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## Genetic and clinical pharmacology studies in GBA1-associated Parkinson's disease

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# Preparing for *GBA1*-targeting Parkinson's disease trials: a biomarker study in patients with *GBA1*-Parkinson's disease and healthy controls

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## Abstract

**Background** A mutation in the *GBA1* gene is the most common genetic risk factor of Parkinson's disease (GBA-PD). *GBA1* encodes the lysosomal enzyme glucocerebrosidase (GCase), which hydrolyzes glucosylceramide (GluCer). GluCer is a basic form of a glycosphingolipid (GSL), and member of a vast network of different complex GSLs. Substrates and products of GCase are potential biomarkers for development of compounds targeting GBA-PD. Here, we compared the variability of various GSLs in plasma, peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) across GBA-PD, idiopathic nonGBA PD (iPD), and healthy volunteers (HVs).

**Methods** Data of five studies were combined. Within-day and between-day variability was assessed, and means were compared of GluCer (various isoforms), LacCer (various isoforms), GluSph, GalSph, GCase activity (using fluorescent 4-methylumbelliferyl- $\beta$ -glucoside) and GCase protein (using enzyme-linked immunosorbent assay) in plasma, PBMCs and CSF if available, in GBA-PD, iPD and HVs. GSLs were measured using validated LC-MS/MS methods. Leukocyte subtypes were isolated separately to assess cell type differences for GluCer, LacCer and GluSph in HVs. Principal component analysis was used to explore global patterns in GSLs, clinical characteristics (Movement Disorders Society-unified Parkinson's disease rating scale part 3, mini mental state exam, *GBA1* mutation type), and participant status (GBA-PD, iPD, HVs).

**Results** Within-subject between-day variability for various molecules ranged from 5.8% to 44.5%, generally lower in plasma than in PBMCs. GluCer levels in plasma were higher in GBA-PD compared to both iPD and HVs. GSLs in the different matrices (plasma, PBMCs, CSF) did not correlate. Both LacCer (various isoforms) and GluSph were at least 20 times as abundant in granulocytes compared to both monocytes and lymphocytes. Absolute levels of various GSL isoforms differ greatly, up to a factor 140, also within cell type. *GBA1* mutation types could not be differentiated based on GSL data.

**Conclusion** GluCer can stably be measured over days both in plasma and PBMCs, and may be used as biomarkers in future clinical trials targeting GBA-PD. GluSph and LacCer 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 GluCer levels in plasma.

## 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.<sup>1</sup> A disease-modifying treatment is still lacking. Through ongoing elucidation of the underlying pathophysiological process, new potential drug targets are being explored.<sup>2,3</sup> 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 Parkinson's disease.<sup>4,5</sup> Approximately 4-15% of PD patients carry a heterozygous *GBA1* variant and in Ashkenazi Jewish PD patients the frequency is approximately 20%.<sup>6-8</sup>

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.<sup>9</sup> GluCer is the most basic form of a glycosphingolipid (GSL), which constitutes a vast network of different complex GSLs, reviewed in great detail elsewhere.<sup>10</sup> In short, GSLs are a class of lipids, essential for e.g. 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 GCase deficiency, the enzyme acid ceramidase can convert GluCer into glucosylsphingosine (GluSph), by removal of the fatty acid chain.<sup>12</sup>

It is not fully understood how the intra- or extracellular levels of these molecules and their ratio are affected in people with Parkinson's disease with (GBA-PD) or without (idiopathic nonGBA PD; for convenience now referred to as iPD) a mutation in the *GBA1* gene, compared to healthy people without PD.

Across different brain regions, studies have found that GCase activity is decreased in GBA-PD and to a lesser extent in iPD.<sup>13-17</sup> Further, in GBA-PD and iPD GCase protein levels were found to be decreased to normal.<sup>13</sup> There is limited evidence for a decrease in mRNA in iPD.<sup>14</sup> There is conflicting evidence regarding accumulation of GluCer, LacCer and GluSph in various brain regions.<sup>15,16,18,19</sup>

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 is limited. GCase activity is decreased in dried blood spots, in monocytes (but not lymphocytes) and in CSF of both GBA-PD and iPD compared to controls.<sup>20-22</sup> GCase protein was unchanged in monocytes and lymphocytes of GBA-PD and iPD compared to controls.<sup>22</sup> GluCer and LacCer were increased in plasma of iPD compared to controls,<sup>23</sup> but this finding was not replicated in serum.<sup>16</sup> In cerebrospinal fluid (CSF), LacCer (total) was increased in iPD compared to controls, but GluCer could not be measured.<sup>16</sup>

As drugs are currently being developed that aim to restore GCase activity as a disease progression modifying 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 report describes the combined data of five studies, evaluating GCase activity, 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).

## Materials and methods

Data of 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 of a phase I single dose clinical trial in HVs (n=40)
- 3 Baseline biomarker data of a phase I multiple dose clinical trial in HVs (n=39)
- 4 Baseline biomarker data of a phase 2a multiple dose clinical trial in GBA-PD (n=40)
- 5 Single blood draw to compare biomarkers between immune cell subsets in HVs (n=6)

Study 1 was used to assess the variability (within- and 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 Supplementary table 1.

## Subjects

For study 1, three groups were enrolled. First, healthy volunteers, 18 to 70 years of age (of which 50% female and of which 50%  $\geq 55$  years of age). Second and third, people with iPD and GBA-PD, respectively. For both PD groups, diagnosis was confirmed by a neurologist and Hoehn & Yahr stage was 1 up to 4. All subjects underwent five blood draws on day 1 (at approximately 11:00h, 13:00h, 15:00h, 17:00h and 19:00h) and a single blood draw on day 5 and on day 8 (both at approximately 13:00h), to assess variability. Only HVs remained fasted from the preceding midnight until after the first blood draw on day 1, to assess a potential food effect.

Studies 2, 3 and 4 were randomized placebo-controlled trials.<sup>24,25</sup> Only data of study subjects on placebo were used for the current report (i. e. baseline data of all subjects and data of subjects randomized to placebo).

For study 2, healthy men and women of non-childbearing potential, 18 to 65 years of age, were enrolled for a clinical trial.<sup>24</sup> Subjects underwent four blood draws on day 1 and a single blood draw on day 2.

For study 3, healthy middle-aged or elderly men and women of non-childbearing potential, 50 to 75 years of age, were enrolled for a clinical trial.<sup>24</sup> Subjects underwent a single blood draw on day 1, day 2, day 8 and day 15. Day 1 and 15 contained a single CSF sample.

For study 4, men and women of non-childbearing potential with confirmed GBA-PD, Hoehn & Yahr stage 1 up to 4,  $\geq 18$  years of age, were enrolled for a clinical trial.<sup>25</sup> Subjects underwent a blood draw on day 1, twice on day 2, day 8, day 15 and day 29. Day 1 and 29 contained a single CSF sample.

For study 5, healthy volunteers,  $\geq 18$  years of age, were enrolled for a single blood draw.

## Plasma, PBMCs and CSF collection

Biomarkers were measured in  $K_2EDTA$  plasma, PBMCs and CSF. PBMCs were isolated from venous blood using cell preparation tubes (CPTs) containing sodium heparin (Becton Dickinson, NJ, USA) according to manufacturer's instructions. In short, CPTs were centrifuged at 1800xg for 30 minutes at room temperature. PBMCs were collected and washed twice with phosphate-buffered saline (PBS) containing 10% heat inactivated human serum (both Gibco, Thermo Scientific, Waltham, MJ, USA). Cells were snap-frozen at  $1 \times 10^7$  cells  $mL^{-1}$  in PBS with 0.1% Bovine Serum Albumin (BSA) buffer and stored at  $-80^\circ C$  until analysis. Cells for GluSph analysis were frozen in glass tubes instead of a cryopreservation tube. In studies 3 and 4, for CSF collection, a Pencan® 25G atraumatic needle was used. The first mL CSF was discarded to prevent contamination with blood, after which 4mL was collected in a 15mL Falcon® tube. CSF was transferred to a glass tube and 0.2% BSA with ascorbic acid was added. This was centrifuged at 2000xg for 3 minutes

at room temperature. Supernatant was transferred to glass tubes, snap frozen and stored at  $-80^{\circ}\text{C}$  until shipment for analysis. Time from collection to freezing did not exceed 60 minutes.

## GCcase activity

GCcase activity was measured in study 1 only. Analysis was performed by Lysosomal Therapeutics, Inc. (Boston, USA). Activity was measured in PBMCs using the fluorescent artificial substrate 4-methylumbelliferyl- $\beta$ -glucoside (4-MUG).  $50\ \mu\text{L}$  of  $1 \times 10^7$  PBMCs / mL was used per analysis. Measurements were performed in duplicate and the average was taken. Technical variability was 45%.

## GCcase protein

GCcase protein was measured in study 1 only. Analysis was performed by Ardena Bioanalytical Laboratory (ABL) (Assen, the Netherlands). The commercial ELISA kit Glucosidase Beta Acid from Cloud Clone Corp. was used. Calibration was  $31.3\text{--}2000\ \text{pg/mL}$ .  $350\ \mu\text{L}$  of  $1 \times 10^7$  PBMCs / mL was suspended 50 times prior to analysis. Output was reported in pg GCcase protein /  $1 \times 10^7$  PBMCs, based on a duplicate measurement. Technical assay variability was 15%.

## GluCer, LacCer, GluSph and GalSph

GluCer was measured in all studies, LacCer and GluSph were measured in studies 3, 4 and 5, and GalSph 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 LC-MS/MS methods, by ABL. Technical assay variability was 15% and all data are based on single measurements, which were only repeated if predefined quality criteria were not met. Assay ranges can be found in Supplementary table 2.

## Leukocyte subtypes

PBMCs constitute of monocytes and lymphocytes, but inherent to isolation methods, some degree of granulocyte contamination is always present,<sup>26</sup> which can vary between blood draws. In study 5, monocytes, lymphocytes and granulocytes were isolated separately. Magnetic cell separation was the method used for all leukocyte subtype isolations. Whole blood from HVS was collected in tubes containing specific anticoagulants. Negative selection assay was optimized for high purity of isolated cells. The negative selection assay consisted of two depletion cycles comprising directly labelling of undesired cell subtypes. Immuno-magnetic cell separation assays comprised the magnetic labelling step and the magnetic capture. Lymphocytes isolation was done by Direct Human Total Lymphocyte Isolation Kit, monocytes isolation by Direct Human Monocyte Isolation Kit, and granulocytes isolation by Direct Human Pan-Granulocyte Isolation Kit according to manufacturer's instructions (StemCell technologies). To verify the purity of the isolated cells, leukocyte subtypes were labelled with specific antibodies; namely CD45, CD3 and CD19 for lymphocytes, CD45 and CD14 for monocytes and CD45, CD66b, CD123 and CD16 for granulocytes; and evaluated by flow cytometry. A density of  $8 \times 10^6$  cells mL<sup>-1</sup> in PBS containing 0.1% BSA was collected in 2× plastic tubes and 2× glass tubes for each leukocyte subtype. Samples were snap frozen and stored at -80°C until shipment for analysis. Time from blood collection to freezing did not exceed 180 minutes.

## Genotyping

The *GBA1* gene was sequenced in all subjects, except for the HVS in study 1 and 5, using saliva derived DNA and methods described previously.<sup>8</sup> In short, next generation sequencing was performed, using long-range polymerase chain reaction and a primer set unique to the functional *GBA1* gene, thereby preventing amplification of the nearby pseudogene. For study 4, all *GBA1* genotypes were confirmed in whole blood.

In study 1 and 4, only *GBA1* variants previously reported in PD or Gaucher's disease (GD) were included. In homozygous state, over 400 variants have

been reported to cause the lysosomal storage disorder GD.<sup>27,28</sup> Some variants, however, do not cause GD in homozygous state, but do increase the risk to develop PD in heterozygous state, e.g. the relatively common variants E326K and T369M. Generally, these latter variants are considered milder and have a higher residual GCase activity. Within GD, some genotypes are considered worse than others, but the genotype-phenotype correlation is variable.

In this report, we applied nomenclature historically used in GD literature, excluding the 39-amino acid signalling peptide.

### **MMSE, H&Y and MDS-UPDRS part III**

In PD subjects, the mini mental state examination (MMSE) (study 1 and 4), Hoehn & Yahr (H&Y) staging (study 1 and 4) and the Movement Disorders Society – unified Parkinson’s disease rating scale (MDS-UPDRS) part 3 (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 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 (measurement 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 within-subject variabilities (standard deviation and coefficient of variation). This was repeated for the variability over days (measurement 2 of day 1 and measurements of 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 study 2, 3 and 4 the

baseline and placebo-treated measurements). Levels are depicted using violin plots. Considering the exploratory nature and extensiveness of measurements, no formal statistical testing was performed to compare means. Means and 95% confidence intervals 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 BMI. For GBA-PD patients only, we also included H&Y, MDS-UPDRS part III 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 available data. Data were normalized prior to performing each PCA, because variables had different units or scales.

- 1 PCA 1 (maximized for GCase activity and protein): Explore patterns in GBA-PD vs iPD vs HVs, using GCase activity, GCase protein, GluCer (multiple isoforms) and GluSph in PBMCs and GluCer (multiple isoforms), GluSph and GalSph in plasma, age and BMI, based on data of study 1.
- 2 PCA 2 (maximized for GluCer, LacCer and GluSph): Explore patterns in GBA-PD vs HVs, using GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, GluSph in plasma and PBMCs, age and BMI, based on data of study 3 and 4.
- 3 PCA 3. 1 (maximized for GBA-PD): Explore patterns in covariates in GBA-PD, using *GBA1* mutation type, MDS-UPDRS part3 total score, H&Y, age at diagnosis, duration of diagnosis, age, BMI, with GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, GluSph in plasma and PBMCs, based on data of study 4.
- 4 PCA 3. 2 (maximized for GBA-PD): same as 3. 1, excluding CSF data.
- 5 PCA 4 (maximized for GluCer): Explore patterns in GBA-PD vs iPD vs HVs, using GluCer (multiple isoforms) in plasma and PBMCs, age and BMI, based on data of study 1, 2, 3 and 4.

PCA is an orthogonal (a.k.a. independent) linear transformation that remodels the data to new latent variables, so called principal components. The greatest variance by some scalar projection of the data is presented on the first component, the second greatest variance on the second component, and so on. This represents the optimal subset of highly correlated original variables with fewer independent variables (i.e., the two principal components). Analysis results are presented using biplots, where projection of vectors (a.k.a. loadings) represent their contribution in the given component of the PCA and the angle between a pair of vectors represents their size of correlation (i.e., vectors in the same direction (small angle) have a positive correlation, vectors at a 90-degree angle are not correlated and vectors in opposite direction are inversely correlated). An indirect interpretation is that a short vector, which has a small contribution to the given component, will therefore only represent correlations with a small magnitude with any other vector.

## 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 two subjects carried the heterozygous T369M variant; one subject withdrew prior to any measurements for personal reasons and was not replaced. In study 4, 40 people with GBA-PD were included. Demography data of all subjects are summarized in Table 1. Genotypes of GBA-PD subjects in study 1 and 4 can be found in Table 2.

### Variability

The within- and between-subject, within- and between-day variability of GluCer (multiple isoforms), GluSph, GalCer, GCase activity and GCase protein in plasma and/or PBMCs, split for HVs, iPD and GBA-PD, are given in Table 3. Generally, plasma measurements were more stable than PBMC

measurements. No diurnal rhythm was observed, based on the samplings from 11:00h to 19:00h in all groups. No acute effect of food intake was observed, based on the first fasted sample in HVs only. GluCer C18:0 (as example), GCASE activity and GCASE protein measurements can be seen in Figure 1.

## 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% CI are discussed. GluCer (multiple isoforms), mean (95% CIs) plasma levels were higher in GBA-PD (C16:0 789.5 [727.5, 851.5]; C18:0 85.9 [78.3, 93.4]; C22:0 1724.9 [1518.4, 1931.4]; C24:0 1881.6 [1649.2, 2113.9]; C24:1 1753.3 [1551.1, 1955.5]), compared to both iPD (C16:0 645.1 [517.9, 772.2]; C18:0 59.4 [47.1, 71.6]; C22:0 900.3 [728.2, 1072.4]; C24:0 845 [729.4, 960.6]; C24:1 758.8 [603.1, 914.5]) and HVs (C16:0 521.9 [497.1, 546.6]; C18:0 53.9 [49.8, 58.1]; C22:0 839.5 [798.2, 880.9]; C24:0 798.8 [756.4, 841.3]; C24:1 787.6 [745.5, 829.7]). Levels in PBMCs and CSF were similar over all groups (no CSF available from iPD), except GluCer C20:0 in CSF, which was higher in GBA-PD (0.17 [0.15, 0.19]) compared to HVs (0.12 [0.10, 0.14]). See Figure 2 (biomarker: GluCer) and Supplementary table 3.

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

GluSph in plasma was elevated in GBA-PD (1.36 [1.21, 1.52]) compared to HVs (0.99 [0.93, 1.06]) and marginally overlapped with iPD (0.93 [0.64, 1.23]), but was similar over all groups in PBMCs. GalSph (plasma only) was

similar in GBA-PD, iPD and HVs. See Figure 2 (biomarker: GluSph and GalSph) and Supplementary table 3.

GCcase activity in PBMCs (using 4-MUG) was generally decreased in GBA-PD (2.29 [1.21, 3.37]) compared to HVs (3.67 [3.32, 4.03]), but still slightly overlapped. Activity in iPD (3.98 [2.22, 5.74]) seemed similar to HVs on average, but varied greatly, with both higher and lower levels compared to HVs. GCcase protein levels (PBMCs only) were increased in iPD (44180 [31613, 56747]) compared to HVs (25871 [20336, 31407]) and to a lesser extent in GBA-PD (35455 [27510, 43400]) compared to HVs. See Figure 2 (biomarker: GCcase activity and GCcase protein) and Supplementary table 3.

## Principal Component Analysis

Different combinations of data of study 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 the supplementary material.

PCA1 (Supplementary figure 1) was maximized for GCcase 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 of study 1 were used, consisting of GBA-PD (n=8), iPD (n=8) and HV (n=7, 1 listwise procedural exclusion of a participant due to missingness of GluCerC22:0 and C24:0 in plasma).

GCcase activity and GCcase protein level in PBMCs are uncorrelated. GCcase activity shows a moderate inverse correlation with GluCer (multiple isoforms), GluSph and GalSph in plasma, but not in PBMCs. GCcase protein is positively correlated to GluCer (multiple isoforms) in PBMCs, but not in plasma.

PCA2 (Supplementary figure 2) was maximized for GluCer and LacCer in plasma, PBMCs and CSF and GluSph in plasma and PBMCs, but also includes age and BMI. Data of 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 (Supplementary figure 3) was maximized for GBA-PD, including *GBA1* mutation type, MDS-UPDRS part III 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 of 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-UPDRS3, duration of diagnosis and age at diagnosis only show weak correlations at best with GSLs.

PCA3. 2 (Supplementary figure 4) 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 of 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 part III and duration of diagnosis seem weakly inversely correlated to LacCer (multiple isoforms) levels in plasma.

PCA4 (Supplementary figure 5) was maximized for GluCer in plasma and PBMCs, but also includes age and BMI. Data of 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 of study 4, effect of granulocyte contamination of isolated PBMCs on LacCer and GluSph levels is depicted in Figure 3. An r-squared of up to 0.67 was seen, suggesting 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 4. Distribution of GluCer over cell types varied per isoform; GluCer C20:0 was twice as abundant in lymphocytes compared to both monocytes and granulocytes, and GluCer C24:0 was more than five times as abundant in monocytes compared to lymphocytes and twice as abundant in monocytes compared to granulocytes. Both GluSph and LacCer (all isoforms) were at least 20 times as abundant in granulocytes compared to 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 Parkinson's disease.<sup>3</sup> Use of biomarkers in early phase clinical trials is crucial for innovative drug development.<sup>29</sup> This paper combined data of five studies to further assess multiple potential biomarkers for GBA-PD. Variability was determined of GluCer (multiple isoforms), GluSph, GalSph, GCase activity (using 4-MUG) 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 heavily between leukocyte subtypes. This shows that it is of 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 likely partly caused by a variable cell type constitution of the PBMC isolation, and/or by contamination of the isolated PBMCs with granulocytes. This variability may be reduced when isolating specific cell types and requires further studies.

A theoretical advantage of using PBMCs as opposed to plasma when measuring GSLs, is that this reflects intracellular levels, which intuitively 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<sup>30</sup> and this intracellular distribution is hard to distinguish. Conversely, measurements in plasma and CSF reflect extracellular GSL, of which the origin is unclear.

To our knowledge, this was 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 to 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. Similarly, for GCase activity, others have shown this is decreased in monocytes, but not in lymphocytes, in patients with PD.<sup>22</sup> In neuronal and astrocytic cell cultures, GCase activity was higher in neuronal cells,<sup>31</sup> but this may also be influenced by the culture medium, with higher glucose content, in which cells are dividing.

GCase activity was only measured in PBMCs, since the enzyme is active within the lysosome. Activity was lower in GBA-PD compared to iPD and HV, but this was not observed in all GBA-PD patients. 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-to-lowest 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 may be caused by the inconsistency in genotype-phenotype correlation known in Gaucher disease.<sup>32</sup> Similarly, in a patient where no *GBA1* variant could be identified (and was therefore classified as *GBA1* wildtype, i. e. iPD in this paper), 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, however, it can be hypothesized that measuring actual GCase activity may be a better predictor. This could also identify patients without a *GBA1* mutation, who could potentially benefit from treatment targeting GCase activity.

Measuring GCase activity remains challenging because current techniques use artificial substrates, of which it is unclear how these interact with the >400 described pathological mutations compared to the endogenous substrate. Additionally, and similar to the fact that LacCer and GluCer differ heavily between leukocyte subtypes, measurements of GCase activity may be influenced by cell type composition, but this remains to be confirmed. This would also explain a degree of variability in the commonly used dried blood spot assessment of GCase activity, in which the cell subtype constitution of a drop of blood is unknown and may vary.

Various chain lengths of different GSLs may have different functions, but this is not yet fully elucidated. Absolute quantity of the different isoforms of a specific GSL differs strongly as well, within a cell type (Figure 4). In multiple previous studies,<sup>16,19</sup> all GSL chain lengths are totaled, meaning the signal of sparse isoforms will be overshadowed by those 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 to consider these different chain lengths, despite their exact function being unknown.

The methodology used for granulocyte isolation can have 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.<sup>33</sup> All activities, from blood collection to the resuspension of isolated granulocytes in buffer, was done at room temperature. It is favorable to not alter the temperature to preserve quantity and function of granulocytes. Granulocytes were isolated within the first hour after blood draw, processed in 3 hours maximum, and immediately snap frozen at -80°C after resuspension in proper buffer.

When comparing GBA-PD to iPD and HV (Figure 2), 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 in GBA-PD in a cross-sectional study. This was not seen in PBMCs or CSF. To a lesser

extent, GluSph was also increased in plasma of GBA-PD compared to iPD and HVs, but not in PBMCs. Mean differences in LacCer (multiple isoforms) were less pronounced, showing mostly similar levels over groups, with some increased and some decreased isoform levels in plasma in GBA-PD compared to HVs. This may reflect distinct metabolisms between LacCer isoforms or could be a chance finding. No clear difference was seen in GalSph in plasma between GBA-PD, iPD and HVs, although this may also be explained by a relatively small sample size. Measurements in PBMCs may have been affected by differences in the composition of leukocyte subtypes in the PBMC isolations.

The GluCer C24:1 isoform in plasma best differentiated GBA-PD from iPD and HVs. Out 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 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, e.g. based on MDS-UPDRS part III, MMSE, duration of diagnosis, age at diagnosis or genotype (e.g. both p. E326K and p. L444P carriers were present in the high and in the 'normal' group).

Using Principal Component Analysis, it was attempted 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. Several patterns based on these biplots are discussed.

Within a matrix (i. e., plasma, PBMCs and CSF), the different isoforms of a GSL (GluCer or LacCer) are interrelated (seen in all PCA biplots). It remains unclear whether these isoforms are affected differently or not when the GSL metabolism is influenced by a pharmacotherapeutic intervention. There is no clear-cut best choice in 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).

gCase activity in PBMCs showed an inverse correlation with plasma GalSph, and to a lesser extent with plasma GluSph and GluCer. gCase activity was weakly related or unrelated to GluCer in PBMCs (Supplementary figure 1). Plasma GluCer was the only gSL measurement that clearly differed between GBA-PD and HVS (although with overlap) (Figure 2, Supplementary figure 5), where the increased plasma GluCer level in GBA-PD could possibly be explained by a chronically lowered intracellular gCase activity level. It may be hypothesized that GluCer in PBMCs (intracellular) better reflects short-term changes.<sup>25</sup>

It was hypothesized that the gCase protein would be upregulated in patients with a low gCase activity, but these were unrelated (Supplementary figure 1). A p. L444P carrier, with the lowest measured gCase activity, did have the highest gCase protein level, possibly due to compensatory upregulation, but two p. E326K carriers with comparable gCase activity, had a two-fold difference in gCase protein, exemplary for the inconsistent relationship with either gCase activity or genotype. gCase protein did positively correlate with GluCer in PBMCs, but not GluSph or any plasma measures. An upregulation of gCase protein may be a response to a relatively higher intracellular GluCer level. gCase protein was measured in PBMCs only, using a commercially available ELISA kit, of which affinity for different mutant proteins is unknown. No differentiation was made between gCase protein in active or in inactive state. Effect of leukocyte subtypes remains to be assessed.

No differentiation between mutation type (Gaucher-associated or PD-only-associated) could be made within the GBA-PD group, based on GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, and GluSph in plasma and PBMCs (Supplementary figure 3, Supplementary figure 4). 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.<sup>34-39</sup> 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. Some genotypes may generally be considered more severe than other genotypes based on GD phenotype, but not exclusively so, and vice versa. 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.

Clinical characteristics assessed in the current *GBA*-PD study (study 4), MDS-UPDRS III, H&Y, MMSE, age at diagnosis and duration of diagnosis, only showed weak correlations with GSL levels (Supplementary figure 3, Supplementary figure 4). MMSE showed an inverse correlation to CSF levels of GluCer and LacCer. MDS-UPDRS III, H&Y and duration of diagnosis were all aligned and inversely correlated to plasma LacCer. However, the magnitude of these correlations is likely small (as indirectly interpreted based on the short projection vectors on components). A long-term follow-up cohort is required to better quantify a possible relationship between GSL measurements and clinical characteristics.

In addition to what was investigated for the current report, there are of course more potential biomarkers for *GBA*-PD. The GSL network is extensive and in a previous report, which assessed 520 lipid species in plasma of iPD and controls, monosialodihexosylganglioside (GM3) gangliosides were most significantly increased in iPD<sup>40</sup>. Furthermore, uric acid has been of interest<sup>41</sup> and knowledge about the role of neuroinflammation is quickly expanding<sup>42</sup>.

In summary, the *GBA1* mechanism is one of the most promising targets for a first disease-modifying treatment for Parkinson's disease. Use of biomarkers during early-phase trials is crucial for more efficient drug-development. To make 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 given bullet-wise in Box 1. Application of biomarkers in clinical trials may provide novel insights and should be published whenever possible.

**Box 1** Bullet-wise summary of the key findings.

**KEY FINDINGS**

- GluCer, GluSph, GalSph, GCASE activity and GCASE protein can be stably measured between-days within-subject, in plasma better than PBMCS.
- Plasma (extracellular) levels of various GluCer isoforms (mainly C24:1 and C24:0) are increased in GBA-PD compared to iPD and HVS.
- GSL levels in plasma (extracellular) are unrelated to levels in PBMCS (intracellular) or CSF (extracellular in central nervous system).
- 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 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 totaled if possible.

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**Table 1 Overview of demographic variables.**

Demographics							
	HVs				Patients		
Study	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/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 part III, 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 of 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)

*BMI=body mass index; max=maximum; MDS-UPDRS=Movement Disorder Society-Unified Parkinson's Disease Rating Scale; min=minimum; MMSE=Mini Mental State Examination; N/A=not applicable; -=not performed in this study.*

**Table 2 Overview of *GBA1* variants. HVs from study 1 and 5 were not sequenced and genotype is therefore unknown.** The *GBA1* allelic names are given, excluding the 39-amino acid signaling peptide. In 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 it is uncertain how these mutations are distributed over alleles.

Study	1	2	3	4	5
<i>GBA1</i> Genotype	HV, iPD, <i>GBA</i> -PD	HV	HV	<i>GBA</i> -PD	HV
<b>Wildtype</b>	8 (iPD)	37	37	0	0
<b>Unknown</b>	8 (HVs)	0	0	0	6
<b>Mutated</b>	8 ( <i>GBA</i> -PD)	3	2	40	0
<i>GBA1</i> 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

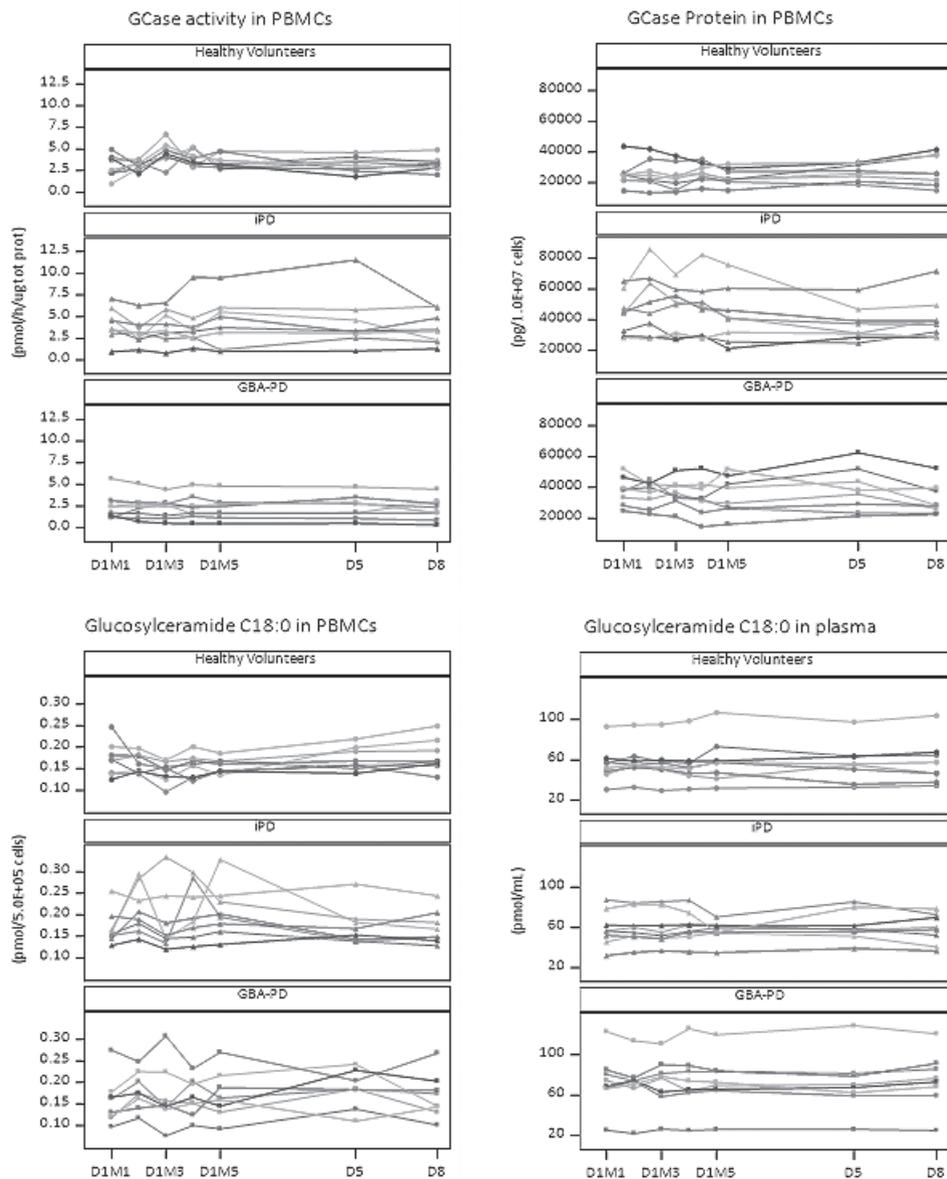
**Table 3 Coefficient of variance (= (SD/mean)\*100) of GluCer (multiple isoforms), GluSph, GalCer, GCase activity and GCase protein in plasma and/or PBMCs, split for HVs, iPD and GBA-PD, for within/day and between/day assessments.** Data is from study 1. Measurements in PBMCs are shaded grey, measurements in plasma are white. PBMCs=peripheral blood mononuclear cells; HV=healthy volunteer; GBA-PD=Parkinson’s disease with a *GBA1* mutation; iPD=idiopathic Parkinson’s disease (i.e. no *GBA1* mutation); GCase=glucocerebrosidase.

Coefficient of Variation (cv) (study 1)					
Variable	Group	Within day		Over days	
		Within subject	Between subject	Within subject	Between subject
<b>PBMCs</b>					
GCase Protein	HV	13.4%	27.4%	17.9%	27.3%
(pg/1.0E+07 cells)	GBA-PD	13.0%	25.9%	19.8%	27.2%
	iPD	11.4%	36.3%	20.0%	32.8%
GCase activity	HV	26.4%	14.0%	14.2%	21.3%
(pmol/h/ug tot prot)	GBA-PD	21.1%	56.2%	17.8%	57.0%
	iPD	21.7%	51.1%	34.2%	53.7%
Glucosylceramide c16:0	HV	20.1%	27.2%	10.8%	15.1%
(pmol/5.0E+05 cells)	GBA-PD	14.9%	15.2%	12.9%	21.1%
	iPD	26.6%	24.3%	18.6%	23.9%
Glucosylceramide c18:0	HV	12.2%	13.3%	10.1%	15.4%
(pmol/5.0E+05 cells)	GBA-PD	12.6%	30.2%	17.2%	21.9%
	iPD	22.4%	22.6%	16.8%	21.1%
Glucosylceramide c22:0	HV	11.1%	7.7%	10.6%	8.1%
(pmol/5.0E+05 cells)	GBA-PD	12.0%	21.0%	13.2%	21.0%
	iPD	21.1%	18.1%	14.3%	13.1%
Glucosylceramide c24:0	HV	13.0%	19.5%	11.9%	19.0%
(pmol/5.0E+05 cells)	GBA-PD	14.4%	23.6%	12.8%	23.8%
	iPD	24.0%	24.1%	15.4%	18.3%
Glucosylceramide c24:1	HV	12.2%	19.8%	12.5%	16.2%
(pmol/5.0E+05 cells)	GBA-PD	12.2%	20.9%	12.9%	25.3%
	iPD	27.1%	27.8%	19.6%	23.8%
Glucosylsphingosine	HV	43.2%	55.3%	35.2%	59.5%
(pmol/5.0E+05 cells)	GBA-PD	32.3%	52.4%	25.9%	51.6%
	iPD	25.9%	38.0%	44.5%	51.7%

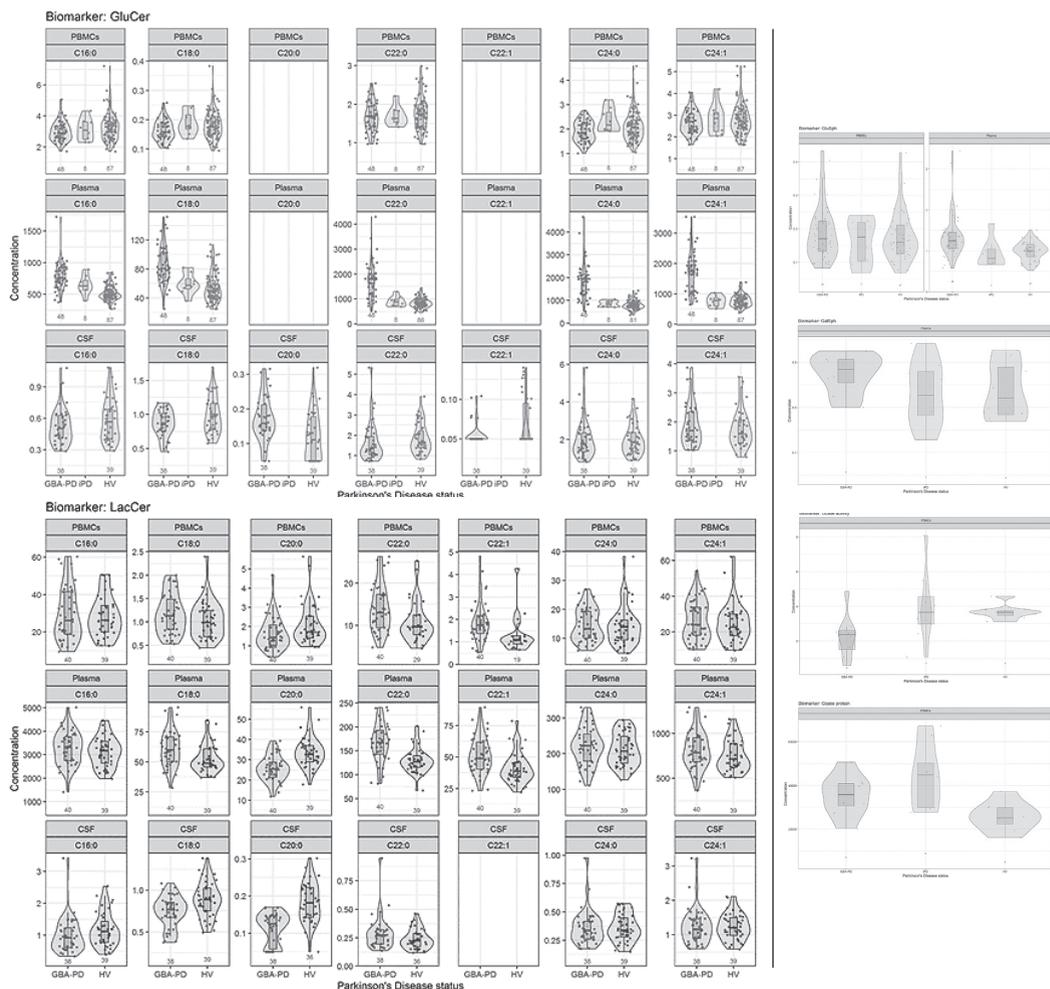
**Table 3** (Continuation of previous page)

Coefficient of Variation (cv) (study 1)					
		Within day		Over days	
Variable	Group	Within subject	Between subject	Within subject	Between subject
<b>PLASMA</b>					
Galactosylsphingosine	HV	5.2%	30.4%	17.5%	27.6%
(pmol/mL)	GBA <sup>-</sup> PD	8.4%	17.1%	10.0%	13.3%
	iPD	7.1%	31.7%	7.7%	32.1%
Glucosylceramide C16:0	HV	4.0%	34.6%	6.8%	36.1%
(pmol/mL)	GBA <sup>-</sup> PD	5.1%	19.7%	6.2%	18.9%
	iPD	5.4%	23.9%	5.8%	23.3%
Glucosylceramide C18:0	HV	6.7%	33.3%	8.5%	34.0%
(pmol/mL)	GBA <sup>-</sup> PD	7.0%	35.5%	8.3%	36.6%
	iPD	9.2%	25.1%	7.3%	25.1%
Glucosylceramide C22:0	HV	8.6%	31.2%	11.4%	30.3%
(pmol/mL)	GBA <sup>-</sup> PD	7.7%	25.7%	9.9%	27.5%
	iPD	10.5%	21.2%	8.7%	25.4%
Glucosylceramide C24:0	HV	8.5%	29.4%	11.8%	29.4%
(pmol/mL)	GBA <sup>-</sup> PD	9.4%	25.0%	12.0%	24.9%
	iPD	10.8%	16.6%	9.1%	17.5%
Glucosylceramide C24:1	HV	6.0%	30.8%	10.1%	31.5%
(pmol/mL)	GBA <sup>-</sup> PD	6.9%	18.0%	7.0%	18.4%
	iPD	6.8%	25.5%	7.4%	24.6%
Glucosylsphingosine	HV	6.3%	29.9%	19.2%	29.4%
(pmol/mL)	GBA <sup>-</sup> PD	7.8%	42.5%	13.9%	37.0%
	iPD	7.8%	39.0%	9.3%	36.0%

**Figure 1** Measurements over time (first five are within day, last two are on separate days) of **gCase activity in PBMCs (top-left)**, **gCase Protein in PBMCs (top-right)**, **GluCer C18:0 in PBMCs (bottom-left)** and **plasma (bottom-right)**, for **HV (top)**, **iPD (middle)** and **GBA-PD (bottom)**. Lines indicate individual participants. PBMCs=peripheral blood mononuclear cells; D=day; M=measurement.



**Figure 2** Violin plots given per biomarker (GluCer, LacCer, GluSph, GalSph, gcase activity, gcase protein) and per matrix (PBMCs, plasma and CSF if available), separate for GBA-PD (left), iPD (middle; not available for LacCer) and HVs (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 \* inter-quartile 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.



**Figure 3** Scatterplots of percentage of granulocytes in a PBMC isolation and the correlation with different LacCer isoforms (pmol per  $0.5 \times 10^6$  cells) and GluSph (pmol per  $1.0 \times 10^6$  cells). R-squared is given per plot.

