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Impaired Sphingolipid Hydrolase Activities in Dementia with Lewy Bodies and Multiple System Atrophy

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Abstract

The synucleinopathies are a group of neurodegenerative diseases characterized by the oligomerization of alpha-synuclein protein in neurons or glial cells. Recent studies provide data that ceramide metabolism impairment may play a role in the pathogenesis of synucleinopathies due to its influence on alpha-synuclein accumulation. The aim of the current study was to assess changes in activities of enzymes involved in ceramide metabolism in patients with different synucleinopathies (Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA)). The study enrolled 163 PD, 44 DLB, and 30 MSA patients as well as 159 controls. Glucocerebrosidase, alpha-galactosidase, acid sphingomyelinase enzyme activities, and concentrations of the corresponding substrates (hexosylsphingosine, globotriaosylsphingosine, lysosphingomyelin) were measured by liquid chromatography tandem-mass spectrometry in blood. Expression levels of *GBA*, *GLA*, and *SMPD1* genes encoding glucoceresobridase, alpha-galactosidase, and acid sphingomyelinase enzymes, correspondently, were analyzed by real-time PCR with TaqMan assay in CD45 + blood cells. Increased hexosylsphingosine concentration was observed in DLB and MSA patients in comparison to PD and controls ($p < 0.001$) and it was associated with earlier age at onset (AAO) of DLB ($p = 0.0008$). *SMPD1* expression was decreased in MSA compared to controls ($p = 0.015$). Acid sphingomyelinase activity was decreased in DLB, MSA patients compared to PD patients ($p < 0.0001$, $p < 0.0001$, respectively), and in MSA compared to controls ($p < 0.0001$). Lower acid sphingomyelinase activity was associated with earlier AAO of PD ($p = 0.012$). Our data support the role of lysosomal dysfunction in the pathogenesis of synucleinopathies, namely, the pronounced alterations of lysosomal activities involved in ceramide metabolism in patients with MSA and DLB.

Keywords Synucleinopathies · Lysosomal storage disorders · Gene expression · Enzyme activity · Substrate concentration

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Introduction

The synucleinopathies belong to a group of neurodegenerative diseases characterized by the accumulation of abnormal protein aggregates of alpha-synuclein in neurons or glial cells. There are three types of synucleinopathies: Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) [1]. All synucleinopathies are characterized by clinical overlap at early stage of disease that makes differential diagnosis challenging [2]. Molecular mechanisms of synucleinopathies are unknown, but multiple hypothesis exist [3]. Understanding of mechanisms underlying pathogenesis of synucleinopathies, especially of MSA as it is rapidly progressive, multisystem, fatal neurodegenerative disease is crucial important.

Over the last decade, the important role of lysosomal dysfunction in the pathogenesis of synucleinopathies was shown. Lysosomal dysfunction firstly was linked to lysosomal storage disorders (LSDs) [4, 5]. LSD is a group of rare inherited disorders caused by the defective function of specific lysosomal enzyme due to mutations in the gene encoding this enzyme [6]. The first evidence of a link between LSDs and neurodegenerative disorders came from the observation of a clinical features of parkinsonism in relatives and patients affected by Gaucher disease (GD), rare LSD, caused by mutations in the *GBA* gene, which result in deficient lysosomal enzyme glucocerebrosidase (GCase) activity [7–9]. Mutations in the *GBA* gene have been reported to be the highest genetic risk factor for PD with an increase of PD risk with a prevalence of 2–30% depending on the population [10–12]. Mutations in another LSD gene, *SMPD1*, encoding the lysosomal enzyme acid sphingomyelinase (ASMase), cause Niemann-Pick disease type A and type B (NPD), and have also been associated with PD risk [13–15]. Mutations in the *GLA* gene, encoding the lysosomal enzyme alpha-galactosidase (GLA), cause X-linked Fabry disease (FD) and were found in PD patients [16]. Previously, decreased GLA and ASMase activities in blood were shown in PD patients [17, 18]. Several studies reported decreased GCase activity in blood and brain samples of sporadic PD, with no mutations in *GBA* [19–21]; meanwhile, the others did not support these findings [22–24]. The only study on postmortem brains of DLB patients showed decreased GCase activity [20]. There are no similar studies on MCA cohort. The assessment of activity of lysosomal enzymes in blood of MSA and DLB patients have not been conducted.

It is worth mentioning that GCase, GLA, and ASMase participate in ceramide metabolism in lysosome due to hydrolyzation of sphingolipids in the lysosome and generate a common product, ceramide, an activator of enzymes taking part in disturbance of homeostasis of which may lead to accumulation and aggregation of alpha-synuclein in the cells [25]. We hypothesized that altered expression of the lysosomal genes, encoding enzymes involved in ceramide metabolism, may affect activities of those enzymes, substrate accumulation, and, therefore, may lead to an increased risk and/or act as modifier of synucleinopathies. Previously, decreased GCase activity and an increase in the concentration of its substrate, hexosylsphingosine (HexSph), both in blood and in postmortem brains, were shown in patients with GBA-PD [20, 26]. Such data make it possible to speculate that the processes associated with impaired metabolism of ceramide in the blood may reflect the pathological processes occurring in the brain. Thus, we extrapolated what was shown in patients with GBA-PD to patients with other synucleinopathies. Here, we aimed to assess the expression levels of the *GBA*, *GLA*, *SMPD1* genes, activity of the correspondent enzymes, and concentration of corresponding enzyme substrates in blood of patients with PD, DLB, and MSA.

Methods

Approval by Institutional Research Ethics Board

This project was approved by ethics committee of Pavlov First Saint-Petersburg State Medical University. A formal written consent form was provided to all included subjects to read and sign prior to the study.

Subjects

A total of 163 sporadic PD patients with no family history of the disease (sPD), 44 DLB patients, 30 MSA patients, and 159 individuals without neurological disorders (controls) were included in the current study. The demographic characteristics of the studied groups are presented in Table 1. Controls had no history of neurological diseases. The diagnosis of PD was established in accordance with the MDS criteria [27]; DLB and MSA were diagnosed according to consortia criteria [28, 29]. To avoid biases and exclude carriers of *GBA* and *LRRK2* mutations from the current study, all participants were screened for the major mutations in the *GBA* gene (L444P, N370S) and in the *LRRK2* gene (G2019S) as previously described [30, 31].

RNA Extraction, cDNA Synthesis, and Quantitative PCR in CD45 + Blood Cells

Homogeneous cell population is believed to provide more precise result in gene expression studies compared to the whole blood due to increase amount of immune blood cells with age and differential gene expression in different

Table 1 Clinical and demographic characteristics of studied participants

Groups	sPD	DLB	MSA	Controls	*p*-value
N	163	44	30	159	-
Age at exam, median (min–max), years	65.0 (38.0–88.0)	73.0 (52.0–90.0)	61.0 (51.0–75.0)	62.0 (23.0–91.0)	$p < 0.001$
Age at onset, median (min–max), years	58.0 (32.0–90.0)	68.0 (51.0–89.0)	56.0 (46.0–71.0)	-	$p < 0.001$
Gender (male:female)	69:94	16:28	14:16	64:95	p = 0.51

sPD sporadic Parkinson's disease, *DLB* dementia with Lewy bodies, *MSA* multiple system atrophy

cell populations [32]. CD45 + peripheral blood cells as all lymphocytes express dopamine (DA) receptors and DA transporter on their plasma membrane [33]. Mononuclear CD45 + peripheral blood cells of individuals from all studied groups were immunomagnetically selected by magnetic cell sorting system (Miltenyi Biotec) according to manufacturer instructions. Total RNA was extracted from CD45 + blood cells using GeneJET RNA Purification Kit (K0731, Thermoscientific, Lithuania) and complementary DNA (cDNA) was synthesized by Revert Acid First cDNA Synthesis kit (K1622, Thermoscientific, Lithuania). *GBA*, *GLA*, and *SMPD1* mRNA levels were analyzed with TaqMan probes in a CFX96 thermocycler (BioRad, USA). Expression levels of studied genes were normalized to the expression levels of *GNB2L1* and *ACTB*. Primers and probes are designed by Primers3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers used for qPCR are presented in Supplementary table 1. Data was analyzed with a threshold set in the linear range of amplification. The value of the expression level relative to the calibrator was determined by the formula $2^{-\Delta\Delta Ct}$ [34].

Enzyme Activity and Substrate Concentration

From each of the study participants, venous blood samples were collected in EDTA tubes. DBS cards were prepared by pipetting 40 µL of whole blood on each spot. DBS were allowed to dry in open air at room temperature for 2 h and then were stored at − 20 °C until extraction. Enzyme activities GCase (EC 3.2.1.45, deficient in Gaucher disease), GLA (EC 3.2.1.22 deficient in Fabry disease), ASMase (EC 3.1.4.12, deficient in Neimann-Pick disease types A and B), and concentration of corresponding substrates (HexSph (glucosylsphingosine (GlcSph) + galactosylsphingosine (GalSph)), globotriaosylsphingosine (LysoGb3), and lysosphingomyelin (LysoSM) were estimated by liquid chromatography tandem-mass spectrometry (LC–MS/MS) in dry blood spots (DBS) as described earlier [35, 36]. During chromatographic separation, GlcSph and its isomer GalSph elute in a single peak named as HexSph. As GalSph blood level is considered negligible in controls and GD patients and does not interfere with GlcSph, HexSph level reflects, primarily, an alterations in GlcSph [37]

Statistical Analysis

χ^2 test was applied to determine gender variables. Conformity of findings to normal distribution was tested using the Shapiro–Wilk test. Gene expression levels, activity of each enzyme, and concentration of each substrate were compared between studied groups using the nonparametric Mann–Whitney *u*-test with Bonferroni correction for multiple comparisons. Given that the Fabry's disease is X-linked, we stratified the analyses by gender. To test the correlation between different gene expression levels, enzymatic activity, and substrate concentrations, Spearman's correlation coefficient was used. For odds ratio, logistic regression analysis was used, in which synucleinopathies status was the outcome and gene expression levels, enzymatic activity, or substrate concentrations were the predictors, adjusted for age and gender; disease duration was performed. To examine the association between gene expression levels, enzymatic activities, substrate concentrations, and age at onset (AAO), linear regression models with the AAO as the dependent variable and age, gender, and gene expression levels or enzymatic activities or substrate concentrations as covariates were performed. We further divided the sPD patient cohort into four groups based on quartiles of gene expression levels, enzymatic activities, and substrate concentrations measured in the control group, and ANOVA was performed to examine the association between estimated parameters and AAO of disease as was performed earlier [17]. Significance with Bonferroni correction for multiple comparisons was established at $p < 0.05$. In Supplementary Fig. 1, all *p*-values are presented before adjustment for multiple comparisons. Statistical analysis was performed using R software (version 3.6.2). Clinical data and experimental data are expressed as the median (min–max).

Results

Expression Levels of Genes Encoding Lysosomal Enzymes in CD45 + Blood Cells

Gene expression was assessed for *GBA*, *GLA*, and *SMPD1* genes in CD45 + blood cells of PD, DLB,

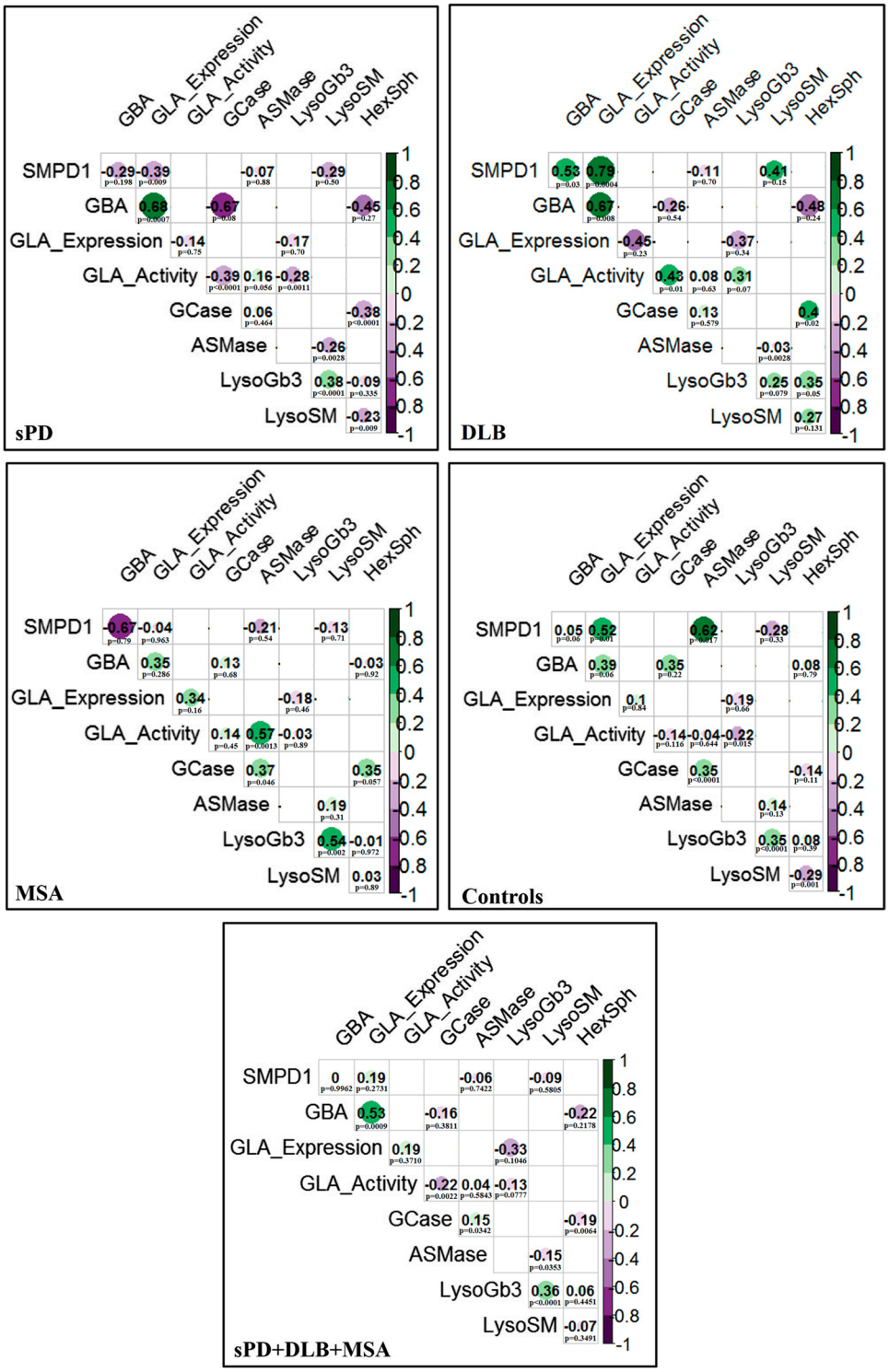

Fig. 1 Correlation matrix of gene expression, enzymes activity, and substrate concentrations in studied groups. The violet and green dots correspond to negative and positive correlations, correspondently. Small dots with light colors represent lower correlations. Larger dots with darker colors correspond to higher correlations

MSA, and controls (Table 2). We stratified the analyses by gender, because the *GLA* gene is X-linked. Among women, *GLA* mRNA level was significantly increased in CD45 + blood cells of DLB patients compared to sPD patients ($p = 0.016$). *SMPD1* gene expression level was significantly lower in MSA than in controls ($p = 0.015$) (Supplementary Fig. 1). There were no differences in *GBA* expression levels between all studied groups ($p > 0.05$).

Table 2 Gene expression levels, activity of lysosomal enzymes, and concentration of lysosphingolipids in blood of sPD, DLB, MSA patients, and controls

	Groups				
	Parameters	DLB	MSA	sPD	Controls
Lysosomal genes, median (min–max)	*GBA*	2.65 (0.04–21.77)	2.74 (0.28–34.33)	1.46 (0.25–16.03)	1.46 (0.20–90.41)
	GLA	0.11 (0.001–45.49)	1.61 (0.04–41.62)	1.64 (0.001–77.62)	0.58 (0.01–99.63)
	GLA (female)	0.04 (0.01–13.99) **p = 0.016***	2.32 (0.04–41.62)	3.11 (0.13–70.01)	0.58 (0.04–38.11)
	GLA (male)	0.73 (0.01–45.49)	1.25 (0.11–18.44)	0.85 (0.01–77.62)	0.61 (0.01–99.63)
	SMPD1	2.84 (0.17–100.12)	0.31 (0.03–45.70), **p = 0.015*****	2.39 (0.10–58.48)	3.59 (0.78–62.14)
Enzyme activities, median (min–max)	GCase	5.43 (1.20–25.76)	6.12 (3.15–11.39)	6.97 (1.73–20.87)	5.56 (1.54–32.13)
	GLA	4.34 (0.94–9.32)	4.92 (2.29–11.00) **p = 0.020*** **p = 0.025*****	3.62 (1.33–13.93)	3.70 (1.03–12.81)
	GLA (female)	3.79 (1.93–8.65)	6.80 (2.83–8.66) **p = 0.015*** **p = 0.008*****	3.38 (1.33–13.93)	3.55 (1.03–10.56)
	GLA (male)	4.91 (0.94–8.64)	4.48 (3.05–10.99)	3.96 (1.68–13.72)	3.94 (1.07–12.81)
	ASMase	3.29 (1.81–7.89) **p = 1.6e-04***	2.73 (1.42–6.16) **p = 1.9e-9*** **p = 3.7e-05*****	5.42 (1.53–13.25)	4.89 (1.47–12.39)
Substrate concentration, median (min–max)	HexSph	3.71 (1.34–11.70) **p = 1.4e-06*** **p = 4.2e-04*****	5.36 (1.69–11.68) **p = 3.9e-08*** **p = 4.9e-06*****	2.35 (0.87–10.71)	2.34 (0.69–10.24)
	LysoGb3	0.71 (0.06–1.27)	0.87 (0.06–1.34)	0.70 (0.04–2.49)	0.76 (0.06–1.84)
	LysoGb3 (female)	0.62 (0.06–1.27)	0.85 (0.06–1.34)	0.75 (0.04–1.85)	0.77 (0.06–1.84)
	LysoGb3 (male)	0.88 (0.40–1.11)	0.85 (0.34–1.32)	0.63 (0.15–2.50)	0.77 (0.15–1.78)
	LysoSM	4.22 (1.81–9.43) **p = 0.04***	3.35 (1.54–5.50) **p = 0.0046**** **p = 0.0036*****	3.74 (1.65–6.36) **p = 0.02*****	4.00 (0.59–11.60)

sPD sporadic Parkinson's disease, *DLB* dementia with Lewy bodies, *MSA* multiple system atrophy, *GCase* glucocerebrosidase, *GLA* alpha–galactosidase, *ASMase* acid sphingomyelinase, *HexSph* hexosylsphingosine, *LysoGb3* globotriaosylsphingosine, *LysoSM* lysosphingomyelin

*Compared to sPD

**Compared to DLB

***Compared to controls

Lysosomal Enzyme (GCase, GLA, ASMase) Activities in Patients with Synucleinopathies

Enzymatic activities of three enzymes (GCase, GLA, ASMase) were measured in DBS of sPD, DLB, MSA patients, and controls (Table 2). GLA activity was increased in MSA patients in comparison to sPD patients and controls ($p = 0.020$, $p = 0.025$, respectively). Among men, GLA activity was not significantly changed in all studied groups ($p > 0.05$). Among women, GLA activity was significantly elevated in MSA patients compared to sPD patients ($p = 0.015$) and controls ($p = 0.008$). We found a decrease in ASMase activity in DLB patients compared to sPD patients ($p < 0.0001$) and in MSA patients compared to sPD and controls ($p < 0.0001$, $p < 0.0001$, respectively). There was no difference between GCase enzymatic activity in all studied groups ($p > 0.05$).

Lysosphingolipid (HexSph, LysoGb3, LysoSM) Concentrations in Patients with Synucleinopathies

Concentrations of three lysosphingolipids, HexSph, LysoGb3, and LysoSM as corresponding substrates for following enzymes, GCase, GLA, and ASMase, were measured in DBS of sPD, DLB, MSA patients, and controls (Table 2). Elevated HexSph concentration was found in DLB and MSA patients compared to sPD patients and controls ($p < 0.0001$). Decreased LysoSM concentration in MSA patients than in DLB patients ($p = 0.0046$) and in controls ($p = 0.0036$) and in sPD patients than in controls ($p = 0.02$) and DLB ($p = 0.04$)

were determined. There were no differences in LysoGb3 concentration between all studied groups ($p > 0.05$).

Correlation Analysis Among Expression of Lysosomal Genes, Lysosomal Enzyme Activities, and Lysosphingolipids Concentrations

Correlation analysis was performed to assess association among expression levels of lysosomal genes, activities of lysosomal enzymes, and lysosphingolipid concentrations shown in Fig. 1.

SMPD1 expression level positively correlated with ASMase activity in controls ($r = 0.62$, $p = 0.017$) while ASMase activity negatively correlated with LysoSM concentration in PD patients ($r = -0.26$, $p = 0.0028$). GCase activity and GLA activity negatively correlated with concentration of their corresponding substrates, HexSph and LysoGb3 in PD patients ($r = -0.38$, $p < 0.0001$; $r = -0.28$, $p = 0.0011$, respectively). Negative correlation was also determined between GLA activity and LysoGb3 concentration in controls ($r = -0.22$, $p = 0.015$) (Fig. 1).

Positive correlation was observed between *SMPD1* and *GLA* mRNA levels in CD45 + blood cells of DLB patients ($r = 0.794$, $p = 0.0004$) and controls ($r = 0.521$, $p = 0.01$). Also, positive correlation was found between *GLA* and *GBA* expression levels in DLB patients ($r = 0.671$, $p = 0.008$) and sPD patients ($r = 0.680$, $p = 0.0007$) as well as between *GBA* and *SMPD1* expression levels in DLB patients ($r = 0.532$, $p = 0.03$). The positive correlation was detected between GCase and GLA activities in DLB ($r = 0.43$, $p = 0.01$) and also between GCase and ASMase activities in MSA patients ($r = 0.37$, $p < 0.0001$) and controls ($r = 0.347$, $p < 0.0001$). Negative correlation was found in sPD patients between GLA and GCase activities ($r = -0.39$, $p = 0.046$) meanwhile positive correlation between ASMase and GLA enzymatic activities was revealed in MSA patients ($r = 0.57$, $p = 0.0013$). We found positive correlation between LysoSM and LysoGb3 concentrations in sPD patients ($r = 0.383$, $p < 0.0001$), MSA patients ($r = 0.54$, $p = 0.002$), and controls ($r = 0.35$, $p < 0.0001$).

Because correlations between expression levels of lysosomal genes, activities of lysosomal enzymes, and lysosphingolipid concentrations were found, the multivariable logistic regression analysis was performed to identify contribution of gene expression, enzymatic activities, and lysosphingolipid concentrations to synucleinopathies status in patients adjusted for age and sex (Table 3). There was no association between expression levels of all genes and synucleinopathies status. At the same time, higher ASMase activity was associated with lower odds of DLB and MSA status (OR = 0.544 95% CI 0.318–0.933; $p = 0.027$; OR = 0.335 95% CI 0.192–0.585; $p = 0.0001$, respectively). Surprisingly, increased GLA activity was found to be associated with increased DLB risk (OR = 1.385; 95% CI 1.017–1.885; $p = 0.039$) and MSA risk (OR = 1.393; 95% CI 1.083–1.790; $p = 0.010$). Higher HexSph concentration was associated with increased risk of DLB (OR = 2.076; 95% CI 1.285–3.352; $p = 0.003$) and MSA (OR = 1.733; 95% CI 1.294–2.320; $p = 0.0002$). Higher LysoSM concentration was linked with lower odds of sPD and MSA status (OR = 0.506; 95% CI 0.360–0.710; $p < 0.0001$; OR = 0.442; 95% CI 0.266–0.735; $p = 0.002$, respectively).

Also, correlation matrix was plotted for of all studied parameters for all our cases taken together (PD, DLB, MSA patients). We observed negative correlation between enzymatic activities and corresponding substrate concentrations that were observed in cohort of PD patients separately. We supposed that this effect was achieved due to prevalence of patients with PD in our study. Data are presented in Fig. 1.

Lysosomal Genes, Enzyme Activities, and Lysosphingolipids Concentrations Are Associated with the Age at Onset of Synucleinopathies

Among DLB patients, increased HexSph concentration was associated with an earlier AAO with adjustment to LysoSM, LysoGb3, and gender (β = −2.626, 95% CI −4.883 to −0.369, $p = 0.044$; i.e., for each 1 nmol/L reduction, age at onset was younger by 2.6 years). Among sPD patients, decreased ASMase activity was associated with an earlier AAO adjustment to GCase, GLA activities, age, and gender (β = 1.108, 95% CI 0.296–1.921, $p = 0.009$; i.e., for each 1 μmol/l/h reduction, age at onset was younger by 1.11 years) (Supplementary table 2). We further divided each studied group with synucleinopathies to four groups, based on expression levels of lysosomal genes quartile, on the enzymatic activity quartiles and lysosphingolipid concentration quartiles in controls (Supplementary table 3). Among DLB patients, increased HexSph concentration was associated with an earlier AAO with significant differences of more than 10 years ($p = 0.000484$). Also, decrease in AAO was linked to decreased of enzymatic activity of ASMase, with significant differences of 6.9 years between the first and last quartiles ($p = 0.012$) in sPD patients (Supplementary table 3).

Also, we examined the association between gene expression levels, enzymatic activities, substrate concentrations, and phenotypic severity according to The Hoehn and Yahr scale for group of PD patients. No significant associations between phenotypic severity and gene expression levels, enzymatic activities, and substrate concentrations were found by regression analysis and ANOVA in PD patients. These results are not presented.

Table 3 The association between gene expression levels, activity of lysosomal enzymes, and concentration of lysosphingolipids in blood and synucleinopathies status

Groups		Odds ratio	95% CI	*p*-value		Odds ratio	95% CI	*p*-value		Odds ratio	95% CI	*p*-value
DLB	***GBA***	0.808	0.439–1.485	0.492	**GCase**	1.073	0.913–1.261	0.394	**HexSph**	**2.076**	**1.285–3.352**	**0.003**
	GLA	0.894	0.489–1.634	0.715	**GLA**	**1.385**	**1.017–1.885**	**0.039**	**LysoGb3**	0.311	0.012–7.767	0.477
	SMPD1	1.038	0.969–1.113	0.286	**ASMase**	**0.544**	**0.318–0.933**	**0.027**	**LysoSM**	1.337	0.784–2.277	0.286
	Age	1.061	0.927–1.213	0.390	**Age**	**1.249**	**1.119–1.395**	**7.91e-05**	**Age**	**1.255**	**1.097–1.436**	**0.001**
	Gender	1.032	0.169–6.322	0.973	**Gender**	0.527	0.103–2.690	0.441	**Gender**	0.802	0.134–4.783	0.809
sPD	***GBA***	0.920	0.836–1.013	0.090	**GCase**	1.024	0.955–1.099	0.504	**HexSph**	0.794	0.629–1.004	0.057
	GLA	1.124	0.978–1.291	0.099	**GLA**	1.023	0.909–1.151	0.699	**LysoGb3**	1.494	0.817–4.623	0.133
	SMPD1	1.033	0.981–1.087	0.220	**ASMase**	1.021	0.897–1.163	0.749	**LysoSM**	**0.506**	**0.360–0.710**	**8.27e-05**
	Age	0.944	0.853–1.044	0.265	**Age**	**1.056**	**1.025–1.088**	**0.0004**	**Age**	**1.075**	**1.039–1.113**	**3.18e—05**
	Gender	0.448	0.095–2.108	0.310	**Gender**	0.790	0.447–1.393	0.415	**Gender**	0.609	0.324–1.144	0.123
MSA	***GBA***	1.148	0.869–1.517	0.330	**GCase**	1.004	0.809–1.246	0.969	**HexSph**	**1.733**	**1.294–2.320**	**0.0002**
	GLA	1.023	0.708–1.479	0.904	**GLA**	**1.393**	**1.083–1.790**	**0.010**	**LysoGb3**	2.641	0.424–16.428	0.298
	SMPD1	0.742	0.481–1.144	0.177	**ASMase**	**0.335**	**0.192–0.585**	**0.0001**	**LysoSM**	**0.442**	**0.266–0.735**	**0.002**
	Age	0.952	0.808–1.122	0.560	**Age**	**1.083**	**1.017–1.152**	**0.013**	**Age**	1.059	0.999–1.123	0.055
	Gender	0.246	0.017–3.374	0.294	**Gender**	**0.246**	**0.068–0.888**	**0.032**	**Gender**	0.596	0.180–1.967	0.395

sPD sporadic Parkinson's disease, *DLB* dementia with Lewy bodies, *MSA* multiple system atrophy, *GCase* glucocerebrosidase, *GLA* alpha−galactosidase, *ASMase* acid sphingomyelinase, *HexSph* hexosylsphingosine, *LysoGb3* globotriaosylsphingosine, *LysoSM* lysosphingomyelin, *95% CI* 95% confidence interval

Discussion

The present study provides a comprehensive assessment of expression levels of lysosomal genes, activities of lysosomal enzymes, and concentrations of their substrates, involved in ceramide metabolism, in patients with synucleinopathies and controls.

Resent data provided evidence about the role of ceramide as bioactive lipid belonging to the class of sphingolipids in the pathogenesis of synucleinopathies [25], in particularly, because of its involvement in activation of cathepsin D, a lysosomal enzyme taking part in alpha-synuclein degradation in the cells [38].

GCase hydrolyzes glucosylceramide to ceramide and glucose. Gradual reduction in GCase activity has been observed earlier and associated with aging, suggesting that a decreased brain GCase activity may contribute to the age-related risk for neurodegenerative disorders [39]. It is worth noting that involvement of alterations in GCase activity in the pathogenesis of sPD remains unclear. Previously, decreased GCase activity was demonstrated in blood and brain samples of PD and DLB patients compared to controls [19–21], and at the same time, we and the other researchers did not reveal significant decrease of GCase activity in blood samples of PD patients [22, 23]. No evidence of *GBA* mRNA level and GCase activity reduction was found in the current study between all studied groups. Among the studies that, like here, have found no decrease of GCase activity in PBMCs of non-GBA-PD, the study of Papagiannakis and colleagues should also be mentioned [24]. Moreover, *GBA* mRNA level do not correlate with GCase activity in all studied groups (PD, DLB, MSA) according to the previous study where authors measured *GBA* expression level and GCase activity in different parts of brain of PD and DLB patients [20]. However, this result was not unexpected, since the same observation has been reported earlier in GD patients [40]. Nevertheless, we found that HexSph concentration was elevated in DLB and MSA compared to PD and controls. So, we conclude that increased HexSph concentration might not correspond with lower GCase activity and altered *GBA* mRNA level. Of interest is that in the current study, we first showed that increased blood HexSph concentration is associated with earlier AAO of DLB. HexSph is a mixture of galactosylsphingosine (GalSph) and glucosylsphingosine (GlcSph, Lyso-Gb1) and its concentration has been recently identified as biomarker of two sphingolipidoses, GD and Krabbe disease. HexSph or Lyso-Gb1 concentration are being discussed as the most reliable marker for GD therapy response [35]. Moreover, Lyso-Gb1 is discussed now as functionally significant biomarker because of its ability to promote a formation of toxic oligomeric alpha-synuclein aggregates in neurons from patients with GD or PD patients [41]. Further studies on larger cohort of DLB and MSA patients are needed to estimate relevance of HexSph as a biomarker.

GLA is closely related to GCase due to contribution both in the conversion of LysoGb3 to ceramide [17]. Previously, decreased GLA activity in blood of PD patients was demonstrated [26, 42–44]. Moreover, recent studies demonstrated decreased GLA activity in postmortem substantia nigra and temporal cortex of PD patients [45, 46]. The decrease in *GLA* mRNA and protein levels in the peripheral leukocytes of sPD patients was also reported [43]. We did not reveal decrease in GLA activity in all groups of patients with synucleinopathies compared to controls in the present study, but unexpectedly, *GLA* expression level and GLA activity were increased in MSA patients compared to controls. Moreover, elevated GLA activity was associated with higher odds of MSA status. The reason for the increased GLA activity is currently unclear. Further research is required to assess the role of GLA in the pathogenesis of synucleinopathies.

Biochemically, GCase and ASMase both play a key role in sphingolipid metabolism [47]. ASMase is a lysosomal enzyme that able to hydrolyze sphingomyelin to produce ceramide; therefore, it plays an important housekeeping role in sphingolipid metabolism and membrane turnover. There is particularly strong evidence for an association of variants in the *SMPD1* gene encoding ASMase with PD [48, 49]. In the current study, we demonstrated that reduced ASMase activity is associated with earlier AAO of PD that corresponds to previous data [18, 49]. We also first revealed decreased ASMase activity in MSA patients compared to controls and PD patients as well as in DLB compared to PD. However, no differences in ASMase activity were found in PD patients and controls as in the study of Alcalay et al. [18]. We detected decreased *SMPD1* expression level in MSA patients compared to controls. Alcalay and coauthors demonstrated *SMPD1* knockdown resulted in decreased ASMase levels and led to increased alpha-synuclein levels in HeLa cell line and BE(2)-M17 dopaminergic cells [18]. We also found increased alpha-synuclein level in CD45 + blood cells in MSA patients compared to controls; in contrast, there were no differences between DBL, PD, and controls [50, 51]. It could be discussed that ASMase activity may influence alpha-synuclein protein level and therefore could play a role in MSA pathogenesis.

In the current study, we found positive correlation between GCase and ASMase activity in MSA patients and controls. It is worth mentioning that in in the preprint of the recent study, a reduction of GCase to ~ 80% as a result of ASMase knockdown by adding transfection reagent has been demonstrated [52]. Moreover, the reduction in ASMase enzymatic activity enforce an increase in GlcCer (Lyso-GB1) concentration in *smpd1*-/- and *gba*-/- double

mutant zebrafish (48). Similarly, to abovementioned data, we showed an increase of HexSph concentration in patients with MSA along with the reduction in ASMase enzymatic activity. This effect could be achieved due to correlation between GCase and GLA with ASMase, and also increased GLA activity in MSA patients that may accelerate the synthesis of GlcCer from its percussor, Lyso-Gb3, which is a substrate for GLA. We also observed an elevated HexSph concentration along with the decreased of ASMase activity and positive correlation between GLA and GCase activities in DLB patients. Previously, Alcalay and coauthors demonstrated positive correlation between GCase, ASMase, and GLA activities in blood of PD patients [17]. We extended this observation on other synucleinopahties. It remains obscure whether *GBA*, *GLA*, and *SMPD1* interact genetically [18, 49]. Taken together, our results may suggest a close relationship between all three hydrolases, GCase, ASMase, and GLA in the pathogenesis of synucleinopahties. The limitations of the current study are sample size of studied groups of patients with synucleinopahties as well as not all groups are matched for age. Another limitation is the lack of replicative group with available activity and/or sphingolipids measurements, due to the rarity of studied synucleinopathies.

Conclusion

Our data indicate that altered activities of sphingolipid hydrolases involved in ceramide metabolism could take part in the pathogenesis of synucleinopathies. Decreased ASMase activity as well as mRNA expression level for *SMPD1* might play the pivotal role in MSA pathogenesis. Future replicative studies are required. It is worth noting that ASMase activity in CSF and in brain tissue of MSA patients has not been studied yet. Moreover, our data allowed to suggest that the decrease of ASMase activity in DLB and MSA could lead to HexSph accumulation, as a consequence of close relation of lysosomal enzymes involved in ceramide metabolism. HexSph may be considered as a possible biomarker for DLB and MSA.

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Author Contribution Project was initiated by TU and SP. KS, MI, KI, LK, IF, DZ, AT, YI, EP, NZ, EG, and AK are doctors who generated the studied groups of patients with MSA, DLB, and PD. TU, AB, KB, AZ, and AE were involved in processing of the collection of biological samples (dry blood spots, CD45 + blood cells). TU and AB were involved in experimental design of study. Execution of biochemical assays have been done by TU, AB, KB, GB, and EZ. Statistical analyses were set up and performed by TU, AB, and KB. Figures were composed by TU and AB. TU, SP, and AB contributed to the interpretation of the data. TU wrote the first draft of the manuscript. The review and critique have been done by SP and KS. The manuscript was edited and finalized by SP and TU.

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Availability of Data and Materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval and Consent to Participate All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by ethics committee of Pavlov First Saint-Petersburg State Medical University.

Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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