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RESEARCH ARTICLE

A Biomarker Study in Patients with GBA1-Parkinson's Disease and Healthy Controls

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ABSTRACT: Background: Molecules related to glucocerebrosidase (GCase) are potential biomarkers for development of compounds targeting *GBA1*-associated Parkinson's disease (GBA-PD).

Objectives: Assessing variability of various glycosphingolipids (GSLs) in plasma, peripheral blood mononuclear cells (PBMCs), and cerebrospinal fluid (CSF) across GBA-PD, idiopathic PD (iPD), and healthy volunteers (HVs).

Methods: Data from five studies were combined. Variability was assessed of glucosylceramide (various isoforms), lactosylceramide (various isoforms), glucosylsphingosine, galactosylsphingosine, GCase activity (using fluorescent 4-methylumbeliferryl-β-glucoside), and GCase protein (using enzyme-linked immunosorbent assay) in plasma, PBMCs, and CSF if available, in GBA-PD, iPD, and HVs. GSLs in leukocyte subtypes were compared in HVs. Principal component analysis was used to explore global patterns in GSLs, clinical characteristics (Movement Disorder Society – Unified Parkinson's Disease Rating Scale Part 3 [MDS-UPDRS-3], Mini-Mental State Examination [MMSE], *GBA1* mutation type), and participant status (GBA-PD, iPD, HVs).

Results: Within-subject between-day variability ranged from 5.8% to 44.5% and was generally lower in plasma

than in PBMCs. Extracellular glucosylceramide levels (plasma) were slightly higher in GBA-PD compared with both iPD and HVs, while intracellular levels were comparable. GSLs in the different matrices (plasma, PBMCs, CSF) did not correlate. Both lactosylceramide and glucosyl-sphingosine were more abundant in granulocytes compared with monocytes and lymphocytes. Absolute levels of GSL isoforms differed greatly. *GBA1* mutation types could not be differentiated based on GSL data.

Conclusions: Glucosylceramide can stably be measured over days in both plasma and PBMCs and may be used as a biomarker in clinical trials targeting GBA-PD. Glucosylsphingosine and lactosylceramide are stable in plasma but are strongly affected by leukocyte subtypes in PBMCs. GBA-PD could be differentiated from iPD and HVs, primarily based on glucosylceramide levels in plasma. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: glycosphingolipid; clinical trial; glucocerebrosidase; neurodegeneration; genetic risk factor; lysosome; glucosylceramide; lactosylceramide; glucosylsphingosine; disease modification

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Introduction

Parkinson's disease (PD; MIM: 168600) is the second most common multifactorial neurodegenerative disorder that results from complex interactions between environmental and (epi)genetic risk factors.¹ A disease-modifying treatment is still lacking. Through ongoing elucidation of the underlying pathophysiological process, new potential drug targets are being explored.^{2,3} One such target is the lysosomal enzyme glucocerebrosidase (GCase; EC 3.2.1.45), encoded by the *GBA1* gene (MIM: 606463). Apart from the common variants in GWAS loci, a variant in the *GBA1* gene is the most common genetic risk factor known to date to develop PD.^{4,5} Approximately 4–15% of PD patients carry a heterozygous *GBA1* variant and in Ashkenazi Jewish PD patients the frequency is approximately 20%.^{6–8}

Knowledge of the involved pathways will contribute to the identification of drug targets and potential biomarkers to evaluate target engagement of new drugs in early phases of development. GCase metabolizes its substrate glucosylceramide (GluCer) by hydrolysis into glucose and ceramide (Cer). This happens at the luminal lysosomal membrane, with assistance of the membrane-bound activator protein saposin C.⁹ GluCer is the most basic form of a glycosphingolipid (GSL), which constitutes a vast network of different complex GSLs, reviewed in great detail elsewhere.¹⁰ In short, GSLs are a class of lipids, essential for membrane functioning and cell signaling, found throughout the body, including the brain. A GSL is built up of three moieties: a saccharide, a fatty acid chain, and a sphingosine chain. Sphingosine and a fatty acid chain together form ceramide. The first upstream GSL to GluCer, by addition of a second saccharide, is lactosylceramide (LacCer). Conversely, GSLs are degraded by stepwise removal of sugar groups (each by a different enzyme) and the eventual hydrolysis by GCase. In case of severe GCase deficiency, when GluCer levels rise, the enzyme acid ceramidase can convert GluCer into glucosylsphingosine (GluSph), by removal of the fatty acid chain.¹¹ GSLs are present in cell membranes, but also get into solution in extracellular space (like plasma) by yet unknown mechanisms. It is not fully understood how the intra- or extracellular levels of these molecules are affected in people with Parkinson's disease with (GBA-PD) or without (idiopathic non-GBA-PD; for convenience now referred to as iPD) a mutation in the GBA1 gene, compared with healthy people without PD. It was recently shown there is no accumulation of GluCer or GluSph in various brain regions in GBA-PD.¹² Note that according to Michaelis-Menten enzyme kinetics, little to no accumulation of the substrate is expected in mild enzyme deficiency (like in GBA-PD).¹³

Whereas brain material provides the most direct measurement of the pathologically affected tissue, for obvious reasons this cannot be collected during clinical trials. Data on peripherally collected material are limited. Measurements of GCase activity generally show a lower activity in GBA-PD compared with controls,^{14–16} but accurate quantification of this activity comes with many methodological challenges, explaining a large variability, recently reviewed elsewhere.¹⁷ GluCer and LacCer (both multiple isoforms) were increased in the plasma of iPD compared with controls,¹⁸ but this finding was not replicated in serum.¹⁹ In cerebrospinal fluid (CSF), LacCer (total) was increased in iPD compared with controls, but GluCer could not be measured.¹⁹

As drugs are currently being developed that aim to restore GCase activity as a disease progressionmodifying strategy in PD, there is a need for further elucidation of these *GBA1*-related molecules in peripherally collected materials, as potential target engagement biomarkers in the clinical trials of these compounds. This article describes the combined data from five studies, evaluating GCase activity (in lysed cells), GCase protein, GluCer (multiple isoforms), LacCer (multiple isoforms), and GluSph, in varying matrices (plasma, peripheral blood mononuclear cells [PBMCs] and/or CSF) in GBA-PD, iPD, and healthy volunteers (HVs).

Subjects and Methods

Data Sources

Data from five studies were combined:

- 1. A biomarker variability study in GBA-PD (n = 8), iPD (n = 8), and HVs (n = 8).
- 2. Baseline biomarker data from a phase 1 single-dose clinical trial in HVs (n = 40).²⁰
- 3. Baseline biomarker data from a phase 1 multipledose clinical trial in HVs (n = 39).²⁰
- 4. Baseline biomarker data from a phase 1b multipledose clinical trial in GBA-PD (n = 40).²¹
- 5. Single blood draw to compare biomarkers between immune cell subsets in HVs (n = 6).

Study 1 was used to assess the variability (withinand between-subject, within- and between-day) of GCase activity, GCase protein, GluCer, GluSph, and galactosylsphingosine (GalSph). Studies 2 (measured GluCer), 3 (measured GluCer, GluSph, and LacCer), and 4 (measured GluCer, GluSph, and LacCer) were used to expand groups for a comparison between GBA-PD and HVs. Study 5 was used to compare GluCer, GluSph, and LacCer between immune cell subsets. At every blood draw, plasma and PBMCs were collected for separate analysis. In studies 3 and 4 CSF was also collected. An overview of measurements per study can be found in Table 1 in Data S1.

Subjects

See supplementary material for details about subject enrolment for each of the five studies.

Plasma, PBMCs, and CSF Collection

Biomarkers were measured in K_2 EDTA plasma, PBMCs, and CSF. PBMCs were isolated from venous blood using cell preparation tubes (CPTs) containing sodium heparin (Becton Dickinson, NJ, USA) according to the manufacturer's instructions. For CSF collection, the first milliliter of CSF was discarded to prevent contamination with blood, after which 4 mL was collected in a Falcon[®] tube. For details see supplementary material.

GCase Activity and Protein

GCase activity and GCase protein were measured in study 1 only in lysed PBMCs. Activity was measured using the fluorescent artificial substrate 4-methylumbeliferryl- β -glucoside (4-MUG). Protein was measured using the commercial ELISA kit Glucosidase Beta Acid from Cloud Clone Corp.

GluCer, LacCer, GluSph, and GalSph

GluCer was measured in all studies, LacCer and GluSph were measured in studies 3, 4, and 5, and Gal-Sph was measured in study 1 only. GluCer and LacCer were measured in plasma, PBMCs, and CSF if available. GluSph was measured in plasma and PBMCs only. GalSph was measured in plasma only. All GSLs were measured using validated liquid chromatography with tandem mass spectrometry (LC–MS/MS) methods.

Leukocyte Subtypes

PBMCs are constituted of monocytes and lymphocytes, but inherent to the isolation method, some degree of granulocyte contamination is often present,²² which can vary between blood draws. In study 5, monocytes, lymphocytes, and granulocytes were isolated separately using magnetic cell separation (see supplementary material for details).

Genotyping

The *GBA1* gene was sequenced in all subjects, except for the HVs in studies 1 and 5, using saliva-derived DNA and methods described previously.⁸ In short, next-generation sequencing was performed, using longrange polymerase chain reaction and a primer set unique to the functional *GBA1* gene, thereby preventing amplification of the nearby pseudogene (see supplementary material for details).

MMSE, H&Y, and MDS-UPDRS-3 (on-state)

In PD subjects, the Mini-Mental State Examination (MMSE) (studies 1 and 4), Hoehn & Yahr (H&Y) staging (studies 1 and 4), and the Movement Disorders Society – Unified Parkinson's Disease Rating Scale Part 3 (MDS-UPDRS-3) in *on*-medication state (motor score) (study 4 only) were performed. Only screening (MMSE and H&Y study 1, MMSE study 4) or baseline (MMSE, H&Y, and MDS-UPDRS-3 study 4) data were used since these ratings might be influenced by placebo effect.

Statistical Testing: Variability

Using study 1 to estimate variabilities within a day (measurements 1 to 5 on day 1), the variables were analyzed with a mixed-model analysis of variance with fixed factors group (HV, iPD, and GBA-PD), measurement and the interaction group by measurement, and a random subject factor. The covariance parameter estimates were used to calculate the between- and withinsubject variabilities (standard deviation and coefficient of variation). This was repeated for the variability over days (measurement 2 on day 1 and measurements on day 5 and day 8), where measurement was replaced by day.

Collated Data of GBA-PD, iPD, and HVs

For every subject, the average per parameter was taken of all available measurements (from study 1 all measurements, from studies 2, 3, and 4 the baseline and placebo-treated measurements). Levels are depicted using violin plots. Considering the exploratory nature and extensiveness of the measurements, no formal statistical testing was performed to compare means. Means and 95% confidence intervals (95% CIs) are given.

Statistical Exploration: Principal Component Analysis

Principal component analysis (PCA) is a statistical technique used to emphasize variation and bring out strong patterns within the data. We aimed to explore multivariate association structures in a set of variables including various GSLs in PBMCs, plasma, and CSF, and age and body mass index (BMI). For GBA-PD patients only, we also included H&Y, MDS-UPDRS-3 total score, MMSE, age at diagnosis, duration of diagnosis, and *GBA1* mutation type. As we did not use any missing data imputation method, we conducted the analysis in four batches (complete cases) to maximize the use of the available data (see supplementary material for details). Analysis results are presented using biplots (supplementary material). Relationships are not quantified using PCA.

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TABLE 1 Overview of demographic variables, including GBA1 variants. Healthy volunteers from studies 1 and 5 were not sequenced and genotype is therefore unknown

Demographics								
Study	HVs				Patients			
	1	2	3	5	1	4	1	
Status	HV	HV	HV	HV	GBA-PD	GBA-PD	iPD	
n	8	40	39	6	8	40	8	
Male/female	4/4	31/9	26/13	1/5	4/4	20/20	7/1	
Age (years), mean (range)	44.4 (22–67)	38.1 (18–64)	67.0 (52–75)	24.3 (23–26)	60.6 (47–72)	61.1 (40-80)	66.0 (54–76)	
BMI (kg/m ²), mean (range)	25.3 (20.4–29.5)	24.1 (18.9–29.8)	25.6 (19.7–31.9)	-	24.0 (18.0–29.1)	24.5 (17.5–35.1)	28.2 (23.2–32.2)	
MMSE, mean (range)	-	_	_	-	27.6 (23–30)	27.8 (20.5–30)	28.4 (26–30)	
Hoehn & Yahr, mean (range)	N/A	N/A	N/A	N/A	2.3 (1-4)	2.3 (1-4)	1.5 (1–3)	
MDS-UPDRS-3, mean (range)	N/A	N/A	N/A	N/A	-	31.4 (14–72)	-	
Age at diagnosis (years), mean (range)	N/A	N/A	N/A	N/A	47.0 (38–53)	55.1 (38–76)	60.7 (48–69)	
Duration diagnosis (years), mean (range)	N/A	N/A	N/A	N/A	14.1 (2.8–25)	6.1 (1.5–17.7)	5.7 (1.8–11.8)	
GBA1 genotype								
Study		1	2		3	4	5	
GBA1	Н	V, iPD, GBA-PD	HV		HV	GBA-PD	HV	
Wildtype		8 (iPD)	37		37	0	0	
Unknown		8 (HVs)	0		0	0	6	
Mutated		8 (GBA-PD)	3		2	40	0	
GBA1 mutations (allelic	name)							
p.R120W		0	0		0	2	0	
p.[D140H;E326K]		2	0		0	6	0	
p.G202R		0	0		0	1	0	
p.[L324P];[T369M]		1	0		0	0	0	
p.G325R		0	0		0	1	0	
p.E326K		2	1		0	9	0	
p.[E326K];[E326K]		1	0		0	1	0	
p.[E326K];[T369M]		0	0		0	1	0	
p.R329C		0	0		0	1	0	
p.T369M		1	2		2	4	0	
p.T369M(;)L444P		0	0		0	2	0	
p.N370S		0	0		0	7	0	
p.L444P		1	0		0	5	0	

Note: The *GBA1* allelic names are given, excluding the 39-amino acid signaling peptide. In the case of two mutations, variants within the staple signs [] are on the same allele, and variants in separate staple signs are on separate alleles. A semicolon in parentheses indicates that it is uncertain how these mutations are distributed over alleles. Abbreviations: HV, healthy volunteers; GBA-PD, Parkinson's disease with a *GBA1* mutation; iPD, idiopathic Parkinson's disease (ie, no *GBA1* mutation); BMI, body mass index; –, not performed in this study; MMSE, Mini-Mental State Examination; N/A, not applicable; MDS-UPDRS-3, Movement Disorder Society – Unified Parkinson's Disease Rating Scale Part 3.

TABLE 2 Coefficient of variation (= $[SD/mean] \times 100$) of glucosylceramide (multiple isoforms), glucosylsphingosine, galactosylsphingosine, glucocerebrosidase (GCase) activity, and GCase protein in plasma and/or peripheral blood mononuclear cells, split for healthy volunteers, idiopathic Parkinson's disease, and GBA1-associated Parkinson's disease, for within-day and between-day assessments

		Coefficient of variation (CV) (study 1) (%)				
	Group	Within-day		Over-days		
Variable		Within- subject	Between- subject	Within- subject	Between- subject	
PBMCs						
GCase protein (pg/1.0 E+07 cells)	HV	13.4	27.4	17.9	27.3	
	GBA-PD	13.0	25.9	19.8	27.2	
	iPD	11.4	36.3	20.0	32.8	
GCase activity (pmol/h/ug tot prot)	HV	26.4	14.0	14.2	21.3	
	GBA-PD	21.1	56.2	17.8	57.0	
	iPD	21.7	51.1	34.2	53.7	
Glucosylceramide C16:0 (pmol/5.0 E+05 cells)	HV	20.1	27.2	10.8	15.1	
	GBA-PD	14.9	15.2	12.9	21.1	
	iPD	26.6	24.3	18.6	23.9	
Glucosylceramide C18:0 (pmol/5.0 E+05 cells)	HV	12.2	13.3	10.1	15.4	
	GBA-PD	12.6	30.2	17.2	21.9	
	iPD	22.4	22.6	16.8	21.1	
Glucosylceramide C22:0 (pmol/5.0 E+05 cells)	HV	11.1	7.7	10.6	8.1	
	GBA-PD	12.0	21.0	13.2	21.0	
	iPD	21.1	18.1	14.3	13.1	
Glucosylceramide C24:0 (pmol/5.0 E+05 cells)	HV	13.0	19.5	11.9	19.0	
	GBA-PD	14.4	23.6	12.8	23.8	
	iPD	24.0	24.1	15.4	18.3	
Glucosylceramide C24:1 (pmol/5.0 E+05 cells)	HV	12.2	19.8	12.5	16.2	
	GBA-PD	12.2	20.9	12.9	25.3	
	iPD	27.1	27.8	19.6	23.8	
Glucosylsphingosine (pmol/5.0 E+05 cells)	HV	43.2	55.3	35.2	59.5	
	GBA-PD	32.3	52.4	25.9	51.6	
	iPD	25.9	38.0	44.5	51.7	
Plasma						
Galactosylsphingosine (pmol/mL)	HV	5.2	30.4	17.5	27.6	
	GBA-PD	8.4	17.1	10.0	13.3	
	iPD	7.1	31.7	7.7	32.1	
Glucosylceramide C16:0 (pmol/mL)	HV	4.0	34.6	6.8	36.1	
	GBA-PD	5.1	19.7	6.2	18.9	
	iPD	5.4	23.9	5.8	23.3	
Glucosylceramide C18:0 (pmol/mL)	HV	6.7	33.3	8.5	34.0	
	GBA-PD	7.0	35.5	8.3	36.6	
	iPD	9.2	25.1	7.3	25.1	

TABLE 2 Continued

		Coefficient of variation (CV) (study 1) (%)			
	Group	Within-day		Over-days	
Variable		Within- subject	Between- subject	Within- subject	Between- subject
Glucosylceramide C22:0 (pmol/mL)	HV	8.6	31.2	11.4	30.3
	GBA-PD	7.7	25.7	9.9	27.5
	iPD	10.5	21.2	8.7	25.4
Glucosylceramide C24:0 (pmol/mL)	HV	8.5	29.4	11.8	29.4
	GBA-PD	9.4	25.0	12.0	24.9
	iPD	10.8	16.6	9.1	17.5
Glucosylceramide C24:1 (pmol/mL)	HV	6.0	30.8	10.1	31.5
	GBA-PD	6.9	18.0	7.0	18.4
	iPD	6.8	25.5	7.4	24.6
Glucosylsphingosine (pmol/mL)	HV	6.3	29.9	19.2	29.4
	GBA-PD	7.8	42.5	13.9	37.0
	iPD	7.8	39.0	9.3	36.0

Note: Data are from study 1 with measurements given for PBMCs and plasma. Some of the variability in PBMCs may be explained by a varying constitution of cell subtypes of the PBMC isolations (mostly for GluSph, and possibly GCase activity and protein).

Abbreviations: PBMC, peripheral blood mononuclear cell; GCase, glucocerebrosidase; HV, healthy volunteer; GBA-PD, Parkinson's disease with a GBA1 mutation; iPD, idiopathic Parkinson's disease (ie, no GBA1 mutation); GluSph, glucosylsphingosine.

Results

Subjects

In study 1, 8 subjects per group (HV, iPD, and GBA-PD) were included. In study 2, 40 HVs were included, of which 2 subjects carried the heterozygous T369M variant and one subject carried the heterozygous E326K variant. In study 3, 39 HVs were included, of which 2 subjects carried the heterozygous T369M variant; 1 subject withdrew prior to any measurements for personal reasons and was not replaced. In study 4, 40 people with GBA-PD were included. Demographic data, including *GBA1* genotypes if available, of all subjects are summarized in Table 1.

Variability

The within- and between-subject, within- and between-day variability of GluCer (multiple isoforms), GluSph, GalSph, GCase activity, and GCase protein in plasma and/or PBMCs, split for HVs, iPD, and GBA-PD, are given in Table 2. Generally, plasma measurements were more stable than PBMC measurements. No diurnal rhythm was observed based on the samplings from 11:00 h to 19:00 h in all groups. No acute effect of food intake was observed based on the first fasted sample in HVs only. GluCer C18:0 (as an example), GCase activity, and GCase protein measurements can be seen in Figure 1 in Data S1.

Differences between GBA-PD, iPD, and HVs

Average levels of various GSLs in HVs, iPD, and GBA-PD were compared. Mean values with non-overlapping 95% CIs are discussed. Extracellular GluCer (multiple isoforms), mean (95% CI) levels in plasma were higher in GBA-PD (C16:0789.5 [727.5, 851.5]; C18:0 85.9 [78.3, 93.4]; C22:01724.9 [1518.4, 1931.4]; C24:01881.6 [1649.2, 2113.9]; C24:11753.3 [1551.1, 1955.5]) compared with iPD (C16:0645.1 [517.9, 772.2]; C18:0 59.4, [47.1, 71.6]; C22:0900.3 [728.2, 1072.4]; C24:0845 [729.4, 960.6]; C24:1758.8 [603.1, 914.5]), and/or HVs (C16:0521.9 [497.1, 546.6]; C18:0 53.9 [49.8, 58.1]; C22:0839.5 [798.2, 880.9]; C24:0798.8 [756.4, 841.3]; C24:1787.6 [745.5, 829.7]). Levels in PBMCs and CSF were similar over all groups (no CSF was available from iPD), except GluCer C20:0 in CSF, which was higher in GBA-PD (0.17 [0.15, 0.19]) compared with HVs (0.12 [0.10, 0.14]). See Figure 1 (biomarker: GluCer) and Table 3 in Data S1.

For LacCer (multiple isoforms, GBA-PD and HVs only), levels in PBMCs did not differ between GBA-PD and HVs. Levels in plasma vary, where in GBA-PD these were elevated for LacCerC18:0 (61.0 [56.2, 65.8] vs. 52.3 [48.6, 56.0]), LacCerC22:0 (167.6 [154.8, 180.4] vs. 129.5 [121.2, 137.9]) and LacCerC22:1 (51.5 [46.8, 56.1] vs. 41.3 [37.4, 45.2]), decreased for LacCerC20:0 (25.7 [23.7, 27.7] vs. 33.6 [31.2, 36.0]), and no clear difference for LacCerC16:0, -C24:0, and



FIG. 1. Violin plots given for glucosylceramide (GluCer), per matrix (peripheral blood mononuclear cells [PBMCs], plasma, and cerebrospinal fluid [CSF] if available), separate for Parkinson's disease with a *GBA1* mutation (GBA-PD) (grey, left), idiopathic Parkinson's disease (iPD) (blue, middle) and healthy volunteers (HVs) (green, right). In the boxplots, the horizontal line indicates the median, the box indicates the 25th and 75th percentiles, and the whiskers indicate the largest/smallest value no further than $1.5 \times$ interquartile range from the box. Samples that were (LLOQ were treated as 50% of LLOQ. Some participants have a value between LLOQ and 50% of LLOQ in CSF, if one sample was above LLOQ and one was below (maximum of 2 CSF samples per participant), of which the average was taken. Violin plots for LacCer, GluSph, GalSph, GCase activity and GCase protein can be found in the supplementary material. Concentration in plasma and CSF is expressed in pmol/mL, concentration in PBMCs is expressed in pmol/5.0*10⁵ cells. [Color figure can be viewed at wileyonlinelibrary.com]

-C24:1. In CSF, LacCerC18:0 was decreased in GBA-PD $(0.74 \ [0.68, 0.80])$ compared with HVs $(0.89 \ [0.82, 0.95])$ and LacCerC20:0 was decreased in GBA-PD $(0.113 \ [0.099, 0.127])$ compared with HVs $(0.183 \ [0.165, 0.201])$ but similar in other isoforms (C16:0, C22:0, C24:0, and C24:1). See Figure 2 in Data S1 (biomarker: LacCer) and Table 3 in Data S1.

Extracellular (plasma) GluSph was elevated in GBA-PD (1.36 [1.21, 1.52]) compared with HVs (0.99 [0.93, 1.06]) and marginally overlapped with iPD (0.93 [0.64, 1.23]) but was similar over all groups intracellularly (PBMCs). GalSph (plasma only) was similar in GBA-PD, iPD, and HVs. See Figures 3 and 4 in Data S1 (biomarker: GluSph and GalSph) and Table 3 in Data S1.

GCase activity in lysed PBMCs (using 4-MUG) was generally decreased in GBA-PD (2.29 [1.21, 3.37]) compared with HVs (3.67 [3.32, 4.03]). Activity in iPD (3.98 [2.22, 5.74]) was similar to HVs on average, but varied greatly, with both higher and lower levels compared with HVs. GCase protein levels (PBMCs only) were increased

in iPD (44,180 [31,613, 56,747]) compared with HVs (25,871 [20,336, 31,407]) and to a lesser extent in GBA-PD (35,455 [27,510, 43,400]) compared with HVs. Note that GCase activity and protein were measured in study 1 only, consisting of 8 subjects per group. See Figure 5 and 6 in Data S1 (biomarker: GCase activity and GCase protein) and Table 3 in Data S1.

PCA

Different combinations of data from studies 1–4 were used to conduct five PCAs (PCA1, 2, 3.1, 3.2, and 4) to maximize sample size for certain parameters. Key observations per PCA are described, while the biplots and a more detailed description can be found in Figures 7–11 in Data S1. Note that PCA is suitable for pattern recognition in large datasets, but it does not quantify individual relationships, and these should be interpreted with caution.



FIG. 2. Scatterplots of percentage of granulocytes in a peripheral blood mononuclear cell (PBMC) isolation and the correlation with different lactosylceramide (LacCer) isoforms (pmol per 0.5×10^6 cells) and glucosylsphingosine (GluSph) (pmol per 1.0×10^6 cells). R² is given per plot. [Color figure can be viewed at wileyonlinelibrary.com]

Overall, different isoforms of a GSL (GluCer and LacCer) are interrelated within matrix (plasma, PBMCs, CSF) but not between matrix.

PCA1 (Figure 7 in Data S1) was maximized for GCase activity and protein in PBMCs, but also includes GluCer (multiple isoforms) and GluSph in PBMCs, GluCer, GluSph, and GalSph in plasma, age, and BMI. Data from study 1 were used, consisting of GBA-PD (n = 8), iPD (n = 8), and HV $(n = 7, 1 \text{ listwise procedural exclusion of a participant due to missingness of GluCerC22:0 and C24:0 in plasma).$

GCase activity and GCase protein level in lysed PBMCs are uncorrelated. GCase activity shows a moderate inverse correlation with extracellular (plasma), but not intracellular (PBMCs) GluCer (multiple isoforms), GluSph, and GalSph.

PCA2 (Figure 8 in Data S1) was maximized for GluCer and LacCer in plasma, PBMCs, and CSF and GluSph in plasma and PBMCs, but also includes age and BMI. Data from studies 3 and 4 were used consisting of GBA-PD (n = 40) and HV (n = 39).

All CSF measurements (GluCer and LacCer multiple isoforms) are correlated. CSF measurements do not correlate with either PBMC or plasma measurements and cannot differentiate between GBA-PD and HVs.

PCA3.1 (Figure 9 in Data S1) was maximized for GBA-PD, including *GBA1* mutation type, MDS-UPDRS-3 total score, H&Y, age at diagnosis, duration of diagnosis, age, BMI, GluCer (multiple isoforms), and LacCer (multiple isoforms) in PBMCs, plasma, and CSF and GluSph in plasma and PBMCs. Data from study 4 were used consisting of GBA-PD (n = 38, 2 listwise exclusions due to missing CSF samples).

No differentiation can be made between GBA-PD patients with GD-mutations and non-GD-mutations. Clinical characteristics like MMSE, MDS-UPDRS-3, duration of diagnosis, and age at diagnosis only show weak correlations at best with GSLs.

PCA3.2 (Figure 10 in Data S1) was the same as PCA3.1 but excluding CSF measurements, to emphasize the relation between plasma and PBMC measurements with the clinical GBA-PD covariates. Data from study 4 were used consisting of GBA-PD (n = 40).

No differentiation can be made between GBA-PD patients with GD-mutations and non-GD-mutations. MDS-UPDRS-3 and duration of diagnosis only weakly inversely correlate to LacCer (multiple isoforms) levels in plasma.

PCA4 (Figure 11 in Data S1) was maximized for GluCer in plasma and PBMCs, but also includes age and BMI. Data from studies 1, 2, 3, and 4 were used consisting of GBA-PD (n = 48), iPD (n = 8), and HV (n = 86).

GluCer (multiple isoforms) in plasma and in PBMCs do not correlate. GBA-PD can be differentiated from iPD and HVs based on higher plasma GluCer levels and slightly lower PBMC GluCer levels, however, with some overlap. iPD and HVs cannot be differentiated.

Effect of Cell Subtype

Using data from study 4, the effect of granulocyte contamination of isolated PBMCs on LacCer and GluSph levels is depicted in Figure 2. An R^2 value of up to 0.67 was seen, suggesting that these GSLs are much more abundant in granulocytes than in monocytes and lymphocytes.

Subsequently, GluCer (multiple isoforms), GluSph, and LacCer (multiple isoforms) were determined in separately isolated monocytes (average purity: 84%), lymphocytes (average purity: 94%), and granulocytes (average purity: 94%) from 6 healthy volunteers. Absolute values are presented in Figure 12 in Data S1. Distribution of GluCer over cell types varied per isoform; GluCer C20:0 was twice as abundant in lymphocytes compared with both monocytes and granulocytes, and GluCer C24:0 was more than five times as abundant in monocytes compared with lymphocytes and twice as abundant in monocytes compared with granulocytes. Both GluSph and LacCer (all isoforms) were at least 20 times as abundant in granulocytes compared with both monocytes and lymphocytes, and more abundant in monocytes than in lymphocytes.

Discussion

The GCase mechanism is one of the most promising targets to find a first disease-modifying therapy for PD.³ Use of biomarkers in early-phase clinical trials is crucial for innovative drug development.²³ This article combined data from five studies to further assess multiple potential biomarkers for GBA-PD. Variability was determined of GluCer (multiple isoforms), GluSph, Gal-Sph, GCase activity (using 4-MUG in lysed cells), and GCase protein in plasma and/or PBMCs. Levels were descriptively compared between GBA-PD, iPD, and HVs and effects of various covariates were assessed. GSL levels, primarily of LacCer (multiple isoforms) and GluSph, differ between leukocyte subtypes. This shows that it is of the utmost importance to always consider what tissue and cell type is being used when measuring or comparing GSLs.

A low within-subject between-day variability relative to an expected change is favorable for a biomarker when investigating a long-term effect. Measurements in plasma were generally less variable than in PBMCs. Higher variability in PBMCs was partly caused by a variable cell type constitution of the PBMC isolation, including contamination with granulocytes.

A theoretical advantage of using PBMCs as opposed to plasma when measuring GSLs is that this reflects intracellular (ie, intramembrane) levels, which may be expected to better reflect lysosomal functioning. It should be noted, however, that the majority of the intracellular GSL content is outside of the lysosome,²⁴ so the total intracellular level does not distinguish lysosomal from non-lysosomal. Conversely, measurements in plasma and CSF reflect extracellular GSL, of which the origin is unclear. It may be that the "extracellularization" of GSLs is influenced by factors that themselves are sensitive to membrane composition.

To our knowledge, this is the first study to quantitatively evaluate various GSLs in a cell-specific way. After isolating monocytes, lymphocytes, and granulocytes separately, it was shown that GluSph and LacCer (multiple isoforms) are much more abundant in granulocytes compared with monocytes and lymphocytes. GluCer (multiple isoforms) varied to a lesser extent over cell types, but these differences may still be clinically relevant. Such differences may affect the sensitivity of these measurements as a biomarker because the ratios of cell subtypes in a PBMC isolation can vary between blood draws within the same person.

GCase activity was measured in lysed PBMCs. Average activity was lower in GBA-PD compared with iPD and HV, but the overlap between GBA-PD and iPD and between GBA-PD and HV is considerable. For example, activity was measured in two patients carrying the p.D140H + E326K complex allele, of which one had the highest and the other had the second-tolowest GCase activity within the GBA-PD group. These measurements were consistent within-subject over seven samples and no technical reason could be identified to explain this between-subject inconsistency in GCase activity in patients with the same mutation. This variability is likely caused by polygenic variation affecting local factors like lysosomal pH, adequate trafficking, and related enzymes like saposin C.²⁵ Furthermore, in a patient where no GBA1 variant could be identified (iPD), the second-to-lowest GCase activity was measured of all participants. It is indisputable that being a carrier of certain GBA1 variants is associated with an increased risk of developing PD, but it can be hypothesized that measuring actual GCase activity may be a better predictor (though methodological challenges are considerable¹⁷). This could also identify patients without a GBA1 mutation who could potentially benefit from treatment targeting GCase activity.

Surprisingly, GCase protein was higher in iPD, and to a lesser extent in GBA-PD, compared with HVs (Figure 6 and Table 3 in Data S1). Since this was only measured in 8 participants per group, confirmation in a larger cohort is required. A possible explanation is that GCase is upregulated in PD, irrespective of mutational status, due to lysosomal involvement in PD pathophysiology. Our measurement in GBA-PD may be underestimated because a commercial ELISA kit was used that was not purposefully designed for the various GCase mutants. No differentiation was made between GCase protein in the active or inactive state. The effect of leukocyte subtypes remains to be assessed.

Various GSL chain length isomers may play different roles in GBA-PD progression but this is not yet fully elucidated.^{26,27} The absolute quantity of the different isoforms of a specific GSL also differs widely within a cell type (Figure 12 in Data S1). In multiple previous studies^{19,28} all GSL chain lengths are totaled, meaning the signal of sparse isoforms will be overshadowed by those that are more abundant. For example, LacCerC16:0 is approximately 140 times as abundant as LacCerC20:0 in granulocytes, obscuring any potential change in LacCerC20:0 if these are combined. We therefore advise consideration of these different chain lengths despite their exact function being unknown.

The methodology used for granulocyte isolation can have a great influence on the quality and quantity of the cells. Granulocytes, with special attention to neutrophils, are very fragile and easily activated. Blood collection technique, use of anticoagulant, and processing time are some factors among others which need to be taken in account. The quantity of collected neutrophils is highly dependent on the time of blood storage and time of processing.²⁹ All activities, from blood collection to the resuspension of isolated granulocytes in buffer, were done at room temperature. It is favorable to not alter the temperature to preserve the quantity and function of granulocytes.

When comparing GBA-PD to iPD and HV (Fig. 1) all GluCer isoforms in plasma were elevated in GBA-PD. Apart from a decreased GCase activity in PBMCs, this is the clearest expression of an affected GSL metabolism (of which the unknown process of extracellularization is a part) in GBA-PD in a cross-sectional study. This was not seen in PBMCs or CSF.

A possible explanation for this discrepancy in matrices could be based on GSL shedding and uptake, a process whereby GSLs are released into the extracellular space and may also be taken up into the plasma membrane of other cells (both through a yet unknown mechanism).³⁰ Interestingly, this release is independent of the total amount of that GSL in the cell³¹ (hence we did not measure an increased level in PBMCs). Since plasma and CSF are in different compartments, this can be reasoned two ways: (1) this process is primarily affected systemically and not in the CNS, implying that this aspect of the process may be less relevant for (GBA-)PD pathophysiology or (2) GSL uptake is also higher in the CNS as part of the pathophysiological process, therefore resulting in net no change in CSF levels.

The GluCer C24:1 isoform in plasma best differentiated GBA-PD from iPD and HVs. Of 48 GBA-PD patients, 32 (66.7%) had a higher level of plasma GluCer C24:1 than all HVs, suggesting this may be a suitable marker to show a response. However, since the

Box 1. Summary of the key findings

Key findings

- Glucosylceramide (GluCer), glucosylsphingosine (GluSph), galactosylsphingosine (GalSph), glucocerebrosidase (GCase) activity, and GCase protein can be stably measured between-days withinsubject; within-patient variability is lower in plasma than in peripheral blood mononuclear cells (PBMCs).
- Plasma (extracellular) levels of various GluCer isoforms (mainly C24:1 and C24:0) and GluSph are increased in *GBA1*-associated Parkinson's disease (GBA-PD) compared with idiopathic non-GBA-PD (iPD) and healthy volunteers.
- Glycosphingolipid (GSL) levels in plasma (extracellular) are unrelated to levels in PBMCs (intracellular) or cerebrospinal fluid (extracellular in central nervous system) in a cross-sectional measurement.
- The relationship between GCase activity, GCase protein level, various GSL levels, and *GBA1* genotype is not straightforward, possibly reflecting a complex and variously adaptive GSL metabolism.
- GBA-PD clinical characteristics do not correlate with GSL levels.
- *GBA1* mutation types do not correlate with GSL levels.
- There can be major differences in GSL levels between cell types, so care is advised when using mixes or tissue homogenates (and this may be similarly true for GCase activity and GCase protein).
- GSL isoforms can differ greatly in quantity (within the same cell type) and should not be presented as a composite, or sum, if possible.

origin of this extracellular GluCer is unknown, it remains unclear how fast a response could be expected. The 32 GBA-PD patients with a high plasma GluCer C24:1 level could not otherwise be distinguished from the 16 patients with "normal" levels, for example, based on MDS-UPDRS-3 (*on*-state), MMSE, duration of diagnosis, age at diagnosis, or genotype (eg, both p.E326K and p.L444P carriers were present in the high and in the "normal" groups). It may be hypothesized that GluCer in PBMCs (intracellular) better reflects short-term changes.²¹

A recent paper by Te Vruchte et al also showed that GluCer in plasma is increased in iPD compared with HVs.³² This was measured using normal-phase highperformance liquid chromatography so isoforms could not be differentiated. We did not observe this increase in our iPD group but this consisted of only 8 participants. On the contrary, a recent paper by Surface et al did not show an increase in plasma GluCer (only composite of isoforms given) in GBA-PD compared with iPD or HVs (n = 20 per group).³³ All in all, it remains unclear what the role might be of absolute levels of GSLs in PD. It is also hypothesized that the dynamic flux of the GSL recycling pathway may be more relevant than static absolute levels³⁴ but this requires validation.

Using PCA, an attempted was made to uncover underlying correlation structures within the data. A single PCA biplot is a figurative representation of multivariate correlations, which is a powerful tool to identify global patterns in large datasets. Correlations between individual parameters, as opposed to global patterns, should be interpreted with caution. These patterns per biplot are discussed in the supplementary material.

Overall, within a matrix (ie, plasma, PBMCs, and CSF) the different isoforms of a GSL (GluCer or LacCer) are interrelated (seen in all PCA biplots). There is no clear-cut best choice of matrix since all three investigated matrices have arguments for their usefulness. CSF provides the most direct central nervous system measurement, PBMCs provide the only intracellular measurements, and plasma is the only matrix that showed a clear difference between GBA-PD versus iPD and HVs (for GluCer only).

No differentiation between mutation type (Gaucherassociated or PD-only-associated) could be made within the GBA-PD group based on the measured GSLs in various matrices (Figures 9 and 10 in Data S1). Conflicting results exist related to clinical differences between certain mutations, meaning these are sometimes apparent, but cannot always be reproduced, likely due to a large variability with small samples sizes.³⁵⁻⁴⁰ This lack of differentiation in the current dataset and the conflicting results in clinical impact may be explained by a variation on a molecular level between patients with the same genotype (eg, lysosomal pH and the presence of cofactors like saposin C). Due to the high number of different mutations in the GBA1 gene, the number of patients with a specific genotype may remain too low to provide sufficient power to adequately detect the possibly small molecular or clinical differences between genotypes. Similarly, only weak correlations were seen between GBA-PD clinical characteristics (ie, MDS-UPDRS-3, H&Y, MMSE, age at diagnosis, and duration of diagnosis) and GSL levels (Figures 9 and 10 in Data S1).

The current dataset has some additional limitations. Only 8 iPD participants were included, so a larger group is needed to assess whether there might be subgroups that could benefit from targeted treatment irrespective of mutational status. Second, healthy volunteers in sub-studies 1 and 5 were not genetically tested. This may have influenced analysis of GCase activity and Gcase protein, which were only assessed in substudy 1. However, study 1 was primarily used to assess variability, which was similar across groups. Sub-study 5 (HVs only) was used to confirm GSL cell subtype differences because this was already serendipitously suggested by the GBA-PD data, so genotype was less relevant for this question (and chances are negligible that all HVs happened to be *GBA1* mutation carriers).

It should be noted that no formal statistical testing was performed. The current study is primarily descriptive and emphasizes global patterns. This approach was chosen because of the exploratory nature of the assessments and the otherwise enormous number of direct comparisons which would, in our opinion, not contribute to a robust interpretation.

In addition to what was investigated in the current study, there are of course more potential biomarkers for GBA-PD. The GSL network is extensive, and in a previous study, which assessed 520 lipid species in the plasma of iPD and controls, monosialodihexosylganglioside (GM3) gangliosides were most significantly increased in iPD.⁴¹

In summary, the *GBA1* mechanism is one of the most promising targets for a first disease-modifying treatment for PD. Use of biomarkers during early-phase trials is crucial for more efficient drug development. To make the best use of biomarkers, any noise in the data should be minimized, which requires an in-depth understanding of the physiology and methodological challenges. Our findings may contribute to this understanding, with key findings summarized in Box 1. Application of biomarkers in clinical trials may provide novel insights and should be published whenever possible.

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Ethics Approval and Consent to Participate

The first four studies were approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek), Assen, The Netherlands. These are registered in the Dutch Trial Registry (Nederlands Trial Register, NTR) under study numbers (1) NTR6256, (2) NTR6598, (3) NTR6705, and (4) NTR6960. For all the studies, all subjects signed an informed consent form in accordance with the Declaration of Helsinki prior to any study-related activity. Experiment 5 was covered by an institutional procedure; blood donors provided written informed consent.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.