD patients and iPD, suggesting a common pathological

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pathway based on α-synuclein oligomers. GCase score did not differ in *GBA1*-PD, iPD, and HC in the synaptic terminals, whereas a positive correlation was found between PLA score and GCase score. Moreover, a significant increase in synaptic density was observed in *GBA1*-PD compared to iPD and HC ($P < 0.0001$). Employing ROC curve to discriminate *GBA1*-PD from iPD, we found an AUC value for synaptic density = 0.855 (95 CI 0.749–0.961) with sensitivity = 85.2 (95 CI 66.27 –95.81 ), specificity = 77.1 (95 CI 59.86 –89.58 ), and PPV = 74.19 (60.53 –84.35 ). The highest synaptic density values were observed in p.N409S patients. This work points out to the value of both PLA score and synaptic density in distinguishing *GBA1*-PD from iPD and to their potential to stratify and monitor patients in the context of new pathway-specific therapeutic options.

**KEYWORDS**
autonomic nervous system, biomarker, *GBA1*, Parkinson s disease, skin biopsy, α-synuclein

## 1 | INTRODUCTION

Parkinson s disease (PD) is a multisystem and multifactorial disorder caused by a combination of genetic and environmental agents [1] and characterized by aberrant accumulations of the protein α-synuclein, a major constituent of histopathological lesions in PD [2]. Genetic variants range from common alleles with low penetrance to rarer highly penetrant ones [3]. Because of this multifactorial pathogenesis, PD is clinically heterogeneous and characterized by high variability in the presentation of motor and nonmotor symptoms [4].

The main genetic risk factors for PD are represented by variants in the Glucosylceramidase beta 1 gene (*GBA1*), which are associated with an increased risk in a range between 5- and 8-fold [5, 6]. *GBA1* encodes the -glucocerebrosidase (GCase), a lysosomal enzyme involved in glycosphingolipid metabolism, regulating processes ranging from membrane properties modification to autophagy [6]. Approximately, 300 *GBA1* mutations have been described in Gaucher s disease (GD, a lysosomal storage disorder characterized by biallelic mutations in *GBA1*), many of which are also found in PD [7]. Furthermore, some variants (p.N409S and p.L483P; legacy names p.N370S and p.L444P, respectively) have high allelic frequencies among PD patients. Other variants (p.E365K and p.T408M; legacy names p.E326K and p.T369M, respectively), even if not pathogenic for GD, have been associated with an increased risk for PD [8, 9]. As for genotype–phenotype correlations, multivariable analysis adjusted for sex, age at onset, and disease duration indicated that mutations causing a more severe GD phenotype (e.g., p.L483P-related) were associated with a 3-fold greater risk for dementia in PD patients, but similar mortality risk when compared to less severe mutations [10–13]. Interestingly, α-synuclein aggregation and GCase reduction are mutually linked as revealed by studies performed in different experimental models both in the central and peripheral nervous system [14–16]. Functional loss of GD-linked GCase in neuronal cell models compromises lysosomal protein degradation, affects α-synuclein clearance, and causes its accumulation, thus exacerbating cell stress [16]. On the other hand, α-synuclein aggregates inhibit the normal GCase activity in idiopathic PD (iPD) brain, suggesting that GCase depletion may also contribute to the pathogenesis of iPD [16].

Recently, the search for a reliable biomarker on peripherally collected material (plasma and peripheral blood mononuclear cells) provided evidence that glucosylceramide levels in plasma could be used as a biomarker for selecting and monitoring patients in clinical trials targeting *GBA1*-associated PD (*GBA1*-PD; [17]). Furthermore, the α-synuclein seed amplification assay for analyzing cerebrospinal fluid (CSF) was able to classify both iPD and *GBA1*-PD with high sensitivity and specificity [18]. Besides blood and CSF, the peripheral nervous system could represent an accessible and valuable source for the discovery of novel biomarkers and for better unraveling pathogenic mechanisms involved in PD [19]. We recently identified α-synuclein oligomers in skin biopsy and demonstrated that these aggregates are a reliable biomarker for distinguishing iPD from healthy controls (HC) [20].

In the present study, we focused on a cohort of *GBA1*-PD patients aiming to: (i) understand if α-synuclein oligomer accumulation is a common feature also in PD patients with a strong genetic component, (ii) investigate whether GCase expression changes in synaptic terminals of autonomic structures in skin biopsies, and (iii) unravel indicators that could differentiate patients with specific *GBA1* mutations.

## 2 | METHODS

### 2.1 | Subjects

The population of the present cross-sectional study was composed of 91 subjects: 28 HC, 36 iPD patients, and

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27 PD carriers of *GBA1* gene variants (see Table 1 for details on demographic and clinical data). All subjects were consecutively enrolled from March 2014 to March 2022 at the Parkinson Institute (Milan, Italy) by neurologists experienced in movement disorders and contributing to the Parkinson Institute Biobank [21]. The diagnosis of PD was made according to the UK Brain Bank criteria [22]. As exclusion criteria, we considered a history of diseases or other conditions potentially leading to peripheral neuropathy or general contraindications for performing a skin biopsy. Genetic analysis was performed as previously described: DNA was extracted from peripheral blood using a Chemagic Star workstation (Hamilton, ON, Canada), and the genetic analysis on the *GBA1* gene was performed with a combination of different technologies, ranging from allele-specific PCR [23] to exome sequencing [24]. To ensure the homogeneous assessment of skin biomarkers among the most common *GBA1* variants, specific subgroups were also identified, that is, p.N409S ($n = 7$) and p.L483P ($n = 12$). The remaining 8 patients carried heterogeneous mutations (biallelic mutation p.N409S/p.E365K, $n = 1$; biallelic mutation p.N409S/p.N409S, $n = 1$; p.Q401X, $n = 1$; c.73delC, $n = 1$; p.S38X, $n = 1$; RecNcil, $n = 1$; p.D448H, $n = 1$; p.E388K, $n = 1$). In iPD patients, mutations in *GBA1* and other PD-related genes were excluded. Patients and HC were balanced for age and sex. The following clinical data were collected for all patients: age at PD onset, disease duration, and levodopa equivalent daily dose (LEDD, calculated according to [25]). The Unified Parkinson s Disease Rating Scale (UPDRS) [26] was administered, and the severity of motor symptoms and disease staging were assessed in the medication off state by movement disorder specialists using the UPDRS part III and the Hoehn and Yahr (HY) scale [27]. Other relevant data, including the presence of hyposmia according to medical reports, probable REM sleep behavior disorder (pRBD) according to a cut-off score 6 in the RBD Screening Questionnaire (RBDSQ) [28], and constipation (following Rome III criteria [29]) were collected. The presence of orthostatic hypotension (OH) was confirmed based on specific prescription patterns and/or the detection during clinical examination of a fall of at least 20 mmHg in systolic blood pressure (30 mmHg in patients with supine hypertension) and/or 10 mmHg in diastolic blood pressure within 3 min of standing [30]. Autonomic functions were further investigated using the Composite Autonomic Symptom Score 31 (COMPASS-31) [31], a self-administered questionnaire addressing several autonomic domains (orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, bladder, and pupillomotor). Cognitive functions were examined using the Mini-Mental State Examination (MMSE) [32], the Frontal Assessment Battery (FAB) [33], and the Montreal Cognitive Assessment (MoCA) [34].

## 2.2 | Skin biopsy

Forearm skin biopsies were fixed in Zamboni solution for 24 h at 4°C, then paraffin-embedded and sliced in 3-μm thick serial sections using a microtome (MR2258, Histo-Line Laboratories, Milan, Italy), as previously described [20]. Each sample was processed as follows: (i) one section was stained with hematoxylin and eosin to verify sweat glands presence; (ii) three sections underwent proximity ligation assay (PLA) procedure to localize α-synuclein oligomers and immunofluorescence staining for synaptophysin and (iii) three sections underwent the GCase tyramide signal amplification and synaptophysin staining.

## 2.3 | Proximity ligation assay and immunofluorescence

α-Synuclein oligomers were detected by a PLA commercial Duolink kit (Sigma-Aldrich, St Louis, Missouri, USA) according to the manufacturer s instructions as previously described [20, 35]. In detail, PLUS and MINUS probes were directly conjugated to the rabbit anti α-synuclein antibody S3062 (polyclonal antibody directed against a synthetic peptide within aa 111–132 of human α-Synuclein, Sigma-Aldrich) to obtain α-synuclein-PLUS and α-synuclein-MINUS probes [20, 36, 37]. Once obtained, these were used in the PLA procedure carried out together with classical immunofluorescence as described in details in [20]. Briefly, after deparaffinization and rehydration, sections were pretreated with 10 formic acid (10 min). After a 20-min blocking step with 1 bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS) plus 0.1 Triton X-100, sections were incubated with α-synuclein-PLUS and α-synuclein-MINUS probes (1:100 in PLA diluent) and mouse anti-synaptophysin (clone DakSynap, 1:100; Agilent, Santa Clara, USA) for 2 h at 37°C. The amplification reaction was accomplished and samples were counterstained with Hoechst 33342 (1:1000; Life Technologies, MA, USA) for 10 min and mounted using Mowiol-DABCO. Positive and negative controls were performed as previously described [20]. The polyclonal anti α-synuclein antibody used for probe conjugation is a well characterized α-synuclein antibody able to recognize monomeric α-synuclein in immunofluorescence assay. Indeed, its high specificity was previously verified performing both the staining in a OlaHsd mice, a substrain of C57BL/6J mice carrying a spontaneous deletion of the *SNCA* gene [38], and the preadsorbtion assay [36]. Furthermore, it exhibits also high sensibility as revealed by its typical synaptic pattern in controls and PD patients when used in immunofluorescence assay, while the same antibody used in the PLA assay does not recognize physiological synaptic α-synuclein, but it marks the oligomeric forms in the immediate

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**TABLE 1** Demographic, clinical, and skin biopsy data of *GBA1*-PD, iPD, and HC.

| Variables | *GBA* -PD *N* = 27) | iPD *N* = 36) | HC *N* = 28) | *P*-value |
|---|---|---|---|---|
| Age at skin biopsy, years, mean (SD) | 56.96 (10.15) | 61.94 (9.68) | 60.25 (11.03) | 0.165[a] |
| | | | | 0.052[b] |
| Sex (male), *n* ( ) | 15 (55.6 ) | 21 (58.3 ) | 12 (42.9 ) | 0.208[e] |
| | | | | 1.0[f] |
| Age at PD onset, years, mean (SD) | 45.85 (9.32) | 54.39 (9.86) | — | **0.001**[b] |
| Disease duration, years, median (IQR) | 10 (10) | 6 (11.25) | — | **0.019**[c] |
| LEDD, median (IQR) | 707 (550) | 555.16 (618.75) | — | 0.232[c] |
| HY scale | | | | |
| 1–2, 5, *n* ( ) | 19 (70.4 ) | 23 (63.9 ) | — | 0.563[f] |
| 3–4, *n* ( ) | 8 (29.6 ) | 9 (25 ) | | |
| UPDRS I score, median (IQR) | 2 (4) | 0.5 (3) | — | 0.051[c] |
| UPDRS II score, mean (SD) | 12.14 (8.07) | 7.10 (5.94) | — | **0.013**[b] |
| UPDRS III score (off), mean (SD) | 26.54 (13.06) | 25.52 (16.41) | — | 0.818[b] |
| UPDRS IV score, median (IQR) | 4 (4) | 3 (4) | — | 0.178[c] |
| pRBD, *n* ( ) | 15 (55.5 ) | 13 (36.1 ) | — | 0.136[f] |
| Hyposmia, *n* ( ) | 8 (29.6 ) | 12 (33.3 ) | — | 0.561[f] |
| Constipation, *n* ( ) | 16 (59.2 ) | 28 (77.8 ) | — | 0.134[f] |
| Orthostatic hypotension, *n* ( ) | 14 (51.8 ) | 13 (36.1 ) | — | **0.003**[f] |
| COMPASS-31, orthostatic intolerance, mean (SD) | 15.52 (9.45) | 3.61 (7.50) | 0 (0) | **<0.0001**[d] |
| | | | | **<0.0001**[c] |
| COMPASS-31, vasomotor, mean (SD) | 0.44 (0.85) | 0.58 (1.13) | 0.07 (0.38) | 0.081[d] |
| | | | | 0.774[c] |
| COMPASS-31, secretomotor, mean (SD) | 3.37 (3.70) | 2.75 (3.81) | 1.64 (2.04) | 0.376[d] |
| | | | | 0.488[c] |
| COMPASS-31, gastrointestinal, mean (SD) | 5.25 (4.47) | 3.89 (4.07) | 3.29 (3.52) | 0.147[d] |
| | | | | 0.184[c] |
| COMPASS-31, bladder, mean (SD) | 2.30 (1.68) | 1.36 (1.44) | 0.75 (1.27) | **0.001**[d] |
| | | | | **0.024**[c] |
| COMPASS-31, pupillomotor, mean (SD) | 0.70 (0.87) | 1.36 (1.31) | 0.64 (0.83) | **0.049**[d] |
| | | | | **0.048**[c] |
| COMPASS-31, total, mean (SD) | 28.81 (11.76) | 14.97 (11.75) | 6.39 (5.37) | **<0.0001**[d] |
| | | | | **<0.0001**[c] |
| MMSE score, median (IQR) | 27.60 (4.29) | 26 (3.01) | — | 0.605[c] |
| FAB score, median (IQR) | 14.20 (7.50) | 13.90 (2.40) | — | 0.682[c] |
| MoCA score, mean (SD) | 16.13 (5.73) | 19.53 (4.60) | — | 0.073[b] |
| PLA score, median (IQR) | 259 (394) | 213.82 (256.04) | 27.78 (75.81) | **<0.0001**[d] |
| | | | | 0.429[c] |
| Synaptic density, median (IQR) | 0.048 (0.05) | 0.018 (0.01) | 0.017 (0.02) | **<0.0001**[d] |
| | | | | **<0.0001**[c] |

*Note*: Significant *P*-values ($P < 0.05$) are highlighted in bold.
Abbreviations: COMPASS-31, Composite Autonomic Symptom Score 31; FAB, frontal assessment battery; HC, healthy controls; HY, Hoehn & Yahr scale; iPD, idiopathic Parkinson s disease; LEDD, levodopa equivalent daily dose; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; PLA, proximation ligation assay; pRBD, probable REM sleep behavior disorder; UPDRS, Unified Parkinson s Disease Rating Scale.
[a]ANOVA analysis *GBA1*-PD versus iPD versus HC.
[b]Independent samples *t*-test *GBA1*-PD versus iPD.
[c]Mann–Whitney test *GBA1*-PD versus iPD.
[d]Kruskal–Wallis test *GBA1*-PD versus iPD versus HC.
[e]Fisher s exact test *GBA1*-PD versus iPD versus HC.
[f]Fisher s exact test *GBA1*-PD versus iPD.

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vicinity of pale bodies in *post mortem* human brains [20, 37].

To detect GCase (Rab Mab EPR5142, KO validated, directed against a synthetic peptide within human GCase aa 50–150; Abcam, Milan, Italy) we used the tyramide signal amplification system, as described in the supplementary materials.

Images were collected at 60× magnification (1024 × 1024). The sections were analyzed with a Nikon spinning disk confocal microscope using a silicon-immersion 40× objective. In addition, we used a spinning disk super-resolution by optical pixel reassignment (SoRa) technique using the silicon-immersion 100× objective plus a resolution improvement of 2.8×. PLA score was calculated as previously described [20]: the area of PLA signal within synapses (synaptophysin-positive) was normalized for synaptic density (synaptophysin-positive area/total area of the sweat gland). Similarly, the GCase score was calculated as follows: GCase staining was measured inside synapses (synaptophysin-positive) and normalized for synaptic density (synaptophysin-positive area/total area of the sweat gland).

## 2.4 | Statistical analysis

Based on our previous work [20], we considered the difference in PLA score between PD and HC as the primary outcome measure to calculate the sample size. Expecting an effect size (Cohen s *d*) of 1.18, a two-tailed type I error = 5 and power = 90 , we estimated a sample size of at least 17 patients in each group ( pwr" package, RStudio 2022.07.2 576).

Variables were expressed as counts (percentages) when categorical and as mean (standard deviation, SD) or median (interquartile range, IQR) when continuous according to the normality of distribution. The normality of data was assessed using the Shapiro–Wilk test. Differences between groups were analyzed by ANOVA (multiple groups) adjusting for relevant covariates and the independent samples *t*-test (two groups) or the non-parametric equivalents (Kruskal-Wallis and Mann–Whitney tests, respectively). Based on the assumption of equal or unequal variances, Bonferroni or Games-Howell post hoc corrections for multiple testing were respectively applied. Comparison of categorical variables was assessed using Fisher s exact test. To explore biomarkers discriminatory power, a receiver operating characteristic (ROC) curve analysis was performed, obtaining the area under the curve (AUC) and significance values. AUC interpretation was determined according to Mandrekar and colleagues [39]. Optimal cut-off values were established by coordinate tracing of the ROC curve according to Youden s index analysis. Sensitivity, specificity, positive and negative predictive values (PPV, NPV), were obtained. Correlations between biomarkers and clinical data were investigated using Pearson s or Spearman s analysis as appropriate. Finally, non-collinear independent variables associated with clinical symptoms were investigated using multivariable linear (continuous variables on either a normal or log-transformed scale) and logistic (dichotomous) regression analysis. All tests were two-tailed, and the significance level was set to $P < 0.05$. Analyses were performed using SPSS version 25 (IBM Corporation, Armonk, USA), GraphPad Prism version 8 (GraphPad Software Inc., San Diego, USA), and RStudio 2022.07.2 576.

# 3 | RESULTS

## 3.1 | PLA score as a biomarker to distinguish *GBA* -PD

In agreement with previous results [20], α-synuclein oligomers were increased inside synapses surrounding sweat glands using high-resolution microscopy in iPD (Figure 1A–A ) compared to HC (Figure 1C–C ). Here, we revealed the presence of α-synuclein oligomers also in *GBA1*-PD (Figure 1B–B ). The median values of PLA scores were significantly higher in the *GBA1*-PD than in HC ($P < 0.0001$; Table 1). This finding was confirmed in univariate analysis (with log-transformed PLA score used as dependent variable) and after controlling for the effect of age and sex (adjusted *P*-value 0.00027; Figure 1D). Notably, no difference was found between *GBA1*-PD patients and iPD cases. Looking at the subpopulations of *GBA1*-PD, we did not observe any significant difference in mean PLA score between patients carrying different mutations (Figure 1E). These results were confirmed even when considering p.L483P versus p.N409S mutations alone ($P = 0.627$). ROC curve analysis, used to discriminate *GBA1*-PD from HC, showed an AUC value = 0.927 (95 CI 0.859–0.996, $P < 0.0001$; see Figure 1F). Using a cut-off 98.64, the sensitivity was = 88.9 (95 CI 70.84–97.65), specificity = 88.5 (95 CI 69.85–97.55), PPV = 88.9 (95 CI 73.24–95.90), and NPV = 88.5 (95 CI 72.33–95.74). This cut-off allowed the stratification of our cohort into two groups: subjects with positive PLA score (PLA 98.64) and negative PLA staining (PLA$^-$ <98.64). No significant correlation was found between PLA score and continuous clinical variables (age at PD onset, UPDRS I-II-III-IV, LEDD, disease duration, COMPASS-31, MMSE, FAB, and MoCA scores). In the whole PD cohort, no difference was observed in categorical variables (presence of pRBD, hyposmia, constipation, OH, and HY staging) between PLA versus PLA$^-$ patients. In the iPD subgroup, all pRBD patients ($N = 13$) were PLA , whereas among those without pRBD, 7 (30.4 ) were PLA$^-$ ($P = 0.034$). However, multivariable logistic regression analysis using age, sex, disease duration, PLA, and genetic status as independent variables revealed that the

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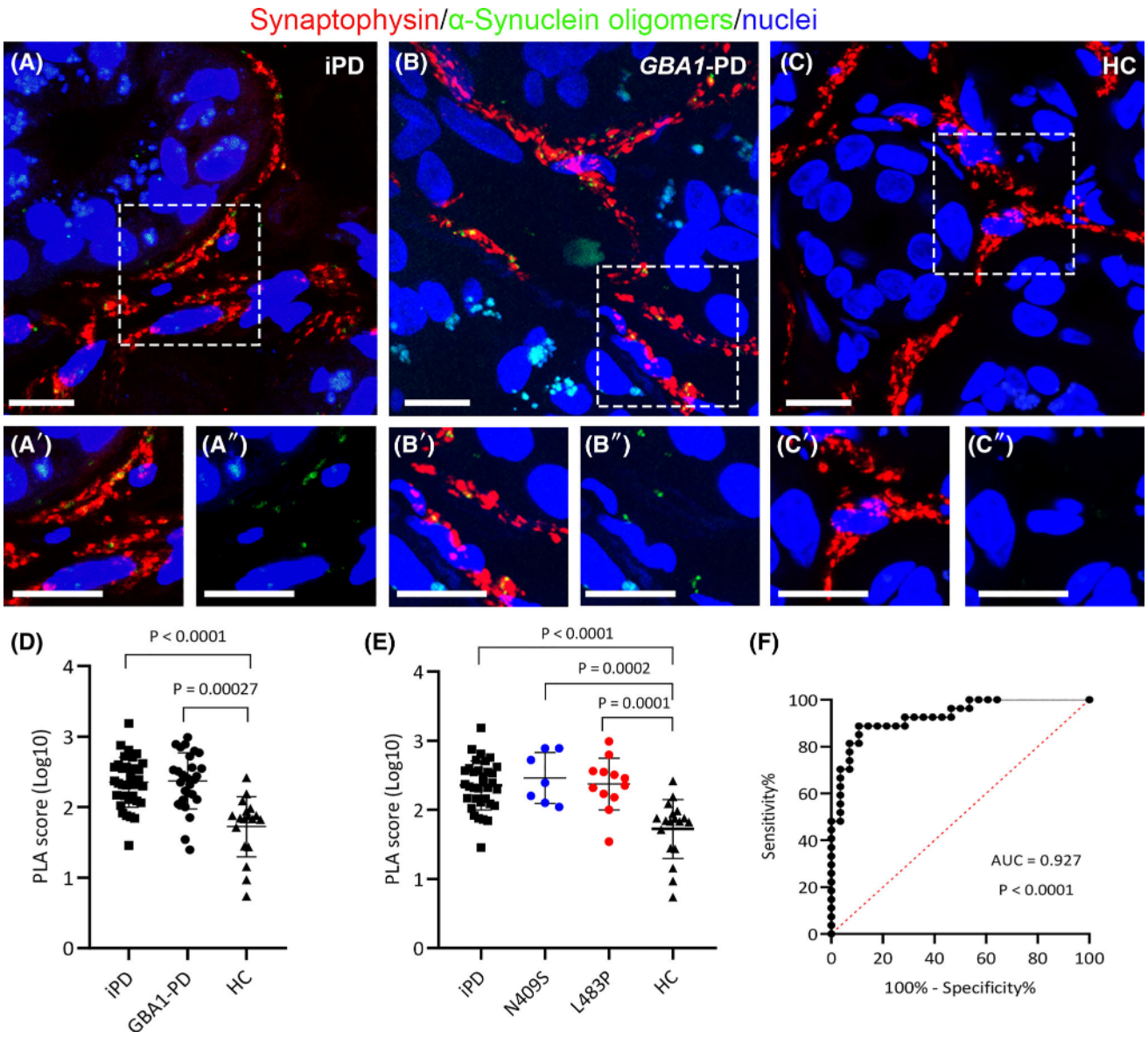


**FIGURE 1** α-Synuclein oligomers in skin biopsies of *GBA1*-PD. (A–C ) Confocal images of sweat glands show α-Synuclein oligomers (PLA signal, green, A, A , B, B , C, C ), synapses (synaptophysin staining, red, A, A , B, B , C, C ), and nuclei (Hoechst, blue, A–C ) in idiopathic PD (iPD, A–A ), *GBA1*-PD (B–B ), and healthy controls (HC, C–C ). Squared insets in (A, B, C) are 2× magnified in A , A , B , B , C , C (high resolution image obtained by SoRa). Scale bar, 10 μm. (D) Univariate analysis to compare mean PLA score values between *GBA1*-PD, idiopathic PD (iPD), and healthy controls (HC). Significant *P*-values (after Bonferroni post hoc correction and adjusted for age and sex) are shown. (E) PLA score (log-transformed) in *GBA1*-PD subgroups. Significant *P*-values (ANOVA analysis with Games-Howell post hoc test) are shown. (F) ROC curve of PLA score to discriminate between *GBA1*-PD and HC.

presence of pRBD was mostly associated with disease duration ($P = 0.054$) and not with PLA staining.

## 3.2 | GCase and PLA score correlate in PD

Given the strict bidirectional pathogenic loop that involves α-synuclein aggregation and GCase reduction in cellular and mouse models [16], we wondered whether GCase expression changes in synaptic terminals of autonomic structures in skin biopsies of *GBA1*-PD where we detected the specific increase of α-synuclein oligomers. We first checked for GCase staining in *post mortem* human brains (Supplementary Figure S1) and found that it decreased in iPD patients compared to controls, as previously reported [40], and localized also at the synapses. Moving to skin biopsies, we found that GCase mostly localizes between the epidermis and the Stratum Corneum, in accordance with what has already been reported [41]. To date, GCase expression within the peripheral nervous system has not been investigated. Here, in a subgroup of 35 PD patients (23 *GBA1*-PD,

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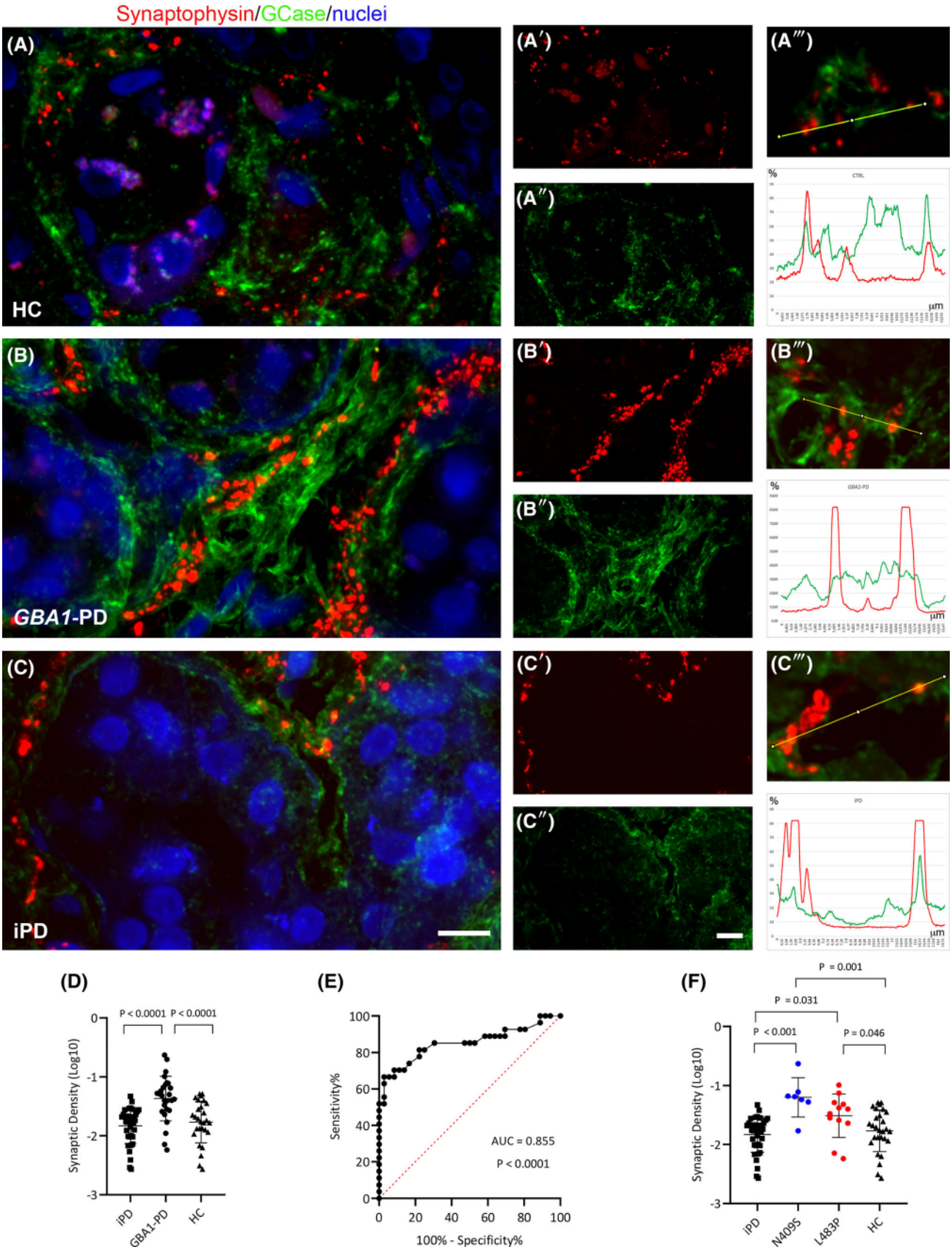


FIGURE 2 Legend on next page.

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12 iPD) and 11 HC, we looked at GCase staining in the synaptic terminals surrounding the secretory coils of sweat glands and observed a diffuse and punctate pattern in all analyzed groups. Interestingly, the GCase signal seemed to be increased in *GBA1*-PD when compared to both control subjects and iPD (Figure 2A–C; A , B , C ). Thus, we focused on the synaptic compartment, and we observed that some synaptic terminals contained GCase, as clearly visible using the intensity profile (Figure 2A , B , C ). We performed the quantitative analysis of the GCase expression inside synapses and normalized for synaptic density to obtain the GCase score and found no significant difference in GCase score between controls, *GBA1*-PD, and iPD (Supplementary Figure S2). This result was confirmed when considering all PD patients versus HC ($P = 0.277$). Interestingly, when analyzing the whole PD population ($N = 35$), we found a direct correlation between PLA score and GCase score ($r = 0.390$, $P = 0.025$), which was mostly driven by the *GBA1*-PD subgroup ($r = 0.479$, $P = 0.021$). This correlation was not confirmed in iPD ($r = 0.073$, $P = 0.832$) and HC ($r = -0.385$, $P = 0.242$). Finally, we performed in the PD group multivariable regression analysis using log-transformed PLA score as the dependent variable and age, disease duration, genetic status, and log-transformed GCase score as independent non-collinear variables. We found that the GCase score was the only variable significantly associated with PLA score ( $= 0.364$, $P = 0.044$; Supplementary Figure S3). No significant difference was detected when stratifying the analysis according to different *GBA1*-PD mutations.

To explore a possible relation between GCase and α-synuclein species other than oligomers, we performed additional stainings for total, phosphorylated, and aggregated, -sheet containing α-synuclein. Looking at total α-synuclein inside the synapses (Supplementary Figure S5A–A ), we observed no differences among HC, iPD, and *GBA1*-PD (Supplementary Figure S5B). We then analysed phosphorylated and aggregated species of α-synuclein using the following specific antibodies and dye: anti-S129 phosphorylated α-synuclein antibody; conformational 5G4 antibody that recognizes α-synuclein aggregates including -sheet conformation; and Thioflavin-S that stains amyloid fibrils (Supplementary Figure S6). As expected, both phosphorylated and aggregated species of α-synuclein were clearly detected inside Lewy bodies in *post mortem* PD human brain (Supplementary Figure S6A,C,E). However, skin biopsies from HC, iPD, and *GBA1*-PD did not show any staining for phosphorylated α-synuclein (Supplementary Figure S6B–B ), -sheet containing aggregated α-synuclein (Supplementary Figure S6D–D ), and amyloid fibrils (Supplementary Figure S6F–F ) inside the synapses.

### 3.3 | The increase in synaptic density identifies *GBA* -PD but not iPD

Although the GCase score resulted unmodified in *GBA1*-PD, the increase of GCase signal prompted us to investigate whether this finding could be caused by changes in synaptic density. Indeed, a rough increase in synaptophysin staining was observed in *GBA1*-PD (Figure 2A , B , C ). Synaptic density was increased as well in *GBA1*-PD compared to both iPD patients and HC (Figure 2D; $P < 0.0001$; Table 1). This statistically significant difference was confirmed in univariate analysis (with log-transformed synaptic density used as the dependent variable) and after controlling for disease duration and age at PD onset (adjusted $P < 0.0001$). ROC curve analysis was employed to discriminate *GBA1*-PD from iPD, and we found an AUC value for synaptic density = 0.855 (95 CI 0.749–0.961, $P = 0.000002$; Figure 2E). A cut-off 0.023 showed a sensitivity = 85.2 (95 CI 66.27 –95.81 ), a specificity = 77.1 (95 CI 59.86 –89.58 ), a PPV = 74.19 (60.53 –84.35 ), and a NPV = 87.10 (72.85 –94.44 ) in detecting *GBA1*-PD. Notably, looking at synaptic density in subpopulations of *GBA1* PD, we observed the highest values in p.N409S patients (Figure 2F), the mild form of *GBA1*-PD.

The increase in synaptic density observed in *GBA1*-PD could rely on a variety of mechanisms including compensatory synaptogenesis in a context of synaptic dysfunction and loss. We addressed this issue by using a well-known marker of sprouting, namely growth associated protein 43 (GAP43) and looking at its distribution at the synapse. The relative amount of GAP43 signal within the synapses (Supplementary Figure S7A–A ) was not significantly different among HC, iPD, and *GBA1*-PD (Supplementary Figure S7B). Interestingly, the analysis of the correlation between synaptic GAP43 and synaptic density revealed an inverse correlation in HC ($r = -0.76$; $P = 0.012$) and iPD ($r = -0.36$; $P = 0.30$), while a positive correlation was a specific feature of the *GBA1*-PD subpopulation ($r = 0.51$; $P = 0.13$; Supplementary Figure S7C).

**FIGURE 2** Synaptophysin and GCase in skin biopsies. Confocal images showing synaptophysin (red), GCase staining (green) and nuclei (Hoechst, blue) in sweat glands of controls (A–A ), *GBA1*-PD (B–B ), and iPD. Scale bar, 15 μm. Intensity profiles show the superimposition of some peaks in a single *z*-plane along the designed line in A , B , C . (D) Univariate analysis to compare mean synaptic density values between *GBA1*-PD, idiopathic PD (iPD), and healthy controls (HC). Significant *P*-values (after Bonferroni post hoc correction and adjusted for age and sex) are shown. (E) ROC curve analysis of synaptic density values suitably allowed the discrimination of *GBA1*-PD from iPD. (F) PLA score (log-transformed) in *GBA1*-PD subgroups. Significant *P*-values (ANOVA analysis with Games-Howell post hoc test) are shown.

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Finally, we decided to explore the association between synaptic density and clinical variables in the overall PD population, and we observed a significant correlation with the orthostatic intolerance subitem ($r = 0.509$, $P < 0.001$; Supplementary Figure S4A) and the total score of the COMPASS-31 ($r = 0.415$, $P = 0.001$; Supplementary Figure S4B). Besides, patients with OH were most likely to have a synaptic density 0.023 (73.1 , $P = 0.02$). Finally, we run multivariable linear regression analyses using age, sex and disease duration as adjusting variables and synaptic density and genetic status as alternate independent predictors. Synaptic density in the overall PD population and genetic status were both associated with both the orthostatic intolerance subitem and the total score of the COMPASS-31. Nonetheless, the analyses showed that the goodness of fit (using the Akaike information criterion as a measure of the relative quality of a model for a given set of data) of the models including synaptic density or genetic status were substantially comparable for both clinical outcomes.

## 4 | DISCUSSION

This study has a twofold importance. First, our results reinforce the concept that α-synuclein oligomers could be a reliable biomarker for distinguishing iPD from control subjects [20] and show that the sensitivity is even higher in the discrimination of *GBA1*-PD patients (88.9 ). Second, we added new information to the pathological pathways characterizing different subpopulations of PD throughout the analysis of synaptic terminals targeting the sweat gland. Here, we observe a positive correlation between α-synuclein oligomers (PLA score) and GCase expression (GCase score) in *GBA1*-PD cohort and report that the *GBA1*-PD cohort exhibits an increase in synaptic density compared to iPD. Notably, the highest values were attributed to variant p.N409S, which is associated with a mild form of *GBA1*-PD. Next, synaptic density displays a moderate correlation with clinical assessment of autonomic dysfunction in the overall PD population.

The increase of α-synuclein oligomers in *GBA1*-PD skin biopsies was expected given that almost all the *post mortem* brains obtained from *GBA1*-PD present Lewy bodies pathology [42]. Thus, the study of the peripheral nervous system likely reflects what happens in the central nervous system [19]. The common pathway of α-synuclein aggregation in iPD and *GBA1*-PD has been supported also by a very recent analysis of the CSF using the α-synuclein seed amplification assay, which gave similar results, obtaining the highest sensitivity in *GBA1*-PD [18].

Focusing on the role of GCase in PD, evidence exists that α-synuclein aggregation and GCase reduction are mutually linked [16]. Previous studies in human brains described a combination of reduced protein levels and catalytic activity, mainly in the *substantia nigra* [15]. On this basis, we expected a decrease in GCase protein levels in the skin and in the synaptic terminals of the *GBA1*-PD and iPD patients. Surprisingly, we observed an overall increase of the GCase signal in the skin of *GBA1*-PD. Given that GCase is a lysosomal ubiquitous enzyme that plays important functions in regulating membrane properties but also extracellular roles beyond the lysosome [41], we may speculate that a compensatory mechanism leads to GCase overexpression in the tissue for counteracting its loss of enzymatic activity. Indeed, *GBA1* mutations affect the endosomal-lysosomal pathway and lead to the accumulation of crowded membranes and dysmorphic organelles, that have already been described in Lewy Bodies [43]. Interestingly, the GCase staining observes in skin biopsy seems to resemble the typical pattern of organelles such as lysosomes. However, we did not see any significant difference in the GCase expression in the synaptic terminals targeting the sweat gland. This discrepancy could be linked to the compensatory sprouting leading to an enrichment of newly formed fibers and, in turn, of GCase in the periphery. To address this point, we evaluated the correlation between synaptic density and GAP43, a sprouting marker. In particular, the inverse correlation in HC suggests that the sprouting decreases in case of high levels of synaptic density, on the contrary, in *GBA1*-PD the sprouting increases with the increase of synaptic density. This phenomenon could be caused by dysfunctional synaptic terminals in *GBA1*-PD patients, probably caused by the described vicious circle that links GCase reduced activity and α-synuclein aggregation [16]. In fact, a direct correlation was found in the *GBA1*-PD subgroup between the GCase score and the PLA score, a finding that supports the presence of a pathogenetic loop involving GCase and α-synuclein aggregation in the periphery. Indeed, a previous study in cell and animal models [16] demonstrated that GCase mutation leads to enzymatic deficiency, that directly induces α-synuclein aggregation by stabilizing soluble oligomers. The α-synuclein aggregates exert an inhibitory effect on GCase activity engendering a vicious circle. This self-propagating positive feedback process proceeds until a pathogenic threshold is surpassed, resulting in neurodegeneration. Given that, GCase could be an attractive target for PD therapy, in order to reduce α-synuclein aggregation also in the peripheral nervous system of *GBA1*-PD.

Since α-synuclein aggregation is a complex process that implies a spectrum of species [44], to get an insight into the correlation of GCase with the process of aggregation of α-synuclein in skin biopsies, we moved beyond α-synuclein oligomers and studied other α-synuclein species, from the monomeric to the aggregated ones. Interestingly, we did not detect any difference in monomeric α-synuclein among HC, iPD, and *GBA1*-PD, indicating that it does not discriminate between patients and controls. Furthermore, we did not observe the presence of

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aggregated -sheet-containing forms, neither with conformational antibody neither with specific dye. In addition, we detect no phosphorylated α-synuclein in our samples. If fibrils and greater assemblies are a later step in the aggregation process, phosphorylation of α-synuclein has also been suggested to feature more advanced stages compared to α-synuclein oligomerization [45, 46], which have been described as an early event in the pathology [35, 47]. This could suggest that in our samples the aggregation process of α-synuclein is at an early stage, or that the intrinsic signal amplification of the PLA technique lets us to unravel the presence of very low amount of oligomers that are not detectable by classical immunofluorescence. Not observing phosphorylated α-synuclein in our samples might appear to contradict previous literature [48]. We cannot rule out the hypothesis that the absence of phospho-synuclein staining in our specimens may be explained by the selection of the forearm as the biopsy site, together with the lower intra-patient sampling in our procedure compared to other studies, as already suggested [49, 50].

Interestingly, we observed that synaptic density, related to the sweat gland secretory coil, in the overall PD population shows a moderate correlation with clinical assessment of autonomic dysfunction, particularly with the total score of the COMPASS-31 and the orthostatic intolerance subitem. This could be explained by the frequent coexistence of excessive sweating and orthostatic hypotension reported in the prodromal phase of PD [51] and by the evidence that sweating is regulated by vasodilation of the vessels surrounding the sweat glands secretory coils [52]. Our results agree with a higher dysautonomic burden in *GBA1*-PD compared to iPD [53], even though the two groups could not be balanced for age at onset and disease duration and these confounders must be taken into account when interpreting our results. However, the increase of synaptic density in the overall *GBA1* PD is unexpected. In fact, we previously reported sweat gland denervation in a higher percentage of iPD subjects compared to HC [20] and others described in iPD patients intraepidermal nerve fiber denervation and loss of autonomic nerves to sweat glands and blood vessels [54]. Furthermore, the deposition of phosphorylated α-synuclein in autonomic fibers was greater in PD patients with autonomic failure [55]. The involvement of the autonomic nervous system is often affecting the cardiac and skin branches in parallel, as proved by the high concordance between $^{123}$I-metaiodobenzylguanidine ($^{123}$I-MIBG) myocardial scintigraphy and skin biopsy data [56]. Focusing on *GBA1* PD, $^{123}$I-MIBG has been reported to be worse than in iPD [57], whilst other studies showed similar uptakes [58]. This discrepancy can be caused by the heterogeneity of the *GBA1* PD variants analyzed. The peculiar profile of autonomic dysfunction in *GBA1*-PD has been further explored in recent research [59], which confirmed through autonomic testing greater cardiovascular and sudomotor dysautonomia in *GBA1*-PD compared with matched PD control subjects.

The relationship between α-synuclein, skin denervation, and clinical features has yielded conflicting results so far. On the one hand, higher ratios of α-synuclein were found to be correlated with reduced sudomotor nerve fiber and both HY scores and sympathetic/parasympathetic function [60]. On the other, some works reported no correlation with age, disease duration, or severity of PD [61], as well as a uniform distribution of phosphorylated α-synuclein not following the side with greater motor impairment [62]. Indeed, in line with the present results, we previously showed [20] high PLA scores both in PD patients with and without autonomic dysfunction and no significant association between PLA and disease duration or the severity of motor features. Several factors could explain these discrepancies in the available literature. First, the moderate association between autonomic symptoms and skin biopsy measures is likely influenced by the use of questionnaires, which may increase the bias of subjective reporting. Previous research observed considerable discordance between overall self-reported scores and electrophysiological test abnormalities [63], as also confirmed in a recent study [59]. Additionally, the prevalence of underrecognized dysautonomia in PD is close to 50 [64]. Last, synaptic density and PLA score may reflect dynamic measures with variable patterns over time and in relation to disease progression. Indeed, synaptic remodeling (pruning and/or sprouting processes) could take place in the sudomotor fibers and compensate for nerve degeneration. Notably, the regeneration property of peripheral nervous system fibers, including both sensory and autonomic skin fibers, has been extensively described [65] and a study by Jeziorska et al. used growth-associated protein 43 (GAP43) to assess altered nerve fiber regeneration in patients with PD [66]. Therefore, it could be argued that compensatory synaptic remodeling may have prevented the elucidation of the exact relationship between peripheral denervation measures and the severity of autonomic symptoms.

This study has indeed some drawbacks, that is, (i) the cross-sectional design which did not allow us to explore a cause-effect relationship between skin biopsy measures and clinical features; (ii) the lack of an instrumental assessment of autonomic symptoms; and (iii) the presence of unbalanced clinical and demographic characteristics in iPD and *GBA1*-PD subgroups. Concerning the last, we have partially addressed the effect of confounders in multivariable analysis.

## 5 | CONCLUSION

Collectively, our research suggests that the assessment of α-synuclein oligomers and synaptic density in the skin has the potential to provide a reliable, accessible, and

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accurate biomarker in iPD and *GBA1*-PD. Our findings may carry preliminary implications for the understanding of peripheral pathophysiological mechanisms underlying nonmotor burden in *GBA1*-PD. A deeper understanding of such mechanisms both in the prodromal and manifest phase of *GBA1*-PD is of paramount importance to address key questions about treatment response in the expanding scenario of pathway-specific therapeutic options, as well as to inform future strategies for more precise patient stratification. In conclusion, we reported the quantitative analysis of α-synuclein oligomers and synaptic density in the skin as a biomarker of iPD and *GBA1*-associated PD.

## AUTHOR CONTRIBUTIONS

Design: SM, ECo, DC, EC, GC, GP, IUI. Execution: SM, ECo, EC, FG, CN, AMC, MJB, FD, LS, VF. Analysis: SM, ECo, EC. Writing: SM, ECo. Editing of final version of the manuscript: EC, RA, GC, GP, IUI.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Source data used for analyses presented in this study are available from the authors upon request.

## ETHICS STATEMENT

This research was carried out in agreement with the principles of the Declaration of Helsinki. All patients gave their written informed consent. Concerning skin biopsy, study procedures were approved by the local Ethics Committee (protocol code 483/18). Human brain samples were obtained from the Nervous Tissue Bank of Milan and the study was approved by the Ethics Committee of the University of Milan (protocol code 66/21).

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