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High Performance Liquid Chromatography-Mass Spectrometry (LC-MS) Based Quantitative Lipidomics Study of Ganglioside-NANA-3 Plasma to Establish Its Association with Parkinson's Disease Patients

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: It is well known that, pathologically, Parkinson's disease is a common neurodegenerative disorder. In Parkinson's disease, the protein which is abundant in the human brain, alpha-synuclein, accumulates inside the nerve cells. In this situation, dysregulation of lipid metabolism performs a crucial role; however, its association with Parkinson's disease is has not yet been explored.

Material/Methods: We performed a high-performance liquid chromatography-mass spectrometry-derived quantitative lipidomics study to analyze the profile of lipidomic plasma obtained from 170 PD patients and 120 controls, taken from our hospital. A logistic regression model was used for analysis in each of the lipid species having all major classes of glycerolipids, sterols, sphingolipids, and glycerophospholipids.

Results: We observed that there are differences in the plasma concentrations of 2 lipid subclasses, triacylglycerides and ganglioside-NANA-3, between control and Parkinson's disease participants. The most significant difference between both the participants was observed in the case of ganglioside-NANA-3 plasma concentration (1.293±0.029 pmol/µl versus 1.488±0.041 pmol/µl, respectively) after normalizing it with respect to total lipid. Further, a group of 22 glucosylceramide and ganglioside-NANA-3 species concentration was used for receiver operating characteristic curve analysis after normalizing it with respect to total lipid. The results were quite consistent with previously reported biomarker results.

Conclusions: Our results show that there is quite good association between high concentration of ganglioside-NANA-3 species and Parkinson's disease. Interestingly, the same metabolic pathway of glucosylceramide, which is a substrate of the enzyme glucocerebrosidase, has been linked with Parkinson's disease, which is at last followed by ganglioside-NANA-3. These results are supported by earlier works in which lower glucocerebrosidase activity has led to risk of the disease.

MeSH Keywords: Chromatography, Liquid • Mass Spectrometry • Parkinsonian Disorders • Phospholipid Transfer Proteins

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Background

The symptoms of rigidity, postural instability, bradykinesia, and resting tremor characterize Parkinson's disease (PD), which is a common, severe, and advanced neurodegenerative disorder. These symptoms result from incremental reduction in dopaminergic neuron input into the striatum [1]. PD is the second most prevalent neurodegenerative disease in China and its prevalence is predicted to continue increasing [2].

Lipid metabolism exhibits a significant impact in various neurodegenerative disorders [3,4]. Further, the contribution of lipid metabolism dysregulation in pathogenesis of PD has also been established in numerous studies [5,6]. The pathogenesis of PD is reflected by the accumulation of alpha-synuclein in Lewy bodies. The interaction of membrane phospholipids with alpha-synuclein is mediated by the amphipathic alpha-helical domain present in its N-terminal [5,7,8]. However, available biophysical evidence shows that alpha-synuclein predominantly binds to the acidic phospholipids rather than to the neutrally-charged lipids; therefore, these bindings potentially modulate the catalytic activity of various cytoplasmic lipid enzymes along with negatively charged lipid-based lysosomal lipases [9,10]. An earlier report about the strong association of mutations in glucocerebrosidase with PD supports the key role of lipid dysregulation in PD and is supported by genetic studies [11]. Other lysosomal proteins such as acid sphingomyelinase [11] and lysosome membrane protein-2 [12] have also been found to be associated with PD.

Additionally, normal lipid function and metabolism including membrane trafficking, particularly with the endolysosomal pathway, has also been shown to be associated with several classical genetic risk factors of PD [13,14]. Furthermore, PTEN-induced putative kinase-1 and parkin protein have shown a link with mitochondria, which is a major cellular station to control the lipid synthesis and metabolism [15], while leucine-rich repeat kinase-2 has been linked with autophagic and endolysosomal function [16]. However, vacuolar protein sorting-associated protein-35, a retromer complex component which is involved in the delivery of lysosomal enzymes to lysosomes, has also been linked to neurodegeneration in PD [17]. Another study has supported an association between several clinical variables in PD and apolipo-protein A1 [18].

Thus, in the background of the aforementioned involvement of lipids PD pathogenesis in PD, the present study was designed to investigate the probable association of plasma lipids with PD. To the best of our knowledge, this is first report in which high-performance liquid chromatography-mass spectrometry (HPLC-MS) lipidomics has been performed on the specific ganglioside-NANA-3 plasma collected from PD patients and controls to compare and establish their relationship.

Material and Methods

Patients

The Institutional Review Board of the East Ward of Sichuan Provincial People's Hospital approved our study. We obtained written informed consent from the enrolled subjects (PD patients) before their inclusion in our study. We recruited 170 PD patients and 120 controls between 2011 and 2016. Controls were caretakers having no blood relation with the patients, which is why mostly spouses were enrolled. PD patients (85 men and 85 women) were recruited personally via regular clinic appointments, and controls (60 women and 60 men) were recruited at the same time. The principal investigator (JZ) or study coordinator (XZ), explained the purpose of the study and administered a structured questionnaire to all participants. All participants were fully sequenced for GBA mutations and screened for the LRRK2 G2019S mutation after telling them that GBA mutations might be risky for PD patients. As per a previously reported questionnaire [19], we designed sample questions on the basis of the genetic information and then interviewed all participants accordingly. Questions involved medical history of the family, demographics, genetic information, and history of any other neurological disorders. All participants were also assessed for cognitive functioning on the basis of the well-known Montreal Cognitive Assessment (MoCA) protocol [20] and for PD via the Unified Parkinson's Disease Rating-based Scale. We interviewed the PD patients and controls simultaneously except during the MoCA-based examination.

HPLC-MS lipidomics study

A modified Bligh/Dyer extraction procedure was used for preparation of lipid extracts. We used 10-mL ethylenediamine tetraacetic acid-based Vacutainer tubes used to collect blood, which was centrifuged and aliquoted to 1-mL plasma aliquots within 60 min of collection. Samples were stored in a –80°C freezer until processing. To each 200 µL plasma sample, we added 1600 µL organic solvents ($CHCl_3$-CH_3OH; 5: 3 v/v) and 200 µL KCl (1 M), and then the samples were centrifuged at 4500 RPM for 5 min. The organic phase (lower section) was collected and then dried using a speed vacuum.

Lipid extracts were then spiked with appropriate respective internal standards, and then the samples were analyzed using the 6490 Triple Quadrupole LC-MS system (Agilent Technologies, Santa Clara, CA). The LC separation of lipid extracts prior to MS analysis is an important component of the analysis, as it prevents signal suppression of low-abundance lipid species such as phosphatidic acid (PA), bis(monoacylglycero)phosphate (BMP), and GM3 by lipids that are typically found in higher abundance, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). An Agilent Zorbax Rx-Sil column

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(2.1×100 mm) was used under normal-phase conditions for separation of glycerophospholipids and sphingolipids keeping the following conditions as: mobile phase A ($CHCl_3$-CH_3OH-H_2O-NH_4OH (1 M), 60: 34.9: 5: 0.1, v/v) and mobile phase B ($CHCl_3$-CH_3OH-NH_4OH (1 M), 84.5: 15: 0.1, v/v); flow rate 300 µL/min; 5% A for 2 min, then shifted to 70% A over 20 min and then kept unaffected for 4 min and then again shifted to 10% A over 2 min and held for 7 min. Eventually, the gradient elution condition was shifted to the native ratio for 6 min and kept unaffected for 7 min for column re-equilibration. DAG, TAG, and CE were separated with an isocratic mobile phase ($CHCl_3$-CH_3OH-CH_3COONH_4 (0.1 M), 24: 24: 2, v/v) in reverse-phase HPLC using an Agilent Zorbax Eclipse XDB- C18 column (4.6×100 mm) at a flow rate of 250 µL/min. For the quantification of DAG species, 4-methyl 16: 0 diether DAG (Avanti Polar Lipids, Alabaster, AL) was taken as an internal standard. Multiple reaction monitoring transition methods were used for the quantification of lipid species [21]. Dioctanoyl phosphatidylinositol (PI) (Echelon Biosciences, Inc., Salt Lake City, UT) was used for quantitation of lysophosphatidylinositol. Except for PI, all other internal standards were purchased from Avanti Polar Lipids (Alabaster, AL). The list of internal standards used for quantification of lipid species is shown in Table 1. In total, more than 500 unique lipid species belonging to around 40 lipid subclasses were measured in this study. In a few cases (e.g., GB-3 and ganglioside-NANA-3) we were unable to procure lipid standards; therefore, we chose the closest lipid internal standards in elution gradient. To present the concentrations of individual lipid species, we chose pmol/µL of plasma sample as a unit. The differences in total lipid amount between samples were corrected by calculating the lipid concentrations as mol% of the total lipid concentration for each sample. By averaging across all samples with the same PD status and/or gender and calculating the standard error of mean, we averaged the concentration of individual lipid species.

Statistical analysis

We used the Fisher exact test, chi-square test, and *t* test to compare clinical characteristics and demographics between PD patients and the control group. Both the units (pmol/µL and mol%) of measuring lipid concentrations were quantile-normalized followed by fitting with a logistic regression model (R version 3.2.2, package 'preprocessCore') by keeping lipid concentration as independent variables and PD patient status as the dependent variable while controlling for body mass index (BMI), age, statin usage, and sex. On the basis of Wald's χ^2-test, the statistical significance of residuals was evaluated for each lipid to predict PD patient status. As per a previous report [22], q-values were evaluated by controlling the false discovery rate at a level of 0.05 (R package 'q-value'). It is important to mention here that in our study the sample size was small and we included samples from different Spot participants and identified significant differences (data not shown) in ganglioside-NANA-3 concentration between both types of participants. We presented here a conservative statistical analysis, without considering our initial hypothesis of differential ganglioside-NANA-3 concentrations in PD patients and control participants. We randomly separated the samples in a 90/10 split into validation and development cohorts in the classification analysis. Along with the development cohort, a support vector machine having a radial basis kernel function was trained and then evaluated with the validation cohort. Additionally, for both the cohorts, quantile normalization was applied with respect to separate lipid measures. To estimate the expected area under the curve w.r.t. receiver operating characteristic, a random resampling procedure was used.

Results

Features of PD patients and control participants

Table 2 shows the comparison of clinical features and demographics of between both types of participants. We designed the study to avoid significant differences in sex ratio, age, or statin use between groups. Both groups had similar median MoCA scores and similar median BMI. The median levodopa equivalent dose was 450 mg (range 100–1500 mg), median UPDR Scale-I+II score was 10 (range 0–31), median UPDR Scale-III score was 15 (range 0–48), and median disease duration of PD patients was 6 years (range 0–27 years).

Lipid biomarker identification

Using the LC-MS procedure (as described in "Materials and Methods") we analyzed the extracted plasma lipid samples with respect to clinical features. We identified 2 lipid subclasses – ganglioside-NANA-3 and triglycerides – and found significant differences for 28 lipid species between groups (Table 3). For this purpose, during this study we tested 34 lipid subclasses having 503 lipid species. We found that the ganglioside-NANA-3 levels were about 15% higher in PD patients in comparison to control participants, and this was the most striking difference between the 2 groups (Figure 1). In this case, concentrations were 1.615±0.039 pmol/µL (PD patients) and 1.401±0.038 pmol/µL (control participants) (p-value=5.820E-04; q-value=4.795E-02). This happened because ganglioside-NANA-3 levels were affected by several ganglioside-NANA-3 species, including the most prominent ones: d18: 1/20: 0 and d18: 1/22: 1 species. As shown in Table 3, the concentrations of ganglioside-NANA-3 d18: 1/20: 0 were 0.019±0.002 pmol/µL (PD patients) and 0.015±0.002 pmol/µL (control participants) (p-value=2.295E-03; q-value=4.795E-02) and ganglioside-NANA-3 d18: 1/22: 1 were 0.134±0.004 pmol/µL (PD patients) and 0.111±0.003 pmol/µL (control participants) (p-value=1.304E-03;

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Table 1. The lipid internal standards (IS) spiked to quantify and match the specific lipid species.

Spiked IS	Lipid species quantified
PA 14:0/14:0	**PA** 30:0; 32:0; 32:1; 34:0; 34:1; 34:2; 36:1; 36:2; 36:3; 36:4; 38:0; 38:1; 38:3; 38:4; 38:5; 38:6; 40:6; 40:7
PC 14:0/14:0	**PC** 30:0; 32:0; 32:1; 34:0; 34:1; 34:2; 36:1; 36:2; 36:3; 36:4; 38:2; 38:3; 38:4; 38:5; 38:6; 40:3; 40:4; 40:5; 40:6; 40:7; 42:1; 42:2; 42:3; 42:4; 42:5; 42:6; 42:7; 44:2; 44:3; 44:4; 44:5; 44:6; 44:7; 46:1; 46:2; 46:3; 46:4; 46:5; 46:6; 46:7; 48:5; 48:6; 48:7 **PCe** 30:0; 32:0; 32:1; 34:0; 34:1; 34:2; 36:2; 36:3; 36:4; 38:0; 38:1; 38:3; 38:4; 38:5; 38:6; 40:0; 40:1; 40:2; 40:3; 40:4; 40:5; 40:6; 40:7; 42:0; 42:1; 42:2; 42:3; 42:4; 42:5; 42:6; 42:7; 44:0; 44:1; 44:2; 44:3; 44:4; 44:5; 44:6; 44:7; 46:0; 46:1; 46:2; 46:3; 46:4; 46:5; 46:6; 46:7; 48:0; 48:1; 48:2; 48:3; 48:4; 48:5; 48:6; 48:7; 50:5; 50:6; 50:7; 52:6; 52:7
PE 14:0/14:0	**PE** 32:1; 34:1; 34:2; 36:1; 36:2; 36:3; 36:4; 38:3; 38:4; 38:5; 38:6; 40:4; 40:5; 40:6; 40:7 **PEp** 32:0; 32:1; 34:0; 34:1; 34:2; 36:1; 36:2; 36:3; 36:4; 38:1; 38:2; 38:3; 38:4; 38:5; 38:6; 40:4; 40:5; 42:5; 42:6; 42:7
PG 15:0/15:0	**PG** 30:0; 32:0; 32:1; 34:0; 34:1; 34:2; 36:0; 36:1; 36:2; 36:3; 36:4; 38:0; 38:1; 38:2; 38:3; 38:4; 38:5; 38:6; 40:6; 40:7; 42:7
PI 12:0/13:0	**PI** 30:0; 32:1; 34:0; 34:1; 34:2; 36:1; 36:2; 36:3; 36:4; 38:2; 38:3; 38:4; 38:5; 38:6; 40:4; 40:5; 40:6; 40:7; 42:5; 42:6 **Ganglioside-NANA-3** d18:0/16:0; d18:0/18:0; d18:0/20:0; d18:0/20:1; d18:0/22:0; d18:0/22:1; d18:0/24:0; d18:0/24:1; d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/20:1; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1; d18:1/26:0; d18:1/26:1
PS 14:0/14:0	**PS** 36:0; 36:1; 36:2; 36:3; 38:1; 38:2; 38:3; 38:4; 38:5; 40:4; 40:5; 40:6; 40:7
BMP 14:0/14:0	**BMP** 32:1; 34:0; 34:1; 34:2; 36:0; 36:1; 36:2; 36:3; 36:4; 38:4; 40:7
APG 14:0/14:0/14:0	**APG** 16:0–34:0; 16:0–34:1; 16:0–34:2; 16:0–36:0; 16:0–36:1; 16:0–36:2; 16:0–36:3; 16:0–36:4; 16:0–38:1; 16:0–38:2; 16:0–40:5 **NAPE** 20:4–18:1/18:1 **NAPEp** 20:4–16:0/22:4; 20:4–18:0/18:1; 20:4–18:0/20:4; 20:4–18:0/22:4; 20:4–18:0/22:6; 20:4–18:1/20:4 **NAPS** 16:0–36:1; 16:0–36:2; 16:0–36:4; 16:0–38:0; 16:0–38:4; 16:0–38:5; 16:0–40:4; 16:0–40:5; 16:0–40:6
LPC 13:0	**LPC** 16:0; 16:1; 18:0; 18:1; 20:0; 20:1; 20:2; 20:3; 20:4 **LPCe** 16:0; 16:1; 18:0; 18:1; 20:0; 20:1; 20:2; 20:3; 20:4
LPE 14:0	**LPE** 16:0; 18:0; 18:1; 20:4 **LPEp** 16:0; 18:0; 18:1
LPI 13:0	**LPI** 16:1; 18:0; 18:1; 20:2; 20:3; 20:4
Cer d18:0/17:0	**Cer** d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/20:1; d18:1/22:0; d18:1/22:1; d18:1/26:0; d18:1/26:1 **dhCer** d18:0/16:0; d18:0/16:1; d18:0/18:0; d18:0/18:1; d18:0/20:0; d18:0/20:1; d18:0/22:0; d18:0/22:1; d18:0/26:0
SM d18:1/12:0	**SM** d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/20:1; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1
dhSM d18:0/12:0	**dhSM** d18:0/16:0; d18:0/16:1; d18:0/18:0; d18:0/18:1; d18:0/20:0; d18:0/20:1; d18:0/22:0; d18:0/22:1; d18:0/24:0; d18:0/24:1; d18:0/26:0; d18:0/26:1
GalCer d18:1/12:0	**GalCer** d18:1/16:0; d18:1/18:0; d18:1/20:0; d18:1/22:0; d18:1/24:0; d18:1/24:1; d18:1/26:0; d18:1/26:1
Sulf d18:1/12:0	**dhSulf** d18:0/16:0; d18:0/22:0; d18:0/24:0; d18:0/24:1 **Sulf** d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/20:1; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1
GlcCer d18:1/12:0	**GlcCer** d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/20:1; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1; d18:1/26:0; d18:1/26:1
LacCer d18:1/12:0	**LacCer** d18:0/16:0; d18:0/22:0; d18:0/24:0; d18:0/24:1; d18:1/16:0; d18:1/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1; d18:1/26:0; d18:1/26:1 **GB-3** d18:0/16:0; d18:0/16:1; d18:1/18:0; d18:1/18:1; d18:1/20:0; d18:1/22:0; d18:1/22:1; d18:1/24:0; d18:1/24:1

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Table 1. The lipid internal standards (IS) spiked to quantify and match the specific lipid species.

Spiked IS	Lipid species quantified
D7-Cholestrol	FC
CE 17:0	**CE** 16:0; 16:1; 18:0; 18:1; 18:2; 20:0; 20:1; 20:2; 20:3; 20:4; 22:2; 22:3; 22:4; 22:5; 22:6; 24:2; 24:4; 24:5; 24:6
MG 17:0	**MG** 16:0; 18:0
4ME 16:0 diether DG	**DG** 28:0/14:0; 30:0/14:0; 30:1/14:0; 32:0/16:0; 32:1/16:0; 32:2/16:1; 34:0/16:0; 34:1/16:0; 34:2/16:0; 34:2/16:1; 36:0/18:0; 36:1/18:0; 36:2/18:0; 36:2/18:1; 36:3/18:1; 36:4/18:0; 38:1/18:0; 38:2/18:0; 38:2/18:1; 38:3/18:0; 38:3/18:1; 38:4/18:0; 38:4/18:1; 40:4/18:0; 40:4/18:1; 40:5/18:0; 40:5/18:1; 40:6/18:0; 40:6/18:1
D5-TG 16:0/18:0/18:0	**TG** 48:0/16:0; 48:1/16:0; 50:0/16:0; 50:1/16:1; 50:2/16:1; 50:3/16:1; 52:0/18:0; 52:1/18:0; 52:2/18:0; 52:3/18:1; 52:4/18:1; 52:5/18:1; 52:5/20:4; 54:0/18:0; 54:1/18:0; 54:2/18:0; 54:3/18:0; 54:4/18:1; 54:4/20:4; 54:5/18:1; 54:5/20:4; 54:6/18:1; 54:6/20:4; 54:7/18:1; 54:7/20:4; 56:3/18:1; 56:4/18:1; 56:4/20:4; 56:5/18:1; 56:5/20:4; 56:6/20:4; 58:5/20:4; 58:6/20:4; 58:8/22:6; 58:9/22:6; 60:7/22:6; 60:8/22:6; 60:9/22:6

Phosphlipids: PA – phosphatidic acid; PC – phosphatylcholine; PCe – ether phosphatidylcholine; PE – phosphatidylethanolamine; PEp – plasmalogen phosphateidylethanolamine; PG – phosphatidylglycerol; Pl – phosphatidylinositol; PS – phosphatidylserine; BMP – Bis(monoacylglycero)phosphate; APG – acyl phosphatidyl glycerol; NAPE – *N*-acylphosphatidylethanolamine; NAPEp – *N*-acyl plasmogenphosphatidylethanolamine; NAPS – *N*-acyl phosphatidylserine; LPC – lysophosphatidyl choline; LPCe – ether lysophosphatidylcholine; LPE – lysophosphatidylethanolamine; LPEp – plasmalogen lysophosphatidylethanolamine; LPl – lysophosphatidylinositol;
Sphingolipids: Cer – ceramide; dhCer – dihydroceramide; SM – sphingomyelin; dhSM – dihydrosphingomyelin; GalCer – galactosylceramide; dhSulf – dihydrosulfatide; Sulf – sulfatide; GlcCer – glucosylceramide; LacCer – lactosylceramide; Ganglioside-NANA-3 – monosialodihexosylganglioside; GB-3 – globotriaosylceramide;
Neutral Lipids: FC – free cholesterol; CE – cholesterol ester; MG – monoacylglycerol; DG – diacylglycerol; TG – triacylglycerol.

Table 2. Clinical features and demographics of PD patients and control participants.

Clinical features /demographics	Male		Female		Total	
	PD patients (n=85)**	Controls (n=60)**	PD patients (n=85)**	Controls (n=60)**	PD patients (n=170)**	Controls (n=120)**
Body mass index*	27.5 (20|48)	27.1 (20|45)	24.3 (17|35)	24.5 (17|37)	25.9 (17|48)	25.8 (17|45)
Age (in years)*	68 (46|86)	68.8 (46|91)	69 (45|88)	63 (43|82)	68.5 (45|88)	65.9 (43|91)
Statin using participants (in%)*	15 (17.6%)	17 (28.3%)	23 (27.1%)	11 (18.3%)	19 (22.4%)	14 (23.3%)
PD duration (in years)	6.1 (0|26)		6.5 (0|28)		6.3 (0|28)	
Levodopa (in mg)	533.5 (0|1400)		423.5 (0|1200)		478.5 (0|1400)	
MoCA score#	26.2 (12|31)	27.5 (22|34)	26.8 (18|34)	25.6 (26|34)	26.5 (12|34)	26.6 (26|34)
Scale## I+II	11.2 (0|31)	0.8 (0|8)	8.8 (0|27)	0.7 (0|6)	10.0 (0|31)	0.7 (0|8)
Scale## III	19.6 (4|49)	1.2 (0|11)	15.2 (2|43)	1.2 (0|13)	17.4 (4|49)	1.2 (0|13)

** Mean (Minumum|Maximum); * These features were taken at the time of recruitment of all the participants (for both the genders); # this feature was similar between PD patients and controls both in the analyses including the entire cohort and in analyses stratified by gender; ## Unified Parkinson's disease rating based scale; MoCA – Montreal Cognitive Assessment.

q-value=4.795E-02). Table 3 also shows that 4 GCase products (Cer d18: 0/22: 0; d18: 0/26: 0; d18: 1/20: 1; d18: 1/22: 1) along with triglycerides had significant lower values in PD patients in comparison to those in control participants. Another striking difference between groups was in triglyceride levels (p-value=4.211E-03; q-value=4.878E-02; Table 3). Nevertheless, the statistical significance of the differences in triglyceride plasma levels was not as strong as were the differences in ganglioside-NANA-3 levels, due to a greater variance. This might be because participants were not fasting.

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Table 3. Significantly different concentration between PD patients and control participants for specific lipid species.

	PD patients	Control participants	q-Values	p-Values
Ganglioside-NANA-3	1.615±0.039	1.401±0.038	4.795E-02	5.820E-04
TG	51.669±3.622	72.959±7.029	4.878E-02	4.211E-03
Ganglioside-NANA-3				
d18:1/20:0	0.019±0.002	0.015±0.002	4.795E-02	2.295E-03
d18:1/22:1	0.134±0.004	0.111±0.003	4.795E-02	1.304E-03
TG				
52:2/18:0	1.194±0.079	1.822±0.215	4.795E-02	2.472E-03
52:3/18:1	5.518±0.293	7.443±0.621	4.795E-02	2.255E-03
54:3/18:0	1.173±0.068	1.752±0.228	4.795E-02	1.560E-03
54:4/18:1	2.855±0.169	3.889±0.346	4.795E-02	2.491E-03
54:4/20:4	0.088±0.006	0.102±0.016	4.795E-02	2.229E-03
54:5/18:1	2.412±0.129	3.200±0.229	4.878E-02	3.571E-03
54:5/20:4	0.496±0.034	0.663±0.067	4.878E-02	4.062E-03
54:6/18:1	1.192±0.072	1.532±0.112	4.878E-02	3.967E-03
54:6/20:4	0.722±0.043	0.943±0.079	4.795E-02	2.271E-03
54:7/18:1	0.242±0.021	0.307±0.025	4.878E-02	4.868E-03
56:4/20:4	0.082±0.006	0.110±0.014	4.878E-02	3.671E-03
56:5/18:1	0.269±0.021	0.362±0.033	4.878E-02	4.667E-03
56:5/20:4	0.241±0.016	0.309±0.028	4.795E-02	1.359E-03
56:6/20:4	0.295±0.021	0.404±0.039	4.795E-02	2.659E-03
Cer				
d18:0/22:0	0.0091±0.0003	0.019±0.002	4.795E-02	4.281E-04
d18:0/26:0	0.00059±0.00004	0.0008±0.0002	4.795E-02	3.011E-04
d18:1/20:1	0.0024±0.0002	0.0039±0.0004	4.878E-02	4.761E-03
d18:1/22:1	0.029±0.002	0.041±0.004	4.795E-02	2.619E-04
DG 36:2/18:0	0.269±0.017	0.343±0.031	4.878E-02	4.522E-03
PC				
34:2	33.792±0.427	35.381±0.711	4.878E-02	4.551E-03
46:2	0.0073±0.0004	0.0091±0.0003	4.795E-02	2.611E-03
PCe 46:3	0.015±0.002	0.019±0.002	4.885E-02	2.991E-03
PE 34:2	1.203±0.049	1.433±0.081	4.878E-02	4.561E-03
PS 40:4	0.023±0.002	0.029±0.001	4.878E-02	4.621E-03
SM d18:1/20:1	4.298±0.065	4.019±0.056	4.795E-02	1.671E-03
dhSM d18:0/18:0	0.739±0.033	0.944±0.062	4.878E-02	3.919E-03

* In pmol/μL; Ganglioside-NANA-3 – monosialodihexosylganglioside; TG – triacylglycerol; Cer – ceramide; DG – diacylglycerol; PC – phosphatylcholine; PCe – ether phosphatidylcholine; PE – phosphatidylethanolamine; PS – phosphatidylserine; SM – sphingomyelin; dhSM – dihydrosphingomyelin.

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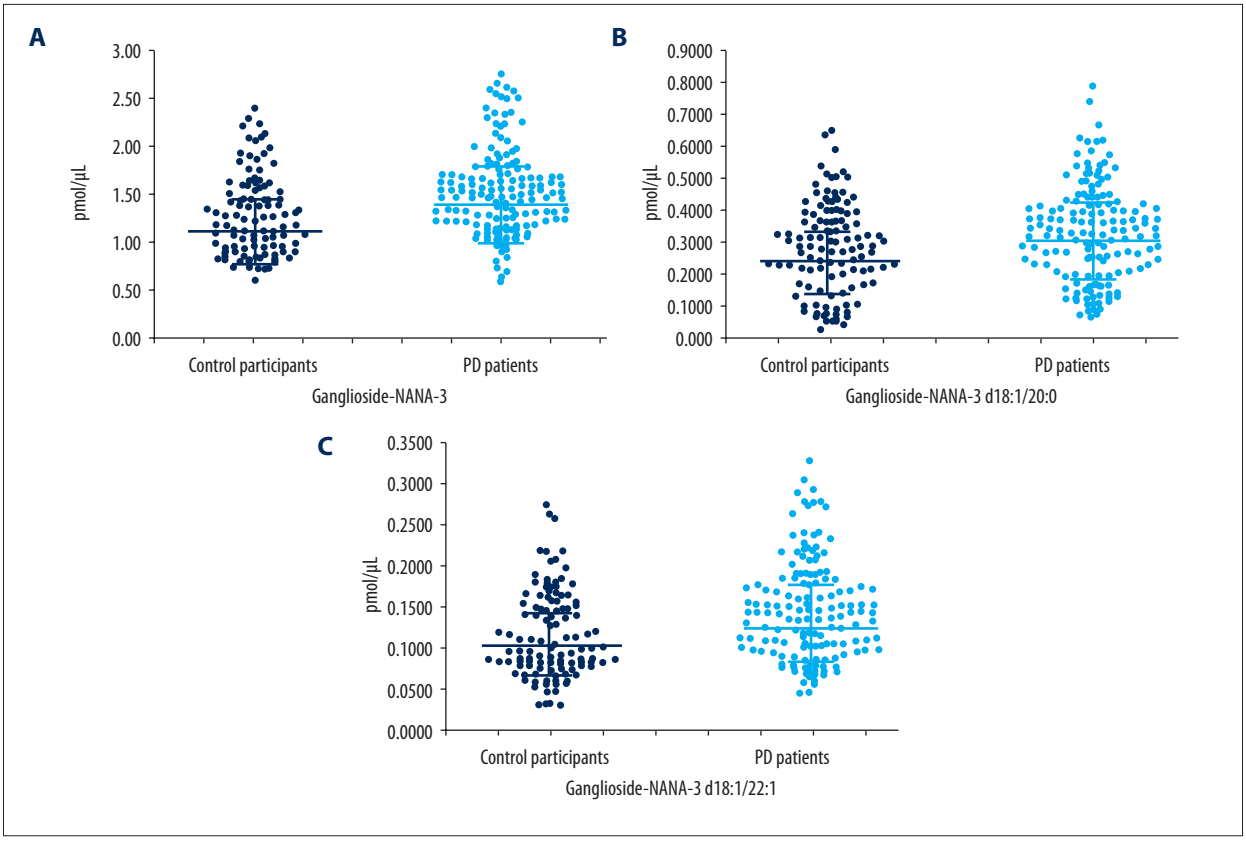

Figure 1. Scatter plots of: (**A**) total ganglioside-NANA-3 participants; (**B**) total ganglioside-NANA-3 d18: 1/20: 0 species participants, and (**C**) total ganglioside-NANA-3 d18: 1/22: 1 species.

Sexual effects on lipid levels

We stratified our analyses by sex as per a previously reported work [23] in which several sphingolipid subclasses were demonstrated to vary by participant sex. The concentrations of ganglioside-NANA-3 for males were 1.621±0.039 pmol/µL (PD patients) and 1.341±0.048 pmol/µL (control participants) (p-value=0.003; q-value=0.04), and for females they were 1.609±0.052 pmol/µL (PD patients) and 1.461±0.056 pmol/µL (control participants) (p-value=0.038; q-value=0.044). This means that, with respect to concentration of ganglioside-NANA-3, there were higher differences (between groups) in the males than in females. There were other significant lipid subclass alterations in male PD patients with respect to higher concentrations of NAPEp and lower concentrations of DG, while female PD patients remained unaltered in any lipid subclass in comparison to female control participants.

As per earlier reports [24,25] which showed the dependence of development of PD on GCase activity risk factor, we selected 2 lipid subclasses – GlcCer and ganglioside-NANA-3 – for receiver operating curve (ROC) analysis using non-linear classifiers. ROC analysis was conducted to evaluate the utility of 18 specific lipid species of 2 chosen subclasses in the discrimination of PD patients from control participants (data not shown). The area under the ROC analysis curve was 0.761 for men and 0.638 for women.

Discussion

This was a comparative study of 503 plasma lipid species belonging to 34 subclasses of lipid in 170 PD patients and 120 control participants after sequencing and screening for GBA and/or LRRK2 G2019S mutations. We found a significantly higher ganglioside-NANA-3 plasma concentration in PD patients as compared to control participants. The simplest member of the ganglioside family is ganglioside-NANA-3, which is composed of glycosphingolipid (ceramide backbone) and mono *N*-acetylneuraminic acid (NANA, sialic acid; at position-3) and served as precursors to brain-abundant complex gangliosides of the a- and b-series [26]. The Golgi apparatus synthesizes gangliosides (including ganglioside-NANA-3) and then the synthesized ganglioside-NANA-3 is delivered to the plasma membrane so that they may contribute to cellular signalling. Furthermore, through the GCase-based pathway, the internalized ganglioside-NANA-3 plasma is metabolized into ceramide and monosaccharide after targeting them via the endocytic

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pathway to the lysosome. Many studies [27–29] reported associations between PD and the reduced GCase activity in the periphery and in the central nervous system, but the mechanism behind the association of PD with reduced GCase activity is unknown. We believe that finding of increased ganglioside-NANA-3 plasma levels in PD patients in comparison to healthy control participants is due to the alteration of lipids metabolized by GCase. As reported elsewhere [30], other lipids (GlcCer and GalCer) in the GCase pathway were found at higher levels in PD patients than in healthy controls, and we consider our work as proof to support it. Thus, our work further strengthens the known association of PD pathogenesis and the GCase pathway since we found a close association between the GCase pathway and lipid metabolites.

To date, there are few models to explain the potential mechanism of alteration of ganglioside-NANA-3 plasma levels in PD patients. One such reported model [31] postulates a ganglioside-binding domain in the marked preference to ganglioside-NANA-3, a-synuclein. It includes residue E46, which is further mutated in E46K (a familial form of PD). Hence, the deleterious effect of the channel mutation was reversed in ganglioside-NANA-3-enriched vesicles.

There are some limitations to the present study. First, we could not establish a relationship between ganglioside-NANA-3 plasma levels in PD patients and their brains. Second, we did not properly understand and document the degree of natural variation in blood lipids between and within individual PD patients and the control participants. Third, we used surrogate standards to reference ganglioside-NANA-3 plasma levels since suitable synthetic reference standards are not available, although establishing reference levels of specific lipids is essential prior to their use in a clinical setting.

Conclusions

We have succeeded in highlighting the potential role of ganglioside-NANA-3 plasma in PD. Our work has shown a future research pathway to examine the association of complex gangliosides like ganglioside-NANA-2 and ganglioside-$(NANA)_2$-3, which are derived from ganglioside-NANA-3 in its biosynthetic pathway with PD patients by using our lipid panel. However, the interactions between these glycosphingolipids and a-synuclein should be further explored for better understanding of the pathophysiology of ganglioside-NANA-3 in PD patients.

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