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SMPD1 mutations, activity and α -synuclein accumulation in Parkinson's disease

Roy N. Alcalay, MD MSc^{1,2,*}, Victoria Mallett, BA^{3,*}, Benoît Vanderperre, PhD^{4,*}, Omid Tavassoly, PhD⁴, Yves Dauvilliers, MD PhD⁵, Richard Y.J. Wu^{3,6}, Jennifer A. Ruskey, MSc^{3,7}, Claire S. Leblond, PhD^{3,8}, Amirthagowri Ambalavanan, PhD^{3,8}, Sandra B. Laurent, BSc^{3,7}, Dan Spiegelman, MSc^{3,7}, Alexandre Dionne-Laporte, MSc^{3,7}, Christopher Liang, BA¹, Oren A. Levy, MD PhD¹, Stanley Fahn, MD¹, Cheryl Waters, MD¹, Sheng-Han Kuo, MD¹, Wendy K. Chung, MD PhD^{9,10}, Blair Ford, MD¹, Karen S. Marder, MD MPH¹, Un Jung Kang, MD¹, Sharon Hassin-Baer, MD^{11,12,13}, Lior Greenbaum, MD PhD^{11,14,15}, Jean-

Corresponding author: **Ziv Gan-Or**, Montreal Neurological Institute, The Department of Human Genetics, McGill University, 1033 Pine Avenue, West, Ludmer Pavilion, room 327, Montreal, QC, H3A 1A1, Tel: +1-514-398-5845, Fax: +1-514 398-8248, ziv.gan-or@mcgill.ca.

Author roles:

- 1) Research project: A. Conception, B. Organization, C. Execution;
- 2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique;
- 3) Manuscript: A. Writing of the first draft, B. Review and Critique.

Roy N. Alcalay: 1A, 1B, 1C, 2C, 3A.

Victoria Mallett: 1B, 1C, 3B, 2A

Benoît Vanderperre: 1A, 1B, 1C, 2A, 2B, 3A, 3B

Omid Tavassoly: 1A, 1B, 1C, 2A, 2B, 3A, 3B

Yves Dauvilliers: 1B, 2C, 3B

Richard Y.J. Wu: 1B, 1C, 3B

Jennifer A. Ruskey: 1B, 1C, 3B

Claire S. Leblond: 1C, 3B

Amirthagowri Ambalavanan: 1C, 2B, 2C, 3B

Sandra B. Laurent: 1B, 1C, 3B

Dan Spiegelman: 1C, 3B

Alexandre Dionne-Laporte: 1C, 3B

Christopher Liang: 1B, 3B

Oren A. Levy: 1C, 3B

Stanley Fahn: 1C, 3B

Cheryl Waters: 1C, 3B

Sheng-Han Kuo: 1B, 3B

Wendy K. Chung: 2C, 3B

Blair Ford: 1C, 3B

Karen S. Marder: 1C, 3B

Un Jung Kang: 1C, 3B

Sharon Hassin-Baer: 1B, 3B

Lior Greenbaum: 1B, 3B

Jean-Francois Trempe: 1B, 1C, 3B

Pavlina Wolf: 1B, 1C, 3A, 3B

Petra Oliva: 1A, 1B, 3B

Xiaokui Kate Zhang: 1A, 1B, 3B

Lorraine N. Clark: 3B

Melanie Langlois: 1C, 3B

Patrick A. Dion: 1B, 3B

Edward A. Fon: 1A, 3B

Nicolas Dupre: 1A, 1B, 3B

Guy A. Rouleau: 1B, 3B

Ziv Gan-Or: 1A, 1B, 2A, 2B, 2C, 3A, 3B

*These authors contributed equally to the work

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Francois Trempe, PhD¹⁶, Pavlina Wolf, BSc¹⁷, Petra Oliva, PhD¹⁷, Xiaokui Kate Zhang, PhD¹⁷, Lorraine N. Clark, PhD^{2,18,19}, Melanie Langlois, MD^{20,21}, Patrick A. Dion, PhD^{3,7}, Edward A. Fon, MD⁴, Nicolas Dupre, MD^{20,21}, Guy A. Rouleau, MD PhD^{3,7,8}, and Ziv Gan-Or, MD PhD^{3,7,8}

¹Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, NY, USA.

²Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Columbia University, New York, NY, USA.

³Montreal Neurological Institute, McGill University, Montréal, QC, Canada.

⁴McGill Parkinson Program and Neurodegenerative Diseases Group, Montreal Neurological Institute, McGill University, Montréal, QC, Canada.

⁵Sleep Unit, National Reference Network for Narcolepsy, Department of Neurology Hôpital-Gui-de Chauliac, CHU Montpellier, INSERM U1061, France.

⁶Imperial College School of Medicine, Imperial College London, London, United Kingdom.

⁷Department of Neurology and Neurosurgery, McGill University, Montréal, QC, Canada.

⁸Department of Human Genetics, McGill University, Montréal, QC, Canada.

⁹Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA.

¹⁰Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, NY, USA.

¹¹Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

¹²Department of Neurology, Sheba Medical Center, Tel Hashomer, Israel.

¹³Movement Disorders Institute, Sheba Medical Center, Tel Hashomer, Israel.

¹⁴The Danek Gertner Institute of Human Genetics, Sheba Medical Center, Tel Hashomer, Israel.

¹⁵The Joseph Sagol Neuroscience Center, Sheba Medical Center, Tel Hashomer, Israel.

¹⁶Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada.

¹⁷Translational Science, Sanofi, Framingham, MA, USA.

¹⁸Laboratory of Personalized Genomic Medicine, Department of Pathology and Cell Biology, Columbia University, New York, NY, USA.

¹⁹Department of Pathology and Cell Biology, Columbia University, New York, NY, USA.

²⁰Axe neurosciences du CHU de Québec - Université Laval, Québec, QC, Canada.

²¹Faculty of Medicine, Department of Medicine, Laval University, Québec, QC, Canada.

Abstract

Background: *SMPDI* (acid-sphingomyelinase, ASMase) variants have been associated with Parkinson's disease (PD) in recent studies.

Objective: To further study the role of *SMPD1* mutations in PD.

Methods: *SMPD1* was sequenced in three cohorts (Israel Ashkenazi-Jewish cohort, Montreal/Montpellier and New York), including 1,592 PD patients and 975 controls. Additional data was available for 10,709 Ashkenazi-Jewish controls. ASMase activity was measured by a mass-spectrometry-based assay in the New York cohort. α -Synuclein levels were measured *in-vitro* following CRISPR/Cas9-mediated knock out and siRNA knockdown of *SMPD1* in HeLa and BE(2)-M17 cells. Lysosomal localization of ASMase with different mutations was studied, and *in-silico* analysis of their effect on ASMase structure was performed.

Results: *SMPD1* mutations were associated with PD in the Ashkenazi-Jewish cohort, as 1.4% of PD patients carried the p.L302P or p.fsP330 mutation, compared to 0.37% in 10,709 Ashkenazi-Jewish controls (OR=3.7, 95%CI=1.6–8.2, $p=0.0025$). In the Montreal/Montpellier cohort, the p.A487V variant was nominally associated with PD (1.5% versus 0.14%, $p=0.0065$, not significant after correction for multiple comparisons). Among PD patients, reduced ASMase activity was associated with a 3.5–5.8 years earlier onset of PD in the lowest quartile versus the highest quartile of ASMase activity ($p=0.01$ – 0.001). We further demonstrated that *SMPD1* knockout and knockdown resulted in increased α -synuclein levels in HeLa and BE(2)-M17 dopaminergic cells, and that the p.L302P and p.fsP330 mutations impair the traffic of ASMase to the lysosome.

Conclusions: Our results support an association between *SMPD1* variants, ASMase activity and PD. Furthermore, they suggest that reduced ASMase activity may lead to α -synuclein accumulation.

Keywords

Parkinson's disease; *SMPD1*; acid sphingomyelinase; Genetics; α -synuclein

Introduction

Accumulating genetic and biological data suggest an important role for the lysosome and the sphingolipids metabolic pathway in the pathogenesis of Parkinson's disease (PD).¹ The autophagy-lysosomal pathway is responsible for the degradation of α -synuclein, and dysfunction of this pathway may lead to α -synuclein accumulation and to the development of PD.^{1, 2} Variants in the *GBA* gene, encoding the lysosomal enzyme glucocerebrosidase (GCase), are among the most common risk factors for PD, found in 3–20% of PD patients from different populations.^{3–9} Recently, mutations in another lysosomal gene involved in sphingolipid metabolism, *SMPD1*, which encodes the lysosomal enzyme acid sphingomyelinase (ASMase), has also been associated with an increased risk for PD.^{10–17} In the Ashkenazi-Jewish population, specific Niemann-Pick type A (NPA)-causing *SMPD1* mutations, p.L302P (also called p.L304P) and p.fsP330 (also called p.F333Sfs*52 or c.996delC), were associated with PD in two independent studies.^{11, 15} Additionally, two studies in Chinese populations and two studies in European populations identified additional *SMPD1* mutations and variants associated with PD.^{12, 13, 16, 17}

Both GCase and ASMase hydrolyze sphingolipids in the lysosome and generate a common product, ceramide, suggesting that they may lead to PD in a similar manner.¹ ASMase is responsible for the hydrolysis of sphingomyelin into ceramide and phosphorylcholine, and

biallelic mutations in *SMPD1* lead to NPA or Niemann-Pick type B (NPB),¹⁸ the acute neurovisceral and chronic visceral forms, respectively, of ASMase deficiency. NPA is a severe and rapidly progressive disease of infancy, characterized by hepatosplenomegaly, failure to thrive, psychomotor regression, interstitial lung disease and death by early childhood. NPB has a variable age of onset with a slower disease course, and patients can survive into adulthood.^{1, 19}

In the current study we examined the effects of *SMPD1* mutations on PD risk and onset in two case-control cohorts. We further studied the effect of *SMPD1* mutations on ASMase enzymatic activity, and the association of this activity with risk and clinical characteristics of PD. In addition, we examined whether *SMPD1* deficiency affects α -synuclein accumulation in cellular models, and how specific *SMPD1* mutations affect the lysosomal localization of ASMase. Lastly, we performed an *in-silico* analysis of different *SMPD1* mutations and their effect on ASMase structure.

Materials and Methods

Full version of the materials and methods can be found in the Supplementary Material online.

Study populations

Basic demographic characteristics of the three cohorts that were analyzed in the current study are described in Supplementary Table 1 and the Supplementary Materials. The study population included three cohorts: a) a cohort of 517 unrelated Ashkenazi Jewish (AJ) PD patients recruited at the Sheba Medical Center (termed “Sheba cohort” hereafter), Israel, who were compared to publicly available data including 10,709 AJ controls, b) a cohort of 525 PD patients and 691 controls of French-Canadian/French origin, all unrelated, collected at the Montreal Neurological Institute (“MTL-F cohort”), Montreal, Canada, and c) a cohort of 550 patients and 284 controls, all unrelated, recruited at Columbia University, New York (NY), USA (“NY cohort”). The AJ PD patient population from the Sheba cohort is independent, with no overlap with previous AJ populations in which *SMPD1* was genotyped.^{11, 15} The Montreal and French cohort (termed hereafter MTL-F cohort) was composed of French-Canadian PD patients and controls who were recruited in Quebec, Canada (n=1027), and French PD patients and controls who were recruited in Montpellier, France (n=189). All French-Canadian and French patients and controls underwent genotyping using a genome-wide association study genotyping array (unpublished data, using the OmniExpress array + NeuroX, Illumina), and principal component analysis was performed. Only patients and controls that segregated together with French ancestry were included in the current study. Furthermore, identity-by-descent (IBD) was performed to exclude individuals with cryptic relatedness. Detailed description of the recruitment and demographics of the NY cohort was previously published.^{3, 20} All participants signed informed consent forms prior to their enrollment into the study, and the institutional review boards approved the study protocols.

Sequencing and analysis of *SMPD1* variants

In the Sheba cohort, two *SMPD1* mutations, known to cause NPA and previously shown to be associated with PD were genotyped. The *SMPD1* p.L302P (also known as p.L304P) was genotyped using a TaqMan array (assay ID: C____945630_20, ThermoFisher) according to the manufacturer instructions, and was validated by Sanger sequencing. The p.fsP330 mutation (also known as p.F333Sfs*52 or c.996delC) was screened by Sanger sequencing. The Sanger sequencing chromatograms were analyzed using the Genalys 3.3b software. In the NY cohort, sequencing of the entire *SMPD1* gene was performed using Sanger sequencing (primer sequences and reagents used for PCR amplification and sequencing are available upon request). In the MTL-F cohort, targeted next generation sequencing of the entire coding region of *SMPD1* was performed (Supplementary Table 2 and online supplementary methods).

Acid sphingomyelinase enzymatic activity

Acid sphingomyelinase activity was measured at Sanofi laboratories, and the researchers were blinded to the genetic status of the patients and controls. Dried blood spots were obtained as previously described in the NY cohort.^{21, 22} In brief, blood samples were collected in a 10 ml EDTA tube. Seventy-five microliters of blood was “spotted” on each of five circles on filter paper (Whatman 903 protein savor card, St. Louis, MI) and dried at room temperature for at least 4 hours. Absorbent filter paper was then stored in a sealed plastic bag with desiccants and a humidity indicator in a –20°C freezer and later shipped to the laboratories at room temperature. Upon receipt, the samples were stored at –80°C before analysis. The detailed procedure of enzymatic activity measurement can be found in the online Supplementary Materials.

Knockout of *SMPD1* in HeLa cells using CRISPR/Cas9

To generate *SMPD1* knockout HeLa cells, two guide RNAs, sg1 and sg2, were cloned into the pSpCas9(BB)-2A-Puro vector (pX459_v2, Addgene 62988).²³ Target sequences were (cut site indicated by an asterisk, PAM sequence in parentheses): sg1 AGAGCTGCCCCAGGTCC*GGC (CGG); sg2 CAAAGACATCTCGGAG*CCG (GGG). Further details can be found in the online Supplementary Materials.

Cell cultures, siRNAs and cell transfection

HeLa and BE(2)-M17 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained at 37°C and 5% CO₂. HeLa cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 units/ml penicillin/streptomycin. BE(2)-M17 cells were cultured in a medium containing 50% of Eagle’s Minimum Essential Medium (EMEM), 50% of HAM-F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 units/ml penicillin/streptomycin. Details on siRNAs and cell transfection can be found in the online Supplementary Materials.

Immunoblotting and α -synuclein quantification

Cells were lysed using RIPA lysis buffer with a mixture of protease inhibitors. After adding Laemmli buffer with DTT to the samples, they were boiled for 10 min and separated by SDS-PAGE. Then separated samples were transferred to a nitrocellulose membrane. For α -synuclein, the membrane was incubated with phosphate-buffered saline (PBS) as described previously.²⁴ Membranes were blocked and incubated overnight with primary antibody in PBS-T containing 1% milk. Finally, membranes were washed three times with PBST²⁴ and were developed using ECL substrate.

Immunofluorescence and microscopy

HeLa cells cultured on glass coverslips were transfected for 24 h with plasmids encoding Venus-fused ASMase variants. Cells were then rinsed with PBS, fixed for 10 min at room temperature in 4% paraformaldehyde/PBS, rinsed again with PBS, permeabilized and rinsed in PBS before blocking. Cells were then incubated with mouse anti-LAMP2 primary antibody for 1 h at room temperature in blocking solution, rinsed and incubated with anti-mouse Alexa-555 coupled secondary antibody for 1 h at room temperature. Full details can be found in the online Supplementary Materials.

Molecular cloning

The wild-type human SMPD1 coding sequence was ordered as two synthetic double stranded DNA fragments (gBlocks 1 and 2, Integrated DNA Technologies) and assembled using Gibson Assembly into the BamHI site of the mVenus N1 plasmid, allowing the addition of a C-terminal fluorescent Venus tag. Using gBlock 2 as a template, we used PCR-based mutagenesis to generate the modified coding sequences associated with the p.L302P, p.fsP330 or p.A487V variants, and further assembled these DNA fragments together with gBlock 1 into the BamHI site of the mVenus N1 plasmid by Gibson Assembly. Constructs were verified by Sanger sequencing.

In silico structural analysis

The atomic coordinates of mouse SMPD1 bound to a lipid and to a bisphosphonate inhibitor were downloaded from the Protein Data Bank. The steric clashes induced by each mutation were evaluated using the “mutagenesis” toolbox in PyMol v. 1.5.

Statistical analysis

Statistical analysis is detailed in the online Supplementary Materials.

Results

Association of *SMPD1* mutations with Parkinson’s disease

As the three cohorts (Sheba cohort, MTL-F cohort and NY cohort) were of different origins, they were analyzed separately. Table 1 details the frequencies of the p.L302P and p.fsP330 *SMPD1* mutations in the Sheba cohort and in previous studies in AJ populations.^{11, 15} These populations are independent, and patients do not overlap. The frequency of each mutation was compared to the frequency in publicly available control population of 10,709

individuals of AJ origin. A combined analysis for each mutation and for both mutations combined was further performed. In the Sheba cohort alone, the p.L302P mutation was associated with PD, and the p.fsP330 mutation was not, likely due to reduced power. In the combined analysis of the current AJ population together with previously published AJ populations (Table 1, total number of patients of 1,742 AJ PD patients), carriers of the p.L302P mutation had an OR of 9.0 (95% CI 4.2–19.5, $p < 0.0001$), and carriers of the p.fsP330 had an OR of 2.8 (95% CI 1.4–5.3, $p = 0.003$). In the MTL-F cohort, a total of 29 different *SMPD1* variants that affect the coding sequence or alternative splicing were identified (Supplementary Table 3). Among PD patients, 5.3% ($n = 28$) carried a rare *SMPD1* variant compared to 2.9% ($n = 20$) among controls (OR = 1.88, 95% CI 1.05–3.39, $p = 0.037$). After adjustment for age and sex, this association was not significant, which is likely due to the patients with *SMPD1* variants having a trend towards an earlier AAE (Mann-Whitney U test, $p = 0.079$). The association was mainly driven by a single variant, p.A487V, found in eight (1.5%) patients and in one (0.14%) control (OR = 10.68, 95% CI 1.3–85.6, $p = 0.0065$, not significant after correction for multiple comparisons). In the NY cohort (Table 2), the p.A487V variant was found in three patients (0.55%) and in one control (0.35%, $p = 1.0$). Combining the two cohorts, the p.A487V was found in 11 (1.0%) patients and 2 (0.2%) controls (OR = 5.03, 95% CI 1.11–22.75, $p = 0.024$). None of the *SMPD1* p.A487V mutation carriers was a carrier of *GBA* or the *LRRK2* G2019S mutations. The frequency of rare *SMPD1* variants in the NY cohort was not significantly different between patients and controls, 3.8% versus 5.3%, $p = 0.34$. We further compared the frequency of rare variants that had ASMase reduced activity in patients and controls. Although these variants (p.R291H, p.P331A, p.R378H, p.A487V, p.G492S, p.R498L, p.E517V, p.G530A, c.1829delCGG, p.R610H) were more frequent in patients ($n = 12/550$, 2.2%) than in controls ($n = 4/285$, 1.4%), these differences did not reach statistical significance. Table 2 details the mutations identified in the NY cohort and their enzymatic activity. Interestingly, the p.V114M variant that did not have reduced ASMase activity in our study, has been previously identified in Niemann-Pick patients.¹⁸ This variant was identified in three patients from the MTL-F and NY cohorts, but no carriers were found in the control populations.

ASMase enzymatic activity is associated with specific *SMPD1* variants and the age at onset of PD

Among controls, there was no association between age and sex and ASMase activity ($p = 0.11$ for age, $p = 0.46$ for sex). There was no difference between the average enzymatic activity in patients and controls ($4.64 \mu\text{mol/l/h} \pm 1.68 \mu\text{mol/l/h}$ versus $4.62 \mu\text{mol/l/h} \pm 1.64 \mu\text{mol/l/h}$, respectively, $p = 0.84$). ASMase enzymatic activity was not different between *GBA* variant/mutation carriers and non-carriers ($4.67 \mu\text{mol/l/h} \pm 1.78 \mu\text{mol/l/h}$ versus $4.59 \mu\text{mol/l/h} \pm 1.65 \mu\text{mol/l/h}$, respectively, $p = 0.69$). Interestingly, it was previously reported that GCase activity is increased in PD patients who carry the *LRRK2* p.G2019S mutation.³ Therefore, we examined the ASMase activity in carriers of *LRRK2* mutations. Among PD patients, carriers of the *LRRK2* p.G2019S mutation ($n = 36$) had an average ASMase activity of $5.20 \mu\text{mol/l/h} \pm 1.84$ compared to $4.59 \mu\text{mol/l/h} \pm 1.65$ among non-carriers ($n = 426$, $p = 0.033$, t-test). When including controls in this analysis, the results remained similar, $5.23 \mu\text{mol/l/h} \pm 1.79$ compared to $4.61 \mu\text{mol/l/h} \pm 1.64$, respectively ($n = 38$ and $n = 685$, respectively, $p = 0.025$, t-test).

Among PD patients without *GBA* or *LRRK2* p.G2019S mutations, reduced ASMase enzymatic activity was associated with an earlier AAO (B=1.419, β =0.194, 95% CI for B 0.80–2.04, p <0.001, i.e., for each 1 μ mol/h reduction age at onset was younger by 1.4 years). Further adjustment to GCase activity yielded similar results (B=1.470, β =0.203, 95% CI for B 0.83–2.11, p <0.001). We further divided the cohort to four groups, based on the ASMase enzymatic activity quartiles in controls (Table 3). First, the entire cohort was analyzed, followed by analysis of late-onset PD only (AAO \geq 50 years), and further exclusion of carriers of *GBA* and *LRRK2* p.G2019S mutations that can also affect the AAO. In all analyses, there was a decrease in AAO with the decrease of enzymatic activity, with significant differences of 3.5–5.8 years between the first and last quartiles in the different analyses (Table 3, p 0.01 for all comparisons). Of note, when dividing the patients group into quartiles based on the ASMase activity values in patients only, or patients and controls combined, nearly identical and statistically significant results were obtained. Other demographics and disease characteristics were not different among the quartiles (Supplementary Table 4) among PD patients with late-onset PD who do not carry *GBA* or *LRRK2* mutations. Similar results were obtained when early-onset PD and *GBA* and *LRRK2* mutation carriers were included.

Decreased *SMPD1* expression leads to accumulation of α -synuclein in cellular models

To further investigate the link between ASMase function and PD pathogenesis, a CRISPR/Cas9-mediated knockout of *SMPD1* was performed in HeLa cells. Twelve knockout clones were obtained and compared to wild-type cells. Western blot analysis demonstrated almost complete loss of ASMase expression in all clones, and this was correlated in ten out of twelve clones with increased α -synuclein levels (Figure 1A). Precise quantification in two clones (A6 and A8) done in triplicates showed an 8–10-fold increase (Figure 1B). Quantification of all 12 single clones can be seen in Supplementary Figure 1, and 10 out of the 12 clones demonstrated increase levels of α -synuclein. These results were further confirmed using RNA interference against *SMPD1* in HeLa cells and in the dopaminergic cell line M17 (Supplementary Figure 2). Regardless of the siRNA used, ASMase expression was reduced and this led to an average increase of about 3-fold of α -synuclein levels.

ASMase lysosomal localization is affected by specific *SMPD1* variants

To investigate how *SMPD1* variants could affect ASMase activity, we compared the subcellular localization of ASMase with three variants (p.L302P, p.fsP330 or p.A487V) to that of wild-type ASMase. We transfected all four forms of ASMase (fused to a C-terminal Venus tag) into HeLa cells and performed immunocytochemistry against the lysosomal marker LAMP2 (Figure 2). As expected for a protein that travels through the secretory pathway, wild-type ASMase was mainly detected in a perinuclear network reminiscent of the endoplasmic reticulum (ER). In addition, some puncta were observed closer to the periphery, which colocalized with LAMP2 suggesting that our ASMase-Venus fusion was successfully sorted to the lysosome. Strikingly, ASMase with the p.L302P or p.fsP330 mutations failed to reach the lysosomal compartment, and was almost exclusively localized in the ER. The p.A487V variant seemed unaffected.

***In silico* analysis suggests that mutations in *SMPD1* disrupt enzymatic domain fold and lipid-binding site**

To investigate the effect of *SMPD1* variants on the structure and activity of ASMase, we performed *in silico* structural analysis using the recently published structures of mouse ASMase in the closed and open states, bound to a bisphosphonate inhibitor and a lipid, respectively.²⁵ The p.L302P, p.A487V and p.V114M were modelled, the p.fsP330 was not modeled as it causes early termination of the protein. Superposition of the two structures shows that Leu302 and Ala487 are in the structurally invariant catalytic domain of ASMase, whereas Val114 is located in the lipid-binding domain that undergoes a large conformation change (Supplementary Figure 3A). A proline at position 302 (position 304 in the full transcript) would be incompatible with the helical conformation of Leu304, and thus the mutation L302P would likely unfold the catalytic domain. The mutation A487V would introduce a steric clash in the hydrophobic core of the catalytic domain and would likewise destabilize the domain (Supplementary Figure 3B). The V114M mutation would have two potential effects: the side-chain of Val114 is oriented towards the lipid in the open conformation, and the mutation V114M could interfere with lipid-binding via steric hindrance (Supplementary Figure 3C). On the other hand, the V114M mutation would also introduce severe clashes in the closed state and destabilize this conformation (Supplementary Figure 3D).

Discussion

The current study provides support for the association between *SMPD1*, ASMase activity and PD. The results suggest that specific *SMPD1* variants are associated with PD, and that reduced ASMase activity is associated with earlier AAO of PD. Using two cellular models, we further demonstrated that *SMPD1* knockdown resulted in decreased ASMase levels and led to α -synuclein accumulation. Lastly, we demonstrated that some of the *SMPD1* mutations lead to altered lysosomal localization of ASMase, and that *in silico* analysis suggests deleterious effects on ASMase structure and function. These results suggest that while only a small proportion of PD patients have *SMPD1* variants that increase the risk for PD, ASMase activity may have a role in PD. More studies are necessary to replicate our findings and to elucidate the underlying mechanisms responsible for the effects on AAO and α -synuclein accumulation.

Thus far, not including the current study, *SMPD1* variants have been associated with PD or synucleinopathy risk in seven independent cohorts: two of Ashkenazi-Jewish origin;^{11, 15} two Chinese;^{12, 13} two European;^{16, 17} and one North-American.¹⁰ In these studies, as well as in our cohort, only some of the mutations were associated with PD, while other *SMPD1* mutations, including some that can cause Niemann-Pick type A/B, were not associated with PD. The current study independently shows (i.e. using a different population than the two previous AJ populations that were studied) that the two AJ mutations, p.L302P and p.fsP330, are associated with PD, and that both mutations result in lack of lysosomal localization of ASMase. Together with the experiments demonstrating that reduced ASMase levels lead to α -synuclein accumulation, a hypothesis can be made that these mutations

increase the risk for PD by causing reduced lysosomal localization of ASMase and as a result, accumulation of α -synuclein due to yet unknown mechanism.

It is important to note that when analyzing the AJ cohorts (Table 1), we have performed a combined analysis of both mutations, and a separate analysis of each mutation, which is important for determining their individual effects on risk. At the same time, a combined analysis, especially when analyzing rare mutations, may be crucial for understanding whether a specific gene is important in PD or not. For example, when the association of *GBA* with PD was initially described, there were conflicting results due to one study combining all mutations with a positive association and another report on negative association in which the separate effect of only one mutation was examined.^{26, 27} Subsequent studies that included a combined analysis of various *GBA* mutations have revealed that mutations in *GBA* are indeed associated with PD.^{5, 6, 9} Such combined analyses are also useful for analysis of associations with phenotypes or biomarkers,^{28, 29} and currently burden analyses of multiple genes from the same pathways are routinely performed.^{16, 30} Such recent analysis demonstrated that there is a burden of lysosomal storage disease-causing genes in PD, which was mainly driven by *GBA* but also by *SMPD1*,¹⁶ further supporting the current results.

The role of the third mutation implicated here, p.A487V, is still unclear. In the cellular experiments and *in silico* models, while there was lack of effect of p.A487V on ASMase localization, the *in silico* models suggested that it can destabilize the catalytic domain of ASMase. However, it is still possible that the association of this mutation with PD in our cohort is due to chance alone, and further studies are necessary. Interestingly, it is controversial whether the p.A487V variant causes Niemann-Pick A or B.³¹ Similarly, *GBA* variants that do not cause Gaucher disease, such as the p.E326K variant,³² are important risk factors for PD.³³ These observations may suggest that the mechanisms that lead to PD and accumulation of α -synuclein could be somewhat different than those that lead to either Gaucher or Niemann-Pick types A or B disease. The role of the p.V114M mutation, found in three patients and none in controls, is still unclear, and additional studies are required.

We previously reported that among carriers of the *LRRK2* p.G2019S mutation in the NY cohort, the enzymatic activity of GCase was higher than in non-carriers.³ In the current study, we found a similar association between *LRRK2* p.G2019S genotype and ASMase activity. The mechanism of both associations is still unknown. The reason for the increased activity is currently unclear. We previously suggested that it could be due to lysosomal compartment expansion or effects on the retromer and turnover of GCase/ASMase.³ If another, possibly compensatory, mechanism also plays a role remains to be determined.

Our study has several limitations. Although we studied more than 2500 individuals, due to the rarity of these mutations, we may have been underpowered to test the association of rare mutations with PD and even larger studies will be needed to more accurately determine the role of *SMPD1* variants in PD. Another limitation was that the MTL-F and NY cohorts were not matched for sex and that the MTL-F cohort was not matched for age. However, we adjusted for these differences in the statistical analysis. Of note, the younger age of the control population in the MTL-F cohort is more likely to reduce rather than increase the risk

estimates, since it is possible that control carriers of *SMPD1* variants will develop PD in the future. For example, the control individual with the p.G504X stop mutation was only 25 years old at recruitment, and could develop PD in the future. An additional limitation is that the ASMase enzymatic activity was only available for the NY cohort. Therefore, the association between reduced ASMase activity and earlier AAO requires replication in other cohorts. Another limitation is that our cellular models represent complete or almost complete dysfunction of ASMase, which is not the case in heterozygous carriers of *SMPD1* mutations. However, as can be seen in some of the knockdown and knockout models, there is still residual amount of ASMase, and α -synuclein still accumulates.

The major strength of our study is that we tested the association between *SMPD1* and PD in multiple modalities. First, we conducted a genetic correlation. Then, we tested ASMase activity in PD patients and controls, all of whom were sequenced for *SMPD1* and *GBA* and genotyped for the *LRRK2* G2019S mutation. We further used cellular models to test a potential mechanism for the association between PD and *SMPD1* and performed *in silico* structural analysis of specific *SMPD1* variants. In all modalities, we found suggestive evidence for an association. However, it will be important to further study the functional effects of *SMPD1* mutations in additional cohorts and in human-derived neuronal models.

SMPD1, like many other PD-associated genes, is involved in the autophagy lysosomal pathway, and it is possible that PD could be treated by enhancing specific components of this pathway. Our results, together with the previous studies, suggest that *SMPD1* could be one of these components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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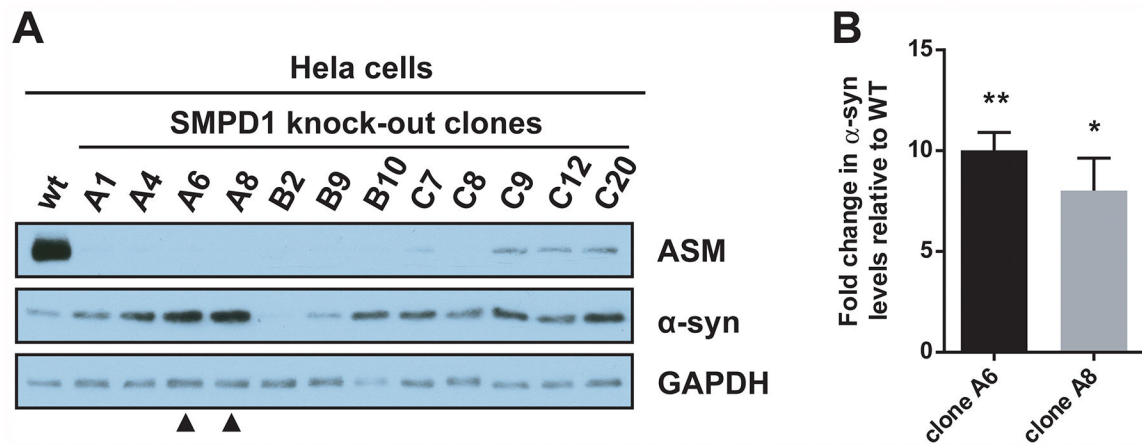


Figure 1. CRISPR/Cas9-mediated knockout of *SMPD1* in HeLa cells leads to α -synuclein accumulation.

(A) Twelve knockout clones were obtained using CRISPR/Cas9, and ASMase and α -synuclein expression were compared with wild-type cells by Western blotting. GAPDH was used as a loading control. Clones A6 and A8 (arrowheads) were further used for quantification. (B) Densitometric quantification of α -synuclein levels in clones A6 and A8 is shown. Data are mean \pm SEM from 3 independent experiments. ** $p < 0.01$, * $p < 0.05$, paired t-test.

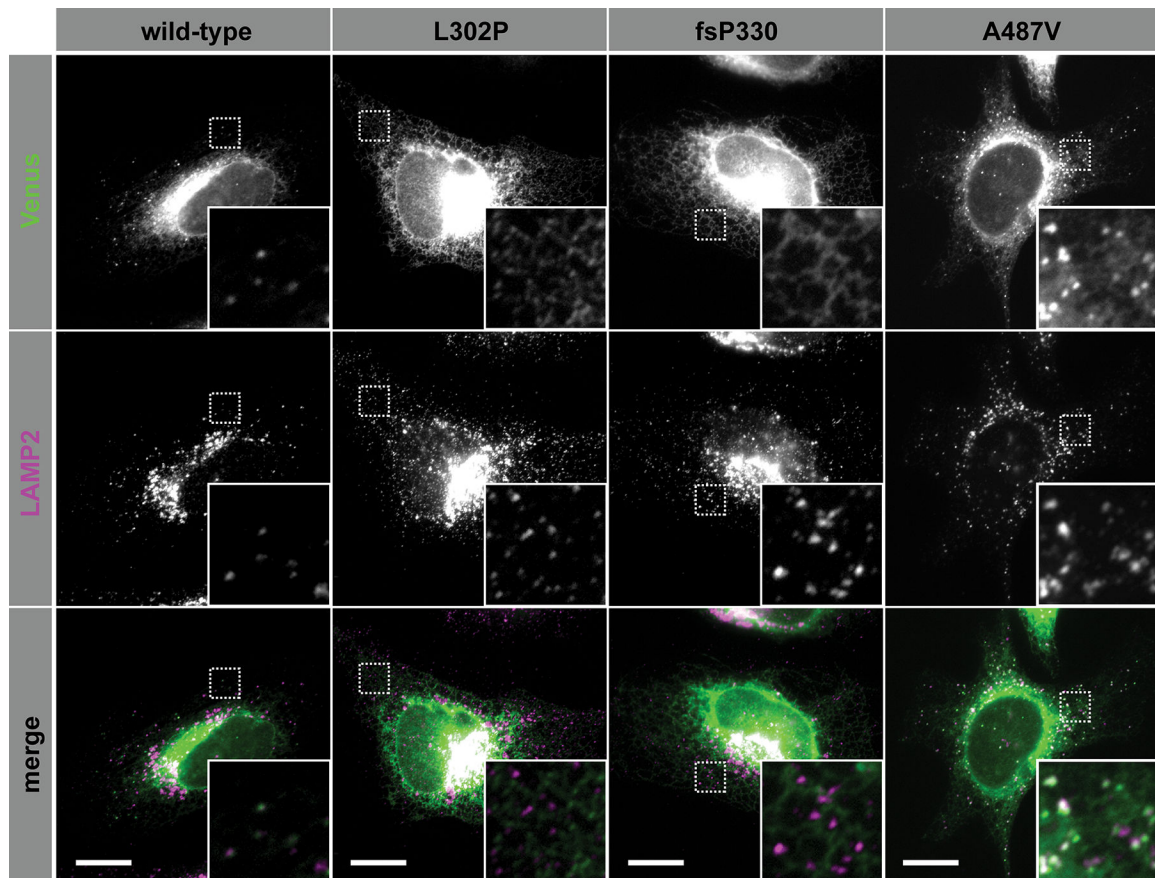


Figure 2. Some *SMPD1* variants are associated with altered lysosomal localization of ASMase. HeLa cells were transfected with plasmids encoding variants of ASMase fused to a C-terminal Venus tag (green), and processed for immunofluorescence using an antibody against the lysosomal marker LAMP2 (magenta) before imaging using an epifluorescence microscope. Scale bar: 10 μ m. The insets correspond to a zoom on the areas delineated by a dashed-square. ASMase with the p.L302P or p.P330fs mutations failed to reach the lysosomal compartment, and was almost exclusively localized in the endoplasmic reticulum. Trafficking of ASMase with the p.A487V variant seemed unaffected.

Table 1.

Association between *SMPD1* mutations in Ashkenazi Jewish PD patient from the current study and previously published studies.

Study	p.L302P		p.fsP330		p.L302P and p.fsP330 combined	
	frequency	p value, OR (95% CI) ^a	frequency	p value, OR (95% CI) ^a	frequency	P value, OR (95% CI) ^a
Current study Sheba cohort PD patients	4/517 (0.8%)	0.0005, 7.6 (2.4–23.9)	3/517 (0.6%)	0.39, 2.1 (0.7–7.1)	7/517 (1.4%)	0.0025, 3.7 (1.6–8.2)
Dagan 2015 PD patients	3/287 (1.0%)	0.0003, 10.3 (2.9–37.0)	5/287 (1.7%)	<0.0001, 6.5 (2.5–17.0)	8/287 (2.8%)	<0.0001, 7.6 (3.5–16.5)
Gan-Or 2013 PD patients	9/938 (1.0%)	<0.0001, 9.4 (3.9–22.8)	5/938 (0.5%)	0.3, 2.0 (0.8–5.1)	14/938 (1.5%)	<0.0001, 4.0 (2.2–7.5)
Combined PD patients	16/1742 (0.9%)	<0.0001, 9.0 (4.2–19.5)	13/1742 (0.7)	0.003, 2.8 (1.4–5.3)	29/1742 (1.7%)	<0.0001, 4.5 (2.8–7.3)
Controls	11/10709 (0.1%)	-	29/10709 (0.3%)	-	40/10709 (0.4%)	-

The table presents the frequencies of the p.L302P and p.fsP330 mutations in the current and previous studies, all in Ashkenazi Jewish PD patients, and compares them to the frequencies in publicly available data from 10,709 controls. OR, odds ratio; CI, confidence interval; PD, Parkinson's disease

^aCompared to the control population at the bottom row.

Table 2.

SMPDI variants and their associated ASMase enzymatic activity in PD patients and controls in the NY cohort

<i>SMPDI</i> Variant	Pathogenicity ^a	Patients (n=550)	Controls (n=284)	ASMase activity ^b (combined PD and controls)	<i>p</i> value, comparing ASMase activity in carriers vs. non-carriers ^c
Rare variants					
p.Q19R	Unknown	4 (0.7%)	2 (0.7%)	6.31 ± 2.67	0.13
p.V114M	Probably pathogenic	2 (0.4%)	0 (0%)	5.04 ± 1.45	0.59
p.P187S	Unknown	1 (0.2%)	3 (1.1%)	5.70 ± 1.43	0.13
p.G269S	Unknown	0 (0%)	1 (0.4%)	4.59	NA
p.R291H	Probably pathogenic	1 (0.2%)	0 (0%)	3.45	NA
p.P331A	Unknown	1 (0.2%)	0 (0%)	2.76	NA
p.R378H	Probably pathogenic	0 (0%)	1 (0.4%)	2.22	NA
p.R389C	Unknown	1 (0.2%)	1 (0.4%)	7.51 ± 3.03	0.08
p.W393G	Probably pathogenic	3 (0.5%)	3 (1.1%)	5.14 ± 1.77	0.54
p.A487V	Unknown	3 (0.5%)	1 (0.4%)	2.85 ± 0.59	0.01
p.G492S	Probably pathogenic	1 (0.2%)	0 (0%)	2.16	NA
p.R498L	Probably pathogenic	2 (0.4%)	0 (0%)	3.32 ± 1.20	0.20
p.E517V	Probably pathogenic	1 (0.2%)	2 (0.7%)	2.38 ± 0.47	0.007
p.G530A	Unknown	1 (0.2%)	0 (0%)	3.00	NA
c.1829delCGG	Unknown	1 (0.2%)	0 (0%)	2.14	NA
p.M613I	Unknown	0 (0%)	1 (0.4%)	7.61	NA
p.R610H	Unknown	1 (0.2%)	0 (0%)	2.14	NA
Common variants					
p.G508R	Not pathogenic	G/G 345 (62.7%)	G/G 196 (69.0%)	4.96 ± 1.73	
		G/R 186 (33.8%)	G/R 80 (28.2%)	4.09 ± 1.37	
		R/R 19 (3.5%)	R/R 8 (2.8%)	3.56 ± 1.18	<i>p</i> <0.001 ^c
Non-carriers^d		529 (96.2%)	269 (94.7%)	4.64 ± 1.64	

ASMase, acid sphingomyelinase

^a A pathogenic variant that may lead to Neimann-Pick type A or B, based on a recently published database of pathogenic mutations.¹⁸

^b ASMase enzymatic activity is measured in μmol/l/h units.

^c Both ANOVA and the non-parametric Kruskal-Wallis tests were performed; in both *p*<0.001.

^d Non-carriers of rare *SMPDI* mutations, not including the p.G508R variant.

Table 3.ASMase activity quartiles^a and the age at onset of PD.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	<i>p</i> value ^b
ASMase activity range (μmol/l/h)	<3.485	3.485–4.46	4.46–5.64	>5.64	
All patients					
N ^c	134	148	146	120	
Average AAO ± SD	58.6 ± 10.3	58.2 ± 12.2	58.2 ± 12.4	62.2 ± 11.0	<i>p</i> =0.01
All patients with late-onset PD (AAO ≥ 50)					
N	115	117	111	104	
Average AAO ± SD	61.5 ± 7.2	63.0 ± 7.5	63.5 ± 8.3	65.0 ± 8.8	<i>p</i> =0.01
All patients excluding <i>GBA</i> and <i>LRRK2</i> mutation carriers					
N	109	119	113	89	
Average AAO ± SD	57.4 ± 10.4	58.2 ± 12.5	58.1 ± 12.9	63.2 ± 11.2	<i>p</i> =0.003
All patients with late-onset PD, excluding <i>GBA</i> and <i>LRRK2</i> mutation carriers					
N	91	93	85	80	
Average AAO ± SD	60.7 ± 7.1	63.4 ± 7.6	64.0 ± 8.2	65.5 ± 9.1	<i>p</i> =0.001

ASMase, acid sphingomyelinase; N, number; AAO, age at onset; SD, standard deviation.

^aQuartiles were determined according to ASMase activity in controls, however nearly identical results were calculated when the quartiles were determined according to patients only, or patients and controls combined.^bAnalysis of variance (ANOVA).^cData on AAO was not available for one patient.