Mutation Update

SMPD1 Mutation Update: Database and comprehensive analysis of published and novel variants

Stefania Zampieri\(^1\), Mirella Filocamo\(^2\), Annalisa Pianta\(^1\), Susanna Lualdi\(^2\), Laura Gort\(^3\), Maria Jose Coll\(^3\), Richard Sinnott\(^4\), Tarekegn Geberhiwot\(^5\), Bruno Bembi\(^1\), Andrea Dardis\(^1\)

\(^1\)Regional Coordinator centre for Rare Diseases, University Hospital Santa Maria della Misericordia, Udine, Italy; \(^2\)Centro di Diagnostica Genetica e Biochimica delle Malattie Metaboliche, Istituto G. Gaslini, Genova, Italy; \(^3\)Sección Errores Congénitos del Metabolismo-IBC, Serv Bioquímica y Genética Molecular, Hospital Clínico; IDIBAPS; CIBERER, Barcelona, Spain; \(^4\)Department of Computing and Information Systems, University of Melbourne, Melbourne, Australia; \(^5\)University of Birmingham and Queen Elizabeth Hospital, Birmingham, UK.

**Corresponding author:** Andrea Dardis

Regional Coordinator Centre for Rare Diseases,

University Hospital Santa Maria della Misericordia, Udine

P.le Santa Maria della Misericordia 15, 33100, Udine, Italy

Tel +39 0432 554472; FAX: +39 0432 554304

Email: dardis.andrea@aoud.sanita.fvg.it

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.22923.

This article is protected by copyright. All rights reserved.
Grant Sponsor:

This work was supported by the EU grant agreement number 2012 1201. An EU rare diseases registry for Niemann Pick Disease type A, B and C (NPDR).
ABSTRACT

Niemann Pick Types A and B (NPA/B) diseases are autosomal recessive lysosomal storage disorders caused by the deficient activity of acid sphingomyelinase (ASM) due to mutations in the SMPD1 gene. Here, we provide a comprehensive updated review of already reported and newly identified SMPD1 variants. Among them, 185 have been found in NPA/B patients. Disease-causing variants are equally distributed along the SMPD1 gene; most of them are missense (65.4%) or frameshift (19%) mutations. The most frequently reported mutation worldwide is the p.R610del, clearly associated with an attenuated NP disease type B phenotype. The available information about the impact of 52 SMPD1 variants on ASM mRNA and/or enzymatic activity has been collected and whenever possible, phenotype/genotype correlations were established. In addition, we created a locus specific database easily accessible at [http://www.inpdr.org/genes](http://www.inpdr.org/genes) that catalogs the 417 SMPD1 variants reported to date and provides data on their in silico predicted effects on ASM protein function or mRNA splicing.

The information reviewed in this paper, providing new insights into the genotype/phenotype correlation, is extremely valuable to facilitate diagnosis and genetic counseling of families affected by NPA/B.

Key words: SMPD1, acid sphingomyelinase, Niemann Pick, lysosomal storage disorder
BACKGROUND

Niemann Pick Types A (NPA; MIM# 257200) and B (NPB; MIM# 607616) are autosomal recessive lysosomal storage disorders caused by the deficient activity of acid sphingomyelinase (ASM; sphingomyelin phosphodiesterase; E.C.3.1.4.12) due to mutations in the SMPDI gene (MIM# 607608) [da Veiga Pereira et al., 1991]. ASM hydrolyzes sphingomyelin to ceramide and phosphocholine in late endosomes and lysosomes. ASM deficiency results in the accumulation of sphingomyelin in the lysosomes and lipid abnormalities in the cell membranes [Schuchman and Desnick, 2001; Schuchman and Wasserstein, 2015].

Clinically, ASM deficiency has been classified as Niemann Pick Type A (NPA) and Niemann Pick type B (NPB) based on the presence or absence of neurological involvement, respectively. NPA is a severe neurodegenerative disorder leading to death within the first three years of life and is characterized by hepatosplenomegaly and psychomotor retardation. In contrast, NPB is a non-neuronopathic disorder characterized by progressive visceral organ abnormalities, including hepatosplenomegaly, pulmonary insufficiency, cardiovascular disease and survival into adulthood. However, the clinical course of NPB patients is quite heterogeneous and intermediate phenotypes have also been reported [Pavlu-Pereira et al., 2005; Mihaylova et al., 2007]. Indeed, a broad spectrum of neurologic abnormalities have been described in patients affected by NPB including cherry red maculae, mental retardation and cerebellar ataxia [Schuchman and Desnick, 2001; McGovern and Schuchman, 2006].

Even though ASM deficiency is panethnic, NPA occurs more frequently among individuals with Ashkenazi Jewish descent [Schuchman and Miranda, 1997].

The SMPDI gene (GenBank NC_000011.10) spans ~5 kb of chromosome 11 (11p15.1–11p15.4) and consists of six exons [da Veiga Pereira et al., 1991; Quintern et al., 1989; Schuchman et al., 1991, 1992]. The 1896bp open reading frame encodes a 631 amino-acid...
protein which is synthesized in the endoplasmic reticulum (ER) as a catalytically inactive 75kDa pre-pro-polypeptide. After the signal peptide (48 amino acids) cleavage, the precursor is processed in the ER-Golgi complex, to a minor non-glycosylated and rapidly-degraded 57 kDa form, and a major catalytically active 70 kDa mature form localized within the lysosome, which is further processed to a 52 kDa form [Ferlinz et al., 1994].

Human ASM contains five N-glycosylation sites [Ferlinz, et al., 1997] and eight disulfide bonds, corresponding to residues Cys91-Cys167, Cys94-Cys159, Cys122-Cys133, Cys223-Cys228, Cys229-Cys252, Cys387-Cys433, Cys586-Cys590 and Cys596-Cys609, which play an important role in protein folding and stability [Lansmann et al., 2003]. The most carboxy-terminal located cysteine at residue 631 is involved in ASM regulation and activation through dimerization of two ASM molecules [Qiu et al., 2003].

Structurally, four functional domains have been identified: a sphingolipid activator protein (SAP) domain, a proline-rich domain, a phosphoesterase domain and a C-terminal domain. Even though 3D structure is still unknown, a model of human ASM active site has been proposed [Seto et al., 2004; Jones et al., 2008]. The SAP domain, located at the N-terminus, is essential for the interaction with the membrane bound lipid substrate [Ponting, 2004], while the catalytic site of the mature form of ASM resides in the phosphoesterase domain [He et al., 1999]. ASM is a zinc-dependent enzyme which contains several highly conserved zinc-binding motifs. In particular, five residues Asp208, Asp280, Asn320, His427 and His459 are supposed to coordinate the zinc ion binding, and a conserved sequence motif, from Asn383 to Asn391, has been predicted to be involved in substrate binding. The enzymatic reaction is catalyzed by the donation of a proton from His321 to Glu390 and determines the release of the products, ceramide and phosphorylcholine [Seto et al., 2004].
The C-terminal domain has been demonstrated to be important for catalytic activity and subcellular localization of ASM, mostly due to several post-translational modifications [Jenkins et al., 2011; Lee et al., 2007].

To date, more than 170 different disease-causing SMPD1 mutations, including missense, nonsense, deletion, insertion, and splice site mutations, have been reported [see the Human Gene Mutation Database (http://www.hgmd.org)].

The Mutation Database presented here aims to provide a comprehensive inventory of all SMPD1 variations in order to facilitate diagnosis and genetic counseling in patients and families with NPA/B. In addition, since residual ASM activity is not a reliable predictor of disease phenotype, this study also aims to provide insights into the molecular mechanisms underlying the phenotypic heterogeneity of the disease.

**SMPD1 MUTATIONS AND DATABASE**

In order to provide an inventory of reported SMPD1 variants, we have developed a Locus-Specific Database (LSDB) for the SMPD1 gene and a website available at http://www.inpdr.org/genes that supports browsing, searching, and sorting the database. In addition, to maintain the database updated, researchers may submit new sequence variants to a database curator. The novel variants will be included in the database only after checking that the nomenclature follows Human Genome Variation Society (HGVS) recommendations [den Dunnen and Antonarakis, 2000; den Dunnen and Antonarakis, 2001]. We note that the databases uses certificates that might cause warnings depending on the browser that is used. These warnings do not restrict access to the registry, which can be accessed if the user clicks the links that appear related to trusting the certificate.
All **SMPD1** variants located in the coding region and in the first or last 50 nucleotides of each intron have been included.

Variants were collected from (i) international peer-reviewed literature published up to April 2015, (ii) the NCBI SNP database ([http://www.ncbi.nlm.nih.gov/SNP](http://www.ncbi.nlm.nih.gov/SNP), dbSNP build 142), (iii) the Leiden Open Variation Database ([https://grenada.lumc.nl/LOVD2/mendelian_genes/docs/index.php](https://grenada.lumc.nl/LOVD2/mendelian_genes/docs/index.php)), (iv) the NHLBI Exome Sequencing Project ([http://evs.gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/)), and (v) the Human Genome Variation Society ([http://www.hgvs.org/central-mutation-snp-databases](http://www.hgvs.org/central-mutation-snp-databases)). Eight novel alleles identified in NPA/B patients have been included as well.

To evaluate the possible functional effects of non-synonymous variants, a prediction of pathogenicity is given by in silico algorithms Sorting Intolerant From Tolerant (SIFT, [http://sift.jcvi.org](http://sift.jcvi.org), [Kumar et al., 2009]) and Polymorphism Phenotyping (PolyPhen-2 [http://genetics.bwh.harvard.edu/pp2](http://genetics.bwh.harvard.edu/pp2), [Adzhubei et al., 2010]). SIFT considers mutations with scores $\leq 0.05$ as damaging, while PolyPhen-2 considers mutations with scores $\geq 0.957$ or between 0.453 and 0.956 as probably or possibly pathogenic, respectively.

Nucleotide changes in the intronic regions were analyzed using a splice site prediction tool ([http://www.fruitfly.org/seq_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)) which finds putative 5’ and 3’ splice sites based on a neural network based approach. Splicing signal sequences of exon and flanking introns have been analyzed using Splicing Regulation Online Graphical Engine (Sroogle, [http://sroogle.tau.ac.il](http://sroogle.tau.ac.il), [Schwartz 2008]) which is an integrated program to analyze and score the four core splicing signals.

The database comprises 417 variants, including 185 variants identified in NPA/B patients (8 being novel) and 4 amino acid substitutions previously reported as polymorphisms. The database provides information on individual sequence variants including chromosome and exon-
intron location, cDNA change, protein change, in silico predicted effect, functional analysis and the reference of the first description of the variant. When available the SNP rsID number has been included. All variants predicted to cause protein truncation have been annotated as “Probably pathogenic”, while missense and intronic mutations were annotated as “Probably pathogenic” or “Probably benign” based on in vitro functional studies, in silico analyses and, when available, the frequency estimated in the general population. Otherwise, they have been annotated as “Unknown significance”.

This paper will be specifically focused on the analysis of the 185 variants reported in NPA/B patients (Supp. Table S1).

**Mutation nomenclature**

*SMPD1* variants are described according to current HGVS mutation nomenclature guidelines ([http://www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen); [den Dunnen and Antonarakis, 2001]), ascribing the A of the first ATG translational initiation codon as nucleotide +1 (GenBank accession number NM_000543.4). (Please note that predicted a termination codon at the protein level has the HGVS-approved symbol “*” but in the literature they are often indicated with an “X”. In the present report, they can appear as both; e.g. p.W32X and p.W32*.) To be noted that many of the *SMPD1* mutations, listed in this report, have been described considering another cDNA reference sequence. The problem affecting *SMPD1* numbering is that the reference sequence contains in exon 1 a highly polymorphic hexanucleotide sequence, hexanucleotide GCTGGC (p.L37_A38 [3_8]), repeated three to eight times. Hence, *SMPD1* mutations located downstream of the nucleotide 142 (aminoacid residue 48), can be found described in two different ways depending on the considered cDNA reference sequence. For instance, the most frequent NPB mutation c.1828-30delCGC (p.R610del) can be found reported as c.1822-24delCGC (p.R608del). To create a practical tool for diagnostic laboratories in the field, disease causing
variants reported in NPA and NPB patients are listed using both numbering in Supp. Table S1.

**Disease-causing SMPD1 variants reported in NPA and NPB patients**

Figure 1 shows the distribution throughout the whole SMPD1 gene of all variants reported in NPA/B patients. Among them, 121 were missense mutations (65.4%), 35 were frameshift mutations (19%), 13 were nonsense mutations (7%), 5 were in frame deletions (2.7%), 5 were intronic variants (2.7%), 4 were mutant alleles bearing two changes (2.2%), 1 duplication (0.5%) and 1 Indel (0.5%) (Figure 2 and Supp. Table S1). No large deletions or insertions have been reported so far.

Considering the criteria described above, 158 mutations found in NPA/B patients were annotated as “Probably pathogenic”, while 23 and 4 mutations were considered of unknown significance or probably benign, respectively.

**SMPD1** variants have been found worldwide. In NPA/B patients from specific populations, such as Italy [Ricci et al., 2004; Pittis et al., 2004]; Spain [Rodriguez-Pascau et al., 2009]; Turkey [Simonaro et al., 2002; Ayuk et al., 2013]; the Czech Republic and Slovakia [Pavlu-Pereira et al., 2005]; China [Zhang et al., 2013], The Netherlands and Belgium [Hollak et al., 2012], the spectrum of SMPD1 mutations have been described. Even though the frequency and distribution of SMPD1 variants vary among different populations and ethnic groups, the most frequently reported mutation worldwide is a 3-base deletion, firstly described by Levran et al [Levran et al., 1991b]. This mutation leads to the loss of the arginine residue Arg610del (p.R610del) and is associated with attenuated NPB phenotype. Indeed, the reported allele frequencies of this mutation among NPB patients were: 100% in Canary Islands [Fernandez-Burriel et al., 2003], 86.6% in Northern-Africa [Vanier et al., 1993], 61.5% in Spain [Rodriguez-Pascau et al., 2009], 12% in patients of multiethnic origin [Simonaro et al., 2002] and
only 9.4% in Italy [Pittis et al., 2004], where the most frequent mutation associated with NPB was the nonsense mutation p.W32X, representing 18.8% of NPB alleles [Pittis et al., 2004]. However, the p.R610del mutation has not been found in patients from China [Zhang et al., 2013] or Czech Republic [Pavlu-Pereira et al., 2005].

Few mutations are more frequently represented among individuals of a particular ethnic group. Indeed, the most frequent alleles accounting for NPA in Ashkenazi Jewish populations are the frame shift mutation p.F333SfsX52 and the missense mutations p.L304P and p.R498L [Levran et al., 1991a; Levran et al., 1992; Levran et al., 1993]. The p.H423Y represents 75% of NPB alleles in Saudi Arabians [Simonaro et al., 2002]. Mutations p.R3AfsX76 and p.H284SfsX7 are frequent in NPA/B patients from China [Zhang et al., 2013]. A possible common origin for the p.A484E allele, the variant most frequently identified among Spanish NPA patients has been suggested by haplotype analysis [Rodriguez-Pascau et al., 2009]. The missense mutation p.W393G was present in 100% of the alleles associated with the intermediate phenotype in the Gipsy population [Mihaylov et al., 2007]. Even though the p.Q294K mutation, which is associated with an intermediate clinical phenotype, has been found in patients from different populations it is highly frequent in patients from Czech and Slovak heritage [Pavlu-Pereira et al., 2005].

**Polymorphic SMPD1 variants**

According to literature and in vitro studies, 4 amino acid substitutions within the SMPD1 coding sequence have been identified that were previously reported as SNPs. The most frequent SNPs are p.V36A, with a MAF of 0.44, and p.G508R with a MAF of 0.15. Besides these SNPs, a polymorphic region coding for the signal peptide of ASM, characterized by a three- to eight-fold repeat of the hexanucleotide GCTGGC (p.L37_A38[3_8]) [Wan and Schuchman 1995] has been described.
Recently, the p.A487V substitution, previously reported as a disease-causing mutation, has been characterized as a polymorphic variant [Rhein et al., 2013].

**GENOTYPE-PHENOTYPE CORRELATION**

The spectrum of reported NPA/B associated *SMPD1* mutations is extremely heterogeneous. Most mutations have been found in single families and in compound heterozygosity. Therefore, it is quite difficult to correlate the genotype with the phenotype. However, some assumptions can be made based on functional analysis of single mutants and for recurrent mutations found in homozygosity.

Table 1 reports all the mutations for which functional analysis has been performed (52 mutations).

**Missense mutations**

Among the 121 missense mutations already reported in NPA/B patients, 40 have been expressed in vitro and the impact of the aminoacid substitution on the ASM activity has been studied. As shown in Table 1, 12 mutations retained a residual enzymatic activity higher than 5% of wild type. Eleven of them were found in patients with NPB disease, as expected. Only the p.F572L retained a residual activity of 30% of wild type and was found in a severe NPA patient in compound heterozygosity with the severe p.G247S mutation [Toth et al., 2011]. However, it was shown that the p.F572L mutation also caused a dramatic reduction of the protein half-life suggesting that the presence of an ASM mutated protein partially inactive and more rapidly degraded may account for the severe phenotype observed in this patient [Toth et al., 2011].

Twenty eight mutations, lead to the synthesis of completely inactive proteins or proteins that retain a very low residual activity (less than 5% of wild type). Most of them, including those
affecting residues that coordinate the zinc ion binding (p.D280A and p.H427R), and a residue located within the substrate binding domain (p.N385S), were found in NPA patients (either in homozygosity or as compound heterozygosity) or in NPB patients in association with mutations that retain a quite high residual activity. Particularly, both p.L304P and p.R498L are frequent Ashkenazi Jewish mutations and have been clearly associated to the severe NPA phenotype in this population [Levran et al., 1992 and Levran et al., 1991a]. However, 12 mutations (p.R230C, p.W246C, p.D253H, p.D253E, p.A283T, p.Q294K, p.L343P, p.A359D, p.M384I, p.H423Y, p.H577R and p.R602P) cause a severe impairment of ASM activity and were found in homozygosity or in association with another severe mutation in NPB patients [Simonaro et al, 2002, Pittis et al., 2004; Pavlu-Pereira et al., 2005; Desnick et al., 2010, Hollak et al., 2012, Acuna et al., 2015]. It is worth noting that 8 of them (p.R230C, p.D253H, p.D253E, p.Q294K, p.L343P, p.M384I, p.H423Y, p.H577R) were found in NPB patients with an intermediate clinical phenotype [Simonaro et al, 2002, Pittis et al., 2004; Pavlu-Pereira et al., 2005; Desnick et al., 2010, Hollak et al., 2012]. This group of mutations includes the p.M384I substitution, which is located within the predicted substrate binding region, very close to the p.N385S. Although in vitro, both mutant proteins are inactive, the p.N385S mutation seems to be associated with a more severe phenotype. This finding might be explained by the nature of the aminoacid changes, since the substitution of asparagine for serine introduces a negative charge within this protein region, whereas the charge is conserved in the p.M384I mutant. Indeed, while the p.N385S was predicted to be pathogenic and damaging by PolyPhen and SIFT programs, respectively, the p.M384I mutation was predicted to be pathogenic by PolyPhen but was tolerated by SIFT.
Particularly significant is the occurrence of the p.Q294K mutation, both in homozygosity and in compound heterozygosity, with the intermediate NPB phenotype [Pavlu-Pereira et al., 2005].

**Nonsense and frameshift mutations**

Mutations which create a premature stop codon, such as nonsense and small deletions or insertions that cause a shift of the open reading frame, would lead to the generation of mRNA species rapidly eliminated by nonsense mediated decay (NMD) or to the synthesis of truncated and likely non functional proteins. Therefore, they were all considered pathogenic.

Five frameshift and 3 nonsense mutations have been expressed in vitro (Table 1). Four frameshift mutations (p.G34AfsX43, p.L180AfsX12, p.S192fsX1, p.A195SfsX14) did not express ASM immunoreactive protein and consequently no enzymatic activity, while the p.V559IfsX19 mutant expressed a completely inactive truncated protein [Dardis et al., 2005]. Even though reported in few families, these mutations may be considered as severe alterations on the basis of the in vitro results.

Although the frameshift mutations p.S192AfsX65 and p.T544PfsX69 have not been expressed in vitro, their occurrence in homozygosity in NPA patients strongly suggest that both are severely detrimental mutations.

None of the three nonsense mutations studied in vitro (p.W32X, p.E260X and p.L263X) expressed catalitically active ASM protein (Table 1). However, while p.E260X and p.L263X have been found in homozygosity in patients affected by NPA disease [Ida et al., 1996; Takahashi et al., 1992], the p.W32X mutation has been unexpectedly found in homozygosity in a patient presenting the less severe type B phenotype [Pittis et al., 2004]. As this mutation introduces a premature stop codon just before the second in frame ATG, it had been hypothe-
sized that initiation of translation at ATG33 would still produce a fairly functional ASM protein. However, successive in vitro experiments have shown that when the first ATG is present, the second one is not used [Ferlinz et al., 1994; Dardis et al., 2005] and therefore, no active ASM is produced in vitro.

The apparent discrepancies between the in vitro studies and the clinical phenotype associated with the p.W32X mutation, suggest that in vivo, when the first ATG is present but unable to produce a canonical transcript, the second initiation codon (ATG33) may be used resulting in the synthesis of a protein missing the first 32 residues of the predicted signal peptide but still partially active.

A similar hypothesis can be proposed for the p.R3AfsX76 mutation predicted to introduce a very premature stop codon. In fact, even in absence of in vitro evidence, this mutation is quite frequently found among NPB Chinese patients even in homozygous status [Zhang et al., 2013].

**In frame deletions**

Only five in frame deletions have been identified so far. Among them, the p.R610del and p.M1_W32del have been characterized in vitro. [Lee et al., 2007; Pittis et al., 2004; Rodriguez-Pascau et al., 2009; Jones et al., 2008] (Table 1).

The most frequent mutation p.R610del, reported to impair the proteolytical maturation of ASM whilst retaining a high residual ASM activity, has been always identified in patients with the non-neuronopathic NPB phenotype [Lee et al., 2007; Rodriguez-Pascau et al., 2009].

The c.2T>G mutation resulting in the substitution of the first methionine to threonine is predicted to inactivate the first in frame translation start site. However, when this mutant was expressed in vitro, a high residual activity was detected (26.9 % of wild type) [Dardis et al., 2005].

This article is protected by copyright. All rights reserved.
confirming that in vitro, in the absence of the first ATG, the second one (ATG33) can serve as a translation initiation site [Ferlinz et al., 1994] and synthesize a protein lacking the first 32 residues. In agreement with the in vitro data, patients homozygous for this mutation displayed a mild form of the disease [Pittis et al., 2004].

Intronic variants

The possible effect of intronic variants on the splicing process has been evaluated in silico using two different programs, NN splice and Sroogle. All of them were predicted to be pathogenic. The impact of two of them (c.1263+4_1257+7delAGGG and c.1486+5G>C) on the SMPD1 splicing process has been characterized (Table 1).

The analysis of SMPD1 transcripts in cultured fibroblasts from a patient carrying the c.1263+4_1257+7delAGGG mutation precludes the use of the exon 3 splice donor site causing the skipping of exon 3 and leading to the synthesis of two mRNA variants lacking exon 3 sequences. One of them had been previously described as a minor transcript expressed in wild type cells, but unable to code for active enzyme. No normal spliced mRNA was transcribed from this allele, therefore the mutation could be considered as a severe [Rodriguez-Pascau et al, 2009].

The effect of the c.1486+5G>C mutation on the mRNA splicing has also been studied in cultured fibroblasts. The mutation seems to influence the strength of donor splice site in intron 5 causing the synthesis of an aberrant transcript which is probably rapidly degraded since no transcripts from this allele were detected in the patient’s cells [Zhang et al., 2013].

Taken together the data presented here suggest that the patients who carry at least one SMPD1 mutant allele leading to the synthesis of a partially active ASM protein present with NPB phenotype. Instead, patients carrying severe mutations in both alleles may display a wider spectrum of clinical presentations ranging from the classical non neurological NPB

This article is protected by copyright. All rights reserved.
phenotype to the severe and fatal neurological NPA phenotype. Therefore, the occurrence of mutations that completely abolish the ASM activity in both alleles makes prognostic interpretation difficult.

**SMPD1 mutations and Parkinson disease**

Over the last years there has been substantial progress in understanding of the genetics of Parkinson disease (PD). Different variants with incomplete penetrance in some lysosomal genes, including SMPD1, have been reported as strong risk factors for PD with a particular prevalence in some populations. In particular, the variants p.L304P and p.R591C have been found associated with increased risk of PD in a cohort of Ashkenazi Jewish and Chinese PD patients, respectively [Gan-Or et al., 2013; Foo et al., 2013].

**Novel SMPD1 variants**

Eight novel alleles (7 alleles bearing single mutations and one with 2 in cis missense mutations) have been identified in 7 unrelated Italian and 2 Spanish NPA/B patients, as described in Supp. Methods. The encountered genotypes are reported in Table 2. Among the alleles bearing single mutations, 4 were missense mutations, 1 was a frameshift mutation, 1 was an in frame deletion and 1 was a nonsense mutation. In addition, the rare variant p.R610C, previously only reported in [http://evs.gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/) (MAF:T=0.0002/1), has been associated with a NPB phenotype in a patient of the present series in cis with the polymorphism p.A487V (Table 2). The in silico analysis of p.R610C using PolyPhen2 and SIFT algorithms, predicted data which do not concur. Therefore, we have classified the mutation as a variant of unknown significance.

The in silico analysis of novel missense mutations, predicted that mutations p.T258I and p.Q598R may exert a pathogenic effect on protein structure. Inconclusive results were ob-
tained for mutations p.R476Q and p.H577D, since also in this case they were predicted to be tolerated by one program and probably damaging by the other one (Table 3). Looking at the patients’s genotype (Table 2) it is worth noting that mutations p.R476Q, and p.R610C have been found in association with severe mutations in patients NP3 and NP6, and both patients presented a very mild form of NPB disease. These data suggest that mutations p.R476Q, and p.R610C may lead to the synthesis of partially active ASM proteins. The mild effect of these missense mutations on ASM protein may also explain the discordant results obtained by in silico analysis. However, it is important to keep in mind that the p.R610C allele also carried the p.A487V. Thus, a “modifier” effect of the polymorphism on the mutant p.R610C function cannot be excluded. In this case, each single mutation may not lead to a significant impairment of protein structure and/or function. However, both being present in cis in the same protein they may cause an impairment of the ASM structure and/or function resulting in a pathological clinical phenotype.

The p.W86X and the p.I528TfsX50 mutations introduce a premature termination codon, which would lead to a truncated and nonfunctional protein. It is reasonable to hypothesize that these mutations would be severe. Indeed, the p.I528TfsX50 mutation has been found in homozygosity in a patients affected by the severe form of the disease.

The p.F482del results in the loss of amino acid phenylalanine at the position 482. The deletion would probably severely affect the ASM protein structure since it has been found in a NPA patient in homozygosity.

Finally, the allele carrying in cis 2 previously reported mutations p.[R230H;R602H], has been identified in homozygosity in a severe NPA patient. While in vitro studies have shown that the p.R602H mutant protein retains a 10% of activity, the effect of the p.R230H mutation on ASM functions has not been studied yet. Therefore, considering the severity of the clinical
phenotype observed in patient PN7, it can be assume that the p.R230H mutation itself would completely abolish the ASM activity or that the concomitant presence of both missense mutations on the same allele would exert a detrimental cumulative effect on the ASM function, ultimately leading to a non functional protein.

FUTURE PROSPECTIVE AND CONCLUSIONS
To date, no specific treatment has been approved for NPA/B disease. However, different therapeutic approaches have been developed; some of them are currently under preclinical and clinical evaluation.
The ASM knock-out (ASMKO) mouse, which recapitulates the main features of NPA disease, has been used as a relevant animal model for testing potential therapies for ASM deficiency [Horinouchi et al., 1995]. In particular, bone marrow (BM) transplantation has been extensively studied in this animal model [Miranda et al., 1998]. The results of these studies have clearly shown a significant improvement of the systemic disease when transplantation was performed during the newborn period. However, neurological involvement continued to progress and the animals ultimately died due to the neurological phenotype. These data suggest that BM transplantation may be considered only in non neurological NP patients.
In humans, BM or stem cell transplantation has been undertaken in few cases. Although systemic improvement such as liver and spleen size reduction has been observed, the shortage of ideal donors and the risk of severe complications due to graft vs host disease, have limited its general use [Victor et al., 2003; Shah et al., 2005].
Autologous hematopoietic stem cell gene therapy using retroviral vector [Miranda et al., 2000] and directed gene therapy using AAV vectors [Passini et al., 2005] have also been evaluated in ASMKO mice. Once again, these approaches resulted in an improvement of vis-
cerebral symptoms but no significant effects were observed in the central nervous system (CNS). However, remarkable effects on CNS pathology were observed when AAV vectors expressing ASM were delivered directly into the CNS. Although these are interesting results, it is worth noting that the enzyme should be delivered into the CNS before the onset of neurological symptoms and that the safety assessment of intracranial injection of AAV vectors in humans is still under discussion.

Enzyme replacement therapy (ERT) with human recombinant ASM (rhASM) has been developed and tested in the ASMKO mouse model [Miranda et al., 2000]. Studies in this animal model have shown that intravenously administration of rhASM prevents lipid storage and inflammatory processes, particularly in liver, spleen and lung in a dose-dependent manner [Miranda et al., 2000].

Based on these results, the FDA approved the evaluation of ERT in non neurological NPB patients and a phase 1 clinical trial has been completed in 2009. A phase 2/3 trial has been planned to start in autumn 2015.

Considering the potential of this therapeutical strategy, an early and accurate diagnosis becomes crucial to ensure a timely therapeutic intervention. In addition, the continuous analysis of possible correlations between the genotype and the clinical course of the disease is essential to improve clinical management and may influence therapeutic choices.

ACKNOWLEDGMENTS

We would like to thank the members of the Niemann Pick Disease Registry (NPDR) Consortium: Toni Mathieson (Niemann-Pick Disease Group -UK); Jim Green and Miriam Evans (European section of the International Niemann-Pick Disease Alliance (INPDA); Ellen Crushell and Eithna Lofty (Children’s University Hospital, Dublin); Eugen Mengel, Hans
Klünnemann, Lisa Peintinger, Pascal Webber, Miriam Stampfer (Niemann-Pick Selbsthilfegruppe Deutschland, Germany); Martin Hrebíček (First Faculty of Medicine, Czech Republic); Isabel Hontanilla, Enrique Pilar, Mercedes Pineda (Fundación Niemann Pick de España, Spain); Marie T. Vanier (INSERM Unit 820, Lyon, France). Some samples were obtained from the “Cell Line and DNA Biobank from patients affected by Genetic Diseases” (Istituto Giannina Gaslini), member of Telethon Network of Genetic Biobanks (project no. GTB12001).

CONFLICT OF INTEREST

The authors declare no conflict of interest.
REFERENCES


SMPD1 mutations causing types A and B Niemann-Pick disease and generation of mutation-

Kumar P, Henikoff S, Ng PC. 2009. Predicting the effects of coding non-synonymous vari-

SMPD1 gene in a Taiwanese patient with type B Niemann-Pick disease. Ann Hematol
88:695-697.

Lansmann S, Schuette CG, Bartelsen O, Hoernschemeyer J, Linke T, Weisgerber J, Sandhoff

Lee CY, Tamura T, Rabah N, Lee DY, Ruel I, Hafiane A, Iatan I, Nyholt D, Laporte F,
sphingomyelinase is critical for its secretion and enzymatic function. Biochemistry 46
:14969-14978.

mutation in the acid sphingomyelinase gene of Ashkenazi Jewish type A and B patients.

This article is protected by copyright. All rights reserved.
Page 26 of 42


Miranda SR, Erlich S, Friedrich VL Jr, Gatt S, Schuchman EH. 2000. Hematopoietic stem cell gene therapy leads to marked visceral organ improvements and a delayed onset of neuro-
logical abnormalities in the acid sphingomyelinase deficient mouse model of Niemann-Pick disease. Gene Ther 7:1768-1776.


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


FIGURE LEGENDS

**Figure 1.** Schematic illustration and overall distribution of *SMPD1* sequence variants identified in NPA/B patients.

Novel mutations are indicated in bold.

# Different nucleotide changes result (c.748A>C and c.750C>A) in the same amino acid substitution (p.S250R).

^*SMPD1* variants found both in single and complex allele.

°*SMPD1* variants found in complex allele.

**Figure 2.** Frequency of mutant alleles identified in patients with NPA/B disease and classified by mutation type.
### Table 1. Residual activity of SMPD1 mutants expressed in vitro

<table>
<thead>
<tr>
<th>Reference Sequence</th>
<th>Protein change (NP_000534.3)</th>
<th>cell system</th>
<th>Residual activity (% of wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_000543.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Missense</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.314T&gt;C</td>
<td>p.L105P</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.395T&gt;C</td>
<td>p.V132A</td>
<td>COS-1 cells/COS-7 cells</td>
<td>13-33%</td>
</tr>
<tr>
<td>c.557C&gt;T</td>
<td>p.P186L</td>
<td>ASM-deficient fibroblasts</td>
<td>43%</td>
</tr>
<tr>
<td>c.631T&gt;C</td>
<td>p.W211R</td>
<td>293-T cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.680T&gt;C</td>
<td>p.L227P</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.688C&gt;T</td>
<td>p.R230C</td>
<td>COS-7 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.730G&gt;A</td>
<td>p.G244R</td>
<td>COS-1 cells</td>
<td>40%</td>
</tr>
<tr>
<td>c.738G&gt;C</td>
<td>p.W246C</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.740G&gt;A</td>
<td>p.G247D</td>
<td>COS-7 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.741_742delinsGC</td>
<td>p.E248Q</td>
<td>COS-7 cells</td>
<td>30%</td>
</tr>
<tr>
<td>c.757G&gt;C</td>
<td>p.D253H</td>
<td>293-T cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.759C&gt;A</td>
<td>p.D253E</td>
<td>ASM-deficient fibroblasts</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.839A&gt;C</td>
<td>p.D280A</td>
<td>ASM-deficient fibroblasts</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.847G&gt;A</td>
<td>p.A283T</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.880C&gt;A</td>
<td>p.Q294K</td>
<td>ASM-deficient fibroblasts</td>
<td>less than 5%</td>
</tr>
</tbody>
</table>

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.22923.

This article is protected by copyright. All rights reserved.
<p>| SNP         | AIY (| AA)  | Cell Type                    | Assay Allele | Assay Frequency | Assay Reference |
|-------------|-------|-----------------------------|--------------|-----------------|-----------------|
| c.911T&gt;C    | p.L304P | ASM-deficient fibroblasts   | less than 5% | J                |
| c.940G&gt;A    | p.V314M | 293-T cells                 | 21%          | D                |
| c.973C&gt;G    | p.P325A | HeLa cells                   | less than 5% | F                |
| c.1028T&gt;C   | p.L343P | ASM-deficient fibroblasts   | less than 5% | F                |
| c.1076C&gt;A   | p.A359D | COS-7 cells                  | Less than 5% | A                |
| c.1106A&gt;G   | p.Y369C | COS-7 cells                  | less than 5% | F                |
| c.1133G&gt;A   | p.R378H | ASM-deficient fibroblasts   | 41%          | F                |
| c.1152G&gt;A   | p.M384I | COS-1 cells                  | less than 5% | T                |
| c.1154A&gt;G   | p.N385S | COS-1 cells                  | less than 5% | T                |
| c.1177T&gt;G   | p.W393G | COS-1 cells                  | 6-10%        | F                |
| c.1267C&gt;T   | p.H423Y | ASM-deficient fibroblasts   | less than 5% | J                |
| c.1280A&gt;G   | p.H427R | 293-T cells                  | less than 5% | D                |
| c.1343A&gt;G   | p.Y448C | COS-1 cells                  | less than 5% | T                |
| c.1406A&gt;C   | p.Y469S | COS-7 cells                  | less than 5% | F                |
| c.1451C&gt;A   | p.A484E | COS-7 cells                  | less than 5% | F                |
| c.1462A&gt;G   | p.T488A | COS-7 cells                  | less than 5% | F                |
| c.1493G&gt;T   | p.R498L | ASM-deficient fibroblasts   | less than 5% | J                |
| c.1564A&gt;G   | p.N522D | 293-T cells                  | 10%          | D                |
| c.1575G&gt;C   | p.Q525H | 293-T cells                  | 64%          | D                |
| c.1693G&gt;T   | p.D565Y | COS-1 cells                  | 10%          | D                |
| c.1716C&gt;G   | p.F572L | COS-7 cells                  | 30%          | T                |</p>
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Allele</th>
<th>Cell Line</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1730A&gt;G</td>
<td>p.H577R</td>
<td>293-T cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.1735G&gt;A</td>
<td>p.G579S</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.1805G&gt;A</td>
<td>p.R602H</td>
<td>COS-1 cells</td>
<td>13%</td>
</tr>
<tr>
<td>c.1805G&gt;T</td>
<td>p.R602P</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>Frameshift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.100delG</td>
<td>p.G34Afs*43</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.538_539delTT</td>
<td>p.L180Afs*12</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.572dupC</td>
<td>p.S192fs*1</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.581dupC</td>
<td>p.A195Sfs*14</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>c.1675_1676delGT</td>
<td>p.V559fs*19</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>In frame deletions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2T&gt;G</td>
<td>p.M1_W32del</td>
<td>COS-1 cells</td>
<td>27%</td>
</tr>
<tr>
<td>c.1829_1831delGCC</td>
<td>p.R610del</td>
<td>COS-7 cells</td>
<td>21.5%</td>
</tr>
<tr>
<td>Intronic variants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1263+4_1257+7delAGGG</td>
<td>NA</td>
<td>Fibroblasts from a patient</td>
<td>no wt transcript</td>
</tr>
<tr>
<td>c.1486+5G&gt;C</td>
<td>p.0 (r.0)</td>
<td>Peripheral blood</td>
<td>no wt transcript</td>
</tr>
<tr>
<td>Nonsense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.W32X</td>
<td>NA</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>p.E260X</td>
<td>NA</td>
<td>COS-7 cells</td>
<td>less than 5%</td>
</tr>
<tr>
<td>p.L263X</td>
<td>NA</td>
<td>COS-1 cells</td>
<td>less than 5%</td>
</tr>
</tbody>
</table>

This article is protected by copyright. All rights reserved.

Page 38 of 42
Table 2. Genotypes encountered in NPA/B patients analyzed in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Ancestry</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1</td>
<td>c.773C&gt;T (p.T258I)</td>
<td>c.1805G&gt;A (p.R602H)</td>
<td>Italian</td>
</tr>
<tr>
<td>NP2</td>
<td>c.872G&gt;A (p.R291H)</td>
<td>c.1729C&gt;G (p.H577D)</td>
<td>Italian</td>
</tr>
<tr>
<td>NP3</td>
<td>c.1630delA (p.T544Pfs*69)</td>
<td>c.1427G&gt;A (p.R476Q)</td>
<td>Italian</td>
</tr>
<tr>
<td>NP4</td>
<td>c.533T&gt;A (p.I178N)</td>
<td>c.1793A&gt;G (p.Q598R)</td>
<td>Italian</td>
</tr>
<tr>
<td>NP5</td>
<td>c.96G&gt;A (p.W32*)</td>
<td>c.258G&gt;A (p.W86*)</td>
<td>Italian</td>
</tr>
<tr>
<td>NP6</td>
<td>c.573delT (p.S192Afs*65)</td>
<td>c.1828C&gt;T (p.R610C)*</td>
<td>Italian</td>
</tr>
<tr>
<td>NP7</td>
<td>c.[683 G&gt;A; 1799G&gt;A] [p.R230H; R602H]</td>
<td>c.[683 G&gt;A; 1799G&gt;A] [p.R230H; R602H]</td>
<td>Italian</td>
</tr>
<tr>
<td>NP8</td>
<td>c.1583_1584delTA (p.I528Tfs*50)</td>
<td>c.1583_1584delTA (p.I528Tfs*50)</td>
<td>Spanish</td>
</tr>
<tr>
<td>NP9</td>
<td>c.1444_1446delTTC (p.F482del)</td>
<td>c.1444_1446delTTC (p.F482del)</td>
<td>Spanish</td>
</tr>
</tbody>
</table>

New mutations are indicated in bold.

* This mutation, previously reported in the [EVS database](http://evs.gs.washington.edu/EVS/), has been associated for the first time with a NPB phenotype in a patient of the present series in cis with the polymorphism p.A487V.

This article is protected by copyright. All rights reserved.
**Table 3. Novel missense variants associated with NPA/B disease**

<table>
<thead>
<tr>
<th>Reference Sequence</th>
<th>Protein change protein accession number</th>
<th>PolyPhen-2</th>
<th>SIFT</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_000543.4</td>
<td>NP_000534.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.773C&gt;T</td>
<td>p.T258I</td>
<td>probably-damaging (0.999)</td>
<td>Damaging (0.04)</td>
<td>Probably pathogenic</td>
</tr>
<tr>
<td>c.1427G&gt;A</td>
<td>p.R476Q</td>
<td>probably-damaging (0.997)</td>
<td>Tolerated (0.08)</td>
<td>Unknown significance</td>
</tr>
<tr>
<td>c.1729C&gt;G</td>
<td>p.H577D</td>
<td>possibly-damaging (0.940)</td>
<td>Tolerated (0.2)</td>
<td>Unknown significance</td>
</tr>
<tr>
<td>c.1793A&gt;G</td>
<td>p.Q598R</td>
<td>possibly damaging (0.560)</td>
<td>Damaging (0.03)</td>
<td>Probably pathogenic</td>
</tr>
</tbody>
</table>

The predicted effect on protein structure has been evaluated in silico using Polyphen-2 and SIFT programs.

SIFT and PolyPhen-2 scores are indicated in brackets. Predicted disruption in protein function were associated with a SIFT score <=0.05 and with a PolyPhen-2 score >=0.957 (Probably damaging) or between 0.453 and 0.956 (possibly damaging).