



Identification and characterization of SMPD1 mutations causing Niemann-Pick types A and B

Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2008-0527.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	
Complete List of Authors:	Rodríguez-Pascau, Laura; Facultat de Biologia, Universitat de Barcelona, Genètica; CIBER de Enfermedades Raras (CIBERER); IBUB Gort, Laura; Institut de Bioquímica Clínica, Hospital Clínic; CIBER de Enfermedades Raras (CIBERER) Schuchman, Edward; Mount Sinai School of Medicine, Genetics & Genomic Sciences Vilageliu, Lluïsa; Facultat de Biologia, Universitat de Barcelona, Genètica; CIBER de Enfermedades Raras (CIBERER); IBUB Grinberg, Daniel; Facultat de Biologia, Universitat de Barcelona, Genètica; CIBER de Enfermedades Raras (CIBERER); IBUB CHABAS, AMPARO; Institut de Bioquímica Clínica, Hospital Clínic; CIBER de Enfermedades Raras (CIBERER)
Key Words:	Niemann-Pick A/B, Acid sphingomyelinase deficiency, <i>SMPD1</i> mutations, heterologous expression, Spanish patients



Identification and characterization of *SMPD1* mutations causing Niemann-Pick types A and B

Laura Rodríguez-Pascau,^{1,2,3*} Laura Gort,^{4,2*} Edward H. Schuchman,⁵
Lluïsa Vilageliu,^{1,2,3} Daniel Grinberg^{1,2,3#} and Amparo Chabás^{4,2}

¹Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

²CIBER de Enfermedades Raras (CIBERER), Barcelona, Spain

³Institut de Biomedicina de la Universitat de Barcelona (IBUB), Barcelona, Spain

⁴Institut de Bioquímica Clínica, Hospital Clínic, Corporació Sanitària Clínic, Barcelona, Spain

⁵Department of Genetics & Genomic Sciences, Mount Sinai School of Medicine, New York, NY, 10029, USA

#Corresponding author: Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain. Tel.: +34 93 4035716, FAX: +34 93 4034420, email: dgrinberg@ub.edu

*These authors contributed equally to the work

ABSTRACT

Niemann-Pick disease (NPD) types A/B is caused by lysosomal acid sphingomyelinase deficiency and presents with an autosomal recessive pattern of inheritance. These two types of the disease were described according to the presence (type A) or absence (type B) of neurological symptoms, although many intermediate cases also have been reported. We present a molecular analysis of 19 Spanish NPD A/B patients and 2 from Maghreb. Eight of the patients had type A and 13 had type B NPD. All mutant *SMPD1* alleles were identified, including 17 different mutations, 10 of which were novel: c.503G>A (p.W168X), c.939C>A (p.Y313X), c.1100A>G (p.Y367C), c.1400A>C (p.Y467S), c.1445C>A (p.A482E), c.1456A>G (p.T486A), c.1159delC (p.R387VfsX7), c.1169_1171delTCT (p.F390del), c.1257+4_1257+7delAGGG, and c.1774_1776delACT (p.T592del). The only frequent mutations in the 21 NPD patients were c.1823_1825delGCC (p.R608del) (38%) and c.1445C>A (p.A482E) (9%). Genotype-phenotype correlations were established for most of the mutations and, in particular, the p.R608del-type B association was confirmed. This mutation accounts for 61.5% of the mutant alleles in the type B subgroup of patients. Expression studies were performed on six of the mutations found in Spanish patients and two other mutations for comparison. All mutant alleles were confirmed to be disease-causing, due to their low enzyme activity. A mutation affecting a non-canonical donor splice site was analysed at the RNA level. Only aberrant mRNAs, corresponding to previously reported minor *SMPD1* transcripts, which do not code for functional enzymes, were produced by this allele. This study is the first exhaustive mutational analysis of Spanish Niemann-Pick A/B disease patients.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

KEYWORDS: Niemann-Pick A/B, Acid sphingomyelinase deficiency, *SMPD1* mutations, heterologous expression, Spanish patients

For Peer Review

INTRODUCTION

1
2
3
4
5
6
7
8 Niemann-Pick disease (NPD) types A/B is an autosomal recessive
9 sphingolipidosis caused by lysosomal acid sphingomyelinase (ASM, E.C.
10 3.1.4.12) deficiency. Type A (MIM# 257200) is an infantile neurovisceral fatal
11 form characterized by massive hepatosplenomegaly and rapidly progressive
12 neurological deterioration, and type B (MIM# 607616) presents a purely visceral
13 form and survival till adulthood. In addition, some patients are described as
14 presenting an intermediate phenotype that includes neurological involvement,
15 cherry-red maculae and mental retardation (Schuchman and Desnick, 2001).
16 Note that Niemann-Pick type C (MIM# 257220), which is outside the scope of
17 this study, is a different disease caused by mutations in other (*NPC1* or *NPC2*)
18 genes. For this reason, Niemann-Pick types A and B are sometimes referred as
19 acid sphingomyelinase deficiency (ASMD).
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 The acid sphingomyelinase gene (*SMPD1*; MIM*607608, GenBank
37 accession number M81780.1) is composed of 6 exons and is located on
38 chromosome 11p15.1-11p15.4 (Schuchman and Desnick, 2001). Three types of
39 human ASM transcripts have been identified. The type 1 transcript is the major
40 ASM species and encodes a catalytically active enzyme. Type 2 and 3 ASM
41 transcripts result from alternative splicing, most likely due to the presence of a
42 weak donor splice site in intron 3. Type 2 and 3 transcripts do not encode
43 functional enzymes (Schuchman and Desnick, 2001).
44
45
46
47
48
49
50
51
52
53
54

55 The human ASM protein is synthesized as a 75 KDa pre-polypeptide,
56 which is converted into a precursor form of 72 KDa. The precursor is subjected
57 to two different processing events. A minor part is cleaved in the endoplasmic
58
59
60

1
2
3 reticulum-Golgi complex, yielding a 57 KDa form, whilst a major part is
4
5 processed to a 70 KDa mature form (Ferlinz, et al., 1994).
6
7

8 To date, nearly one hundred different mutations have been described
9
10 (see Human Gene Mutation Database: <http://www.hgmd.org/>), but only a few
11
12 have been found with a relatively high frequency in specific populations. This is
13
14 the case, for example, of mutation c.1823_1825delGCC (p.R608del), which was
15
16 first described by Levrán et al. (1991) and is always associated with the type B
17
18 phenotype. This mutation has been found with a low frequency in Europe, for
19
20 instance, 9.4% in Italy (Pittis, et al., 2004). In contrast, it was observed in more
21
22 than 85% of the mutant alleles from Northern-African NPD type B patients
23
24 (Vanier, et al., 1993). Mutation spectra for specific populations have been
25
26 published for only a few countries, such as Italy (Ricci, et al., 2004, Pittis, et al.,
27
28 2004) or the Czech Republic and Slovakia (Pavlu-Pereira, et al., 2005). In some
29
30 cases, expression studies have been performed (Dardis, et al., 2005, Pavlu-
31
32 Pereira, et al., 2005, etc.).
33
34
35
36
37

38 Here we present clinical and molecular findings for 21 unrelated NPD
39
40 A/B patients diagnosed in the last twenty years in Spain. In particular, the
41
42 identification of 17 *SMPD1* mutations, ten of which are novel, is reported. To
43
44 confirm their pathogenicity, eight of the mutant alleles were characterized by *in*
45
46 *vitro* expression in COS-7 cells.
47
48
49
50
51
52
53
54

55 SUBJECT AND METHODS

56
57
58
59
60

Patients

1
2
3 Nineteen of the twenty-one unrelated NPD patients were of Spanish
4 origin. Patients 2 and 17 were from Maghreb. According to the clinical
5 presentation and age of onset, eight patients were classified as type A and
6 thirteen patients as type B. Patients were diagnosed at the Institut de
7 Bioquímica Clínica, Hospital Clínic, Barcelona.
8
9
10
11
12
13
14
15
16

17 **DNA extraction and mutation identification**

18
19 Genomic DNA was extracted from patients' leucocytes or cultured
20 fibroblasts using standard protocols. Each exon and the intron boundaries of
21 the *SMPD1* gene were PCR-amplified using self-designed oligonucleotides
22 (primer sequences are available upon request). PCR products were directly
23 sequenced in the forward and reverse directions using the Big Dye Terminator
24 Cycle Sequencing v3.1 Kit (PE Applied Biosystems, Foster City, CA, USA), in
25 an ABI PRISM 3700 DNA analyzer (PE Applied Biosystems, Foster City, CA,
26 USA), following the manufacturer's instructions.
27
28
29
30
31
32
33
34
35
36
37

38 Gene nucleotides were numbered according to sequence RefSeq
39 NM_000543.3, with +1 as A of the ATG start codon. The ATG codon represents
40 +1 for amino acid numbering, according to the preprotein sequence
41 NP_000534.3.
42
43
44
45
46
47
48
49

50 **cDNA and NMD analyses for mutation c.1257+4_1257+7delIAGGG**

51
52 RNA was isolated from patient and control skin fibroblast cultures using
53 QIAshredder (Qiagen, Hilden, Germany) to homogenize the sample, followed
54 by the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity was verified
55 by 1% agarose gel electrophoresis, and the concentration was determined
56
57
58
59
60

1
2
3 spectrophotometrically at OD 260nm. Single-stranded cDNA was synthesized
4
5 using an oligo-dT primer and the M-MLV reverse transcriptase, RNase H Minus
6
7 (Promega, Madison, WI, USA). The polymerase chain reaction was performed
8
9 using Expand High Fidelity Taq polymerase (Roche, Mannheim, Germany) and
10
11 the following primers: NPB14F1 (5'GGCGAATACAGCAAGTGTGA3') in exon 2
12
13 and NPB14R1 (5'TGAGTGTGGCCAAAGAACTG3') in exon 5, for the non-
14
15 specific PCR; NPB14F2 (5'CTGGCTCTATGAAGCGATGG3') in exon 2 and
16
17 NPB14R2 (5'ATCTGGCATCAGGTGGCGG3') in exon 6, for the specific
18
19 amplification of the c.1257+4_1257+7delAGGG allele. The resulting PCR
20
21 fragments were purified with GFX PCR DNA and Gel Band purification kit
22
23 (Amersham Pharmacia Biotech, Amersham, UK) and sequenced. For
24
25 nonsense-mediated mRNA decay (NMD) studies, fibroblasts from the patient
26
27 and control were cultured in the presence of two different concentrations of
28
29 cycloheximide (500 and 1000 microg/ml) for 6h.
30
31
32
33
34
35
36
37
38

39 **Vector construction**

40
41 The entire coding region of the *SMPD1* cDNA was amplified by PCR in
42
43 two fragments. These two fragments were digested, ligated and cloned in a
44
45 pBluescript vector to obtain the whole cDNA, which was confirmed by direct
46
47 sequencing. Mutagenesis was performed on the *SMPD1*-pBluescript plasmid.
48
49 For protein expression, the mutant and wild-type cDNAs were subcloned into
50
51 the pcDNA3.1 expression vector.
52
53
54
55
56
57

58 **Site-directed mutagenesis**

59
60

1
2
3 All mutations were introduced in the wild-type full length *SMPD1* cDNA
4 cloned in pBluescript by PCR-based site-directed mutagenesis using the
5 QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA)
6 according to the manufacturer's instructions. The oligonucleotides used to
7 generate the mutations are listed in Supp. Table S1. All constructs were
8 resequenced to ensure that no spurious mutation had been introduced by the
9 mutagenesis procedure.
10
11
12
13
14
15
16
17
18

22 **Cell culture and transient transfection**

24
25 COS-7 cells were grown in 100mm tissue culture dishes with Dulbecco's
26 modified Eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10%
27 fetal bovine serum and antibiotics (Gibco, Grand Island, NY). For the
28 transfection, 30×10^5 cells were split in antibiotic-free medium and transfected
29 twenty-four hours later (when cells were at 90% confluence) with 3 microg of
30 wild-type or mutant plasmid, using Lipofectamine™ 2000 (Invitrogen, Carlsbad,
31 CA, USA). As a negative control, an empty pcDNA3.1 vector was transfected.
32 Forty-eight hours after transfection, cells were harvested by scraping and
33 centrifuging. Cellular extracts were washed twice with PBS and stored at -80°C
34 until acid sphingomyelinase analysis was performed. Approximately 10% of the
35 cellular pellets volume was put aside for Western blotting.
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

53 **Enzymatic analysis**

55 Acid sphingomyelinase activity was measured in COS-7 transfected cells
56 using the fluorogenic substrate 6-hexadecanoylamino-4-methylumbelliferyl-
57 phosphorylcholine (Moscerdam Substrates, Rotterdam, the Netherlands).
58
59
60

1
2
3 Protein concentration was determined by the Lowry method. In every
4
5 expression experiment, transfections were performed three times for each
6
7 mutation as well as for the wild-type construct and for an empty vector used as
8
9 a negative control. Two independent experiments were carried out for each
10
11 mutant allele. The residual enzymatic activity of the mutant alleles was
12
13 expressed as a percentage of the mean of the activity of the wild-type construct
14
15 transfected in the same experiment. The activity of the negative control was
16
17 subtracted.
18
19
20
21
22
23

24 **SDS-PAGE and Western blot analysis**

25
26 Protein extracts from transfected COS-7 cells (35 microg of protein/lane)
27
28 were subjected to SDS-PAGE (12.5% polyacrylamide) and electrophoretically
29
30 transferred onto nitrocellulose membranes. Each membrane was blocked with
31
32 5% non-fat milk in PBS, containing 0.2% Tween 20 (PBST). The blotted
33
34 membranes were probed with polyclonal anti-human acid sphingomyelinase
35
36 antibody (He, et al., 1999) for 4 h in 3% non-fat milk in PBS with 0.1% Tween
37
38 and then washed 4 times with PBST and another 4 times with PBS. Anti-rabbit
39
40 IgG antibody (Sigma-Aldrich, UK) was used as a secondary antibody, and the
41
42 immunoreactive bands were detected by incubation of the membrane for 2
43
44 minutes in the following solution: 10 ml of 100mM Tris-HCl pH 9, 50 microl of 45
45
46 mM p-coumaric acid, 50 microl of luminal and 10 microl of 30% H₂O₂. Anti-
47
48 alpha-tubulin monoclonal antibody (Sigma-Aldrich, UK) was used to detect
49
50 alpha-tubulin as loading control.
51
52
53
54
55
56
57
58
59
60

Statistical analysis

1
2
3 The Mann-Whitney U test was used to analyze significant differences in
4
5 enzyme activities between pairs of mutant and/or wild-type enzymes.
6
7
8
9

10 11 12 13 14 15 RESULTS

16 17 18 19 Mutation analyses

20 We analysed samples from 21 unrelated NPD patients (8 type A and 13
21 type B) to identify the molecular defects. We identified seventeen different
22 mutations, ten of which were novel. The genotypes and clinical features of the
23 patients are shown in Supp. Table S2. Four of the novel mutations were
24 missense changes [c.1100A>G (p.Y367C), c.1400A>C (p.Y467S), c.1445C>A
25 (p.A482E) and c.1456A>G (p.T486A)], two were nonsense changes [c.503G>A
26 (p.W168X) and c.939C>A (p.Y313X)], and four were small deletions
27 [c.1159delC (p.R387VfsX7), c.1169_1171delTCT (p.F390del),
28 c.1257+4_1257+7delAGGG, and c.1774_1776delACT (p.T592del)]. Of the new
29 mutations, the most frequent was p.A482E, identified in 9.5% (4/42) of the
30 alleles (Table 1). None of the new missense mutations were found in one
31 hundred control alleles. Of the seven previously reported mutations, only
32 p.R608del had a high frequency: 38% (16/42) of total alleles. All the patients
33 carrying this mutation presented with type B of the disease and p.R608del
34 accounted for 61.5% of type B patient alleles.
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

61 62 63 64 65 66 67 68 69 70 Haplotype analyses

1
2
3 Haplotype analyses were performed to determine whether frequent
4 mutations had a unique origin. We analysed twenty previously reported
5 polymorphisms: five in exon 1, seven in exon 2 and eight in exon 6. Only four of
6 them were polymorphic in our patient series: the hexanucleotide repeat
7 polymorphism c.103CTGG(T/C)(G/T)[4_9] (exon 1) reported by Wan and
8 Schuchman (1995) in the signal peptide, and the SNPs c.107C>T (exon 1),
9 c.1516G>A (exon 6), and c.*45G>A (exon 6, 3'UTR). In our series of patients,
10 five out of the six patients homozygous for the p.R608del mutation were also
11 found to be homozygous for the haplotype [7-C-G-G] (alleles of the four
12 polymorphisms were in the order described above). The only exception was
13 patient 13 who was homozygous for [8-C-G-G]. It should be mentioned that in
14 the original work, Wan and Schuchman did not find the 8-repeat allele for the
15 hexanucleotide polymorphism. Patient 11, homozygous for mutation p.A482E,
16 was also homozygous for the haplotype [6-T-G-G]. The haplotypes of patients 6
17 and 20, with genotype p.[R608del] + [A482E], were consistent with those
18 associated with each mutation alone, although the phases could not be
19 confirmed as samples from the parents of these patients were not available.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45

46 **Expression and enzyme activity of wild-type and mutant acid** 47 **sphingomyelinase in COS-7 cells** 48 49

50 The functional effect on enzymatic activity of some of the *SMPD1*
51 mutations reported in Spanish patients was evaluated by in vitro expression in
52 COS-7 cells. Wild type *SMPD1* cDNA or cDNAs bearing the following
53 mutations: p.V130A (c.389T>C), p.L225P (c.674T>C), p.R228C (c.682C>T),
54 p.Y367C (c.1100A>G), p.Y467S (c.1400A>C), p.A482E (c.1445C>A), p.T486A
55
56
57
58
59
60

1
2
3 (c.1456A>G) and p.R608del (c.1823_1825delGCC) were transiently transfected
4 in COS-7 cells. Mutations p.Y367C, p.Y467S, p.A482E and p.T486A were the
5 four novel missense changes, while p.R228C and p.R608del had been
6 described before. The previously studied p.V130A and p.L225P were
7 expressed for comparative purposes. As a negative control, cells were
8 transfected with an empty pcDNA3.1 vector. The mean of wild-type acid
9 sphingomyelinase activity in COS-7 cells was 291.33 ± 48.94 nmol/h/mg,
10 whereas the average endogenous enzyme activity (COS-7 cells transfected
11 with an empty pcDNA3.1) was 5.29 ± 2.79 nmol/h/mg. The residual enzymatic
12 activities for each protein, given as a percentage of that of the wild-type
13 enzyme, are shown in Figure 1. Alleles bearing mutations p.R228C, p.Y367C,
14 p.Y467S, p.A482E and p.T486A resulted in low levels of activity, ranging from
15 1.81 to 4.75%. The changes p.V130A, p.L225P and p.R608del produced
16 enzymes with 33.26, 11.03 and 21.46% of wild type activity respectively. Table
17 2 shows the statistical analysis of the activity values between all pairs of mutant
18 enzymes with detectable levels of activity and between each of them and the
19 wild-type protein. All changes show residual enzymatic activities that were
20 statistically different from those of the wild-type enzyme. No significant
21 differences were detected for p.Y367C, p.Y467S and p.A482E in all pair-wise
22 comparisons. The p.R228C allele activity differed significantly from all the
23 mutants except for p.T486A.

24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53 To confirm the correct synthesis of recombinant proteins in COS-7 cells,
54 immunoblot analysis of the wild-type and mutant proteins was performed. A
55 unique band of 70 KDa, corresponding to the mature ASM protein, was
56
57
58
59
60

1
2
3 detected in all cases using polyclonal anti-human acid sphingomyelinase
4
5 antibody (Fig. 2).
6
7
8
9

10 **Characterization of the c.1257+4_1257+7delAGGG mutation**

11
12 A novel mutation consisting of a 4-bp deletion in the donor splice site of
13
14 intron 3 was identified in the sample from patient 14. In order to test whether
15
16 this mutation affected the splicing process, RNA from the patient was analysed.
17
18 Since the mutation could probably generate an mRNA with a premature stop
19
20 codon and thus be susceptible to degradation by the nonsense-mediated
21
22 mRNA decay (NMD) mechanism, fibroblasts from the patient were cultured in
23
24 the absence or presence of cycloheximide (CHX), an inhibitor of the NMD
25
26 process. Three transcripts were detected in the patient's sample, the smallest of
27
28 which was only visible after CHX treatment (Fig. 3A). Sequencing of the patient
29
30 cDNA from CHX non-treated and treated fibroblasts showed that the two
31
32 additional transcripts corresponded to the type 2 and 3 ASM transcripts
33
34 described by Schuchman et al. (1991). These authors demonstrated the
35
36 existence of three human ASM transcripts due to alternative splicing. Type 1
37
38 transcript is the major ASM species and is the only one that encodes a
39
40 catalytically active enzyme. Type 2 and 3 ASM transcripts result from alternative
41
42 splicing. Exon 3 is skipped in both of them. In type 2 transcripts, a donor site
43
44 located 40 nucleotide downstream of the canonical exon 2 donor site is used. In
45
46 type 3 transcripts, exon 2 is directly joined to exon 4. Mutation
47
48 c.1257+4_1257+7delAGGG precludes the use of the exon 3 donor site and
49
50 causes exon 3 to be skipped, leading to the formation of type 2 and 3 ASM
51
52 transcripts. To confirm that no functional transcript was produced by the
53
54
55
56
57
58
59
60

1
2
3 c.1257+4_1257+7delAGGG allele, an allele-specific PCR reaction was
4 performed. For this purpose, one of the primers included the region of the
5
6 p.R608del mutation, present in the other allele of patient 14. Thus, only alleles
7
8 that did not bear the R608del mutation would be amplified (the
9
10 c.1257+4_1257+7delAGGG allele of the patient and any wild type allele). As
11
12 shown in Figure 3B, no normal-size transcript was observed for the patient. The
13
14 band present in the CHX lane is actually a double band, which contains the
15
16 amplified products of transcripts 2 and 3, as made evident by the digestion of
17
18 the PCR products with the *HinfI* restriction enzyme. The band patterns for
19
20 patient NPB14 indicate the presence of transcripts 2 and 3, the latter only
21
22 visible after CHX treatment (Fig. 3C).
23
24
25
26
27
28
29
30
31
32
33

DISCUSSION

34
35
36 The mutation analysis of 21 NPD patients from Spain, 8 with the type A
37
38 and 13 with the type B, allowed all of the mutant alleles to be identified.
39
40 Seventeen different mutations were detected, 10 of which had not been
41
42 previously described. Only one mutation (p.R608del) was frequent in this
43
44 patient population (16 alleles, 38%). It is also one of the most frequently
45
46 reported mutations in other populations and it was found to be always
47
48 associated with the type B of the disease (Levrán, et al., 1991). Reported
49
50 frequencies of mutation p.R608del in type B NPD patients were: 9.4% in Italy
51
52 (Pittis, et al., 2004), 12% in 324 patients of multiethnic origin (Simonaro, et al.,
53
54 2002), 86.6% in Northern-Africa (Vanier, et al., 1993) and 100% in four patients
55
56 from the Canary Islands (Fernandez-Burriel, et al., 2003). In our series, it was
57
58
59
60

1
2
3 also only found in type B patients and occurred at a very high frequency
4 (61.5%). Moreover, due to the mild phenotype associated with homozygosity for
5
6
7 this mutation, a number of cases might remain undiagnosed.
8
9

10 The novel mutation p.A482E was the second most frequent change in
11 Spanish NPD patients (9.5%). Our results indicate that this new missense
12 mutation is associated with the severe form of the disease, since patient 11, the
13 only one homozygous, was diagnosed as NPD type A due to the early onset
14 and severity of the clinical presentation.
15
16
17
18
19
20
21

22 Six out of the eight type A patients were homozygous for a different
23 mutation each. Thus, it can be concluded that mutations p.W168X, p.Y313X,
24 p.F390del, p.Y467S and p.T592del (as well as the aforementioned p.A482E),
25 found in homozygosity, are associated with this severe form of the disease. The
26 same correlation can be established for mutations p.G245S, p.Y367C and
27 p.H421R, found in compound heterozygotes. Although the effect of the small-
28 deletion mutations p.F390del and p.T592del was not confirmed by expression
29 studies, the fact that they were both found in homozygous type A patients
30 suggests that residues F390 and T592 are more relevant for the ASM protein
31 that R608, responsible for type B disease.
32
33
34
35
36
37
38
39
40
41
42
43
44

45 In the three type B patients not bearing the p.R608del mutation, the
46 mutations responsible for the mild phenotype seemed to be p.R376H, p.R474W
47 and p.R228C, since either the accompanying allele was null or were found in
48 homozygosity. These three mutations had previously been associated with type
49 B disease (Simonaro, et al., 2002).
50
51
52
53
54
55
56

57 Mutation p.T486A is the only novel missense mutation for which no
58 association can be determined since it was found in a patient who was a
59
60

1
2
3 compound heterozygote with p.R608del, responsible for the B phenotype. Only
4
5 the expression analysis could provide some information (see below).
6
7

8 Haplotype analyses were performed in patients carrying the most
9
10 frequent mutations, p.R608del and p.A482E. This analysis suggests a common
11
12 origin for most of the p.R608del alleles in Spain. Similarly, the results are
13
14 consistent with a possible unique origin for mutation p.A482E.
15
16

17 Regarding the *in vitro* expression study, 6 mutations found in Spanish
18
19 patients, 4 of which are novel, were analysed and a significant reduction in the
20
21 enzyme activity was found for all of them. Thus, all can be considered disease-
22
23 causing mutations. Only mutation p.R608del has relatively high residual activity
24
25 (21.5% of wild-type activity), consistent with the less severe B phenotype with
26
27 which it is associated. The other type B mutation, p.R228C presents a much
28
29 lower activity (4.75%) than that of pR608del. In fact, this result is in agreement
30
31 with other studies that showed a wide spectrum of residual activity of NPB
32
33 associated mutations (ranging from 0 to about 30% of the wild type enzyme)
34
35 when expressed *in vitro*. Mutation p.T486A was found in heterozygosity with
36
37 p.R608del in a type B patient and no genotype-phenotype correlation could be
38
39 established. The fact that its activity (4.15%) was similar to that of p.R228C
40
41 suggests that it could be associated with type B phenotype. Mutations p.Y367C,
42
43 p.Y467C and p.A482E were associated with type A, according to the clinical
44
45 features of the patients (see above). These mutations consistently showed
46
47 negligible residual activity. It should be noted that all mutant proteins, including
48
49 those with very low residual activity, were synthesized at normal levels, as
50
51 shown by Western blot analysis. These *in vitro* results are in agreement with
52
53 what was observed in fibroblasts of NPD A/B patients homozygous for
54
55
56
57
58
59
60

1
2
3 p.R608del and other *SMPD1* mutations, where the amount of mutant protein in
4
5 the patients' fibroblasts was found to be similar to that of the wild type (Jones,
6
7 et al., 2008).
8
9

10 Mutations p.V130A and p.L225P were not found in our series of patients
11
12 but were expressed for comparison. We found 33.3% and 11.0% of wild-type
13
14 activity respectively for these mutations. Dardis et al. (2005) had already
15
16 expressed these mutations, identified in Italian type B patients, and found 13%
17
18 and 0% of activity. The differences between the two studies could be due to the
19
20 fact that Dardis et al. used COS-1 cells, whereas we used COS-7 cells.
21
22 However, it is clear that p.V130A retained some residual activity in both studies.
23
24 Moreover, the levels of activity found in the present work seem consistent with
25
26 the type B phenotype presented by the Italian patients.
27
28
29
30

31 Mutation c.1257+4_1257+7delAGGG lies in the intron 3 donor site, but
32
33 does not affect the canonical GT dinucleotide. Thus, the effect on RNA
34
35 processing should be analysed. *In silico* predictions using the Splice Site Score
36
37 Calculation website (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html)
38
39 showed a score reduction from 8 to 1.7 when the mutation was present,
40
41 indicating a possible drastic effect in the splicing process. To confirm this
42
43 hypothesis, cDNA analyses were performed. The results undoubtedly showed
44
45 that no normal mRNA was produced from the mutant allele. In contrast, two
46
47 mRNAs lacking exon 3 were produced, one of them including 40 pb of intron 2.
48
49 These mRNAs have been previously described as minor transcripts of the
50
51 *SMPD1* gene, found in wild-type cells, which do not code for active enzymes.
52
53 One of these transcripts was degraded by the NMD mechanism, as shown in
54
55
56
57
58
59
60

1
2
3 the present study by CHX treatment. All these results showed that the 4-bp
4
5 deletion was a severe, disease-causing mutation.
6
7

8 In summary, this is the first report on the molecular analysis of Spanish
9
10 Niemann-Pick type A/B patients, and one of the few relatively exhaustive
11
12 studies on a specific population reported so far. Ten novel mutations were
13
14 identified and some of them were characterized at the levels of enzyme activity
15
16 or RNA processing. Genotype-phenotype correlations were established for
17
18 most of the mutations and, in particular, the p.R608del-type B association was
19
20 confirmed. These results provide useful information for the management of
21
22 Niemann-Pick A/B patients. Moreover, they may help to establish criteria to
23
24 select individuals with appropriate genotypes for a successful enzyme
25
26 replacement treatment, which will be hopefully available in the near future.
27
28
29
30
31
32
33
34
35

36 **ACKNOWLEDGEMENTS**

37
38 We thank J. Jarque, H. Sellés and A. Sopena for their excellent
39
40 technical assistance, and R. Rycroft for revising the English. We also want to
41
42 thank the patients and physicians who sent samples: Drs. Mateu-Jardí, Caritg
43
44 (Barcelona), Ruiz del Portal (Sevilla), Hierro, López-Herce, Jara, Gutiérrez-
45
46 Solana (Madrid), Álvarez (Vizcaya), Bernaola, Monreal (Pamplona), Cazorla,
47
48 Verdú (Toledo), Galmés (Palma de Mallorca), Peña, Cabrera (Las Palmas de
49
50 Gran Canaria), Cancho (Palencia), Escola, Checa (Badajoz), Arias (Vigo),
51
52 Abeledo, Cabezuelo (Valencia), Benito, González-Valentín (Málaga), Castro-
53
54 Gago (Santiago de Compostela). This work was supported by grants from the
55
56 Spanish Ministerio de Sanidad y Consumo (PI051182; PI051343, REDEMETH
57
58
59
60

1
2
3 2003-REDG054), from the Spanish Ministerio de Educación y Ciencia
4
5 (SAF2006-12276), from the Generalitat de Catalunya (SGR2005-00848), from
6
7 the Fundacion Niemann-Pick de España and from the NIH (NIH 5R01
8
9 HD28607). The CIBER de Enfermedades Raras is an initiative of the ISCIII.
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

27 REFERENCES

- 28
29 Dardis A, Zampieri S, Filocamo M, Burlina A, Bembi B, Pittis MG. 2005.
30
31 Functional in vitro characterization of 14 SMPD1 mutations identified in
32
33 Italian patients affected by Niemann Pick Type B disease. Hum Mutat
34
35 26(2):164.
36
37
38
39
40
41 Ferlinz K, Hurwitz R, Vielhaber G, Suzuki K, Sandhoff K. 1994. Occurrence of
42
43 two molecular forms of human acid sphingomyelinase. Biochem J 301 (
44
45 Pt 3):855-62.
46
47
48
49
50
51 Fernández-Burriel M, Pena L, Ramos JC, Cabrera JC, Martí M, Rodríguez-
52
53 Quinones F, Chabás A. 2003. The R608del mutation in the acid
54
55 sphingomyelinase gene (SMPD1) is the most prevalent among patients
56
57 from Gran Canaria Island with Niemann-Pick disease type B. Clin Genet
58
59 63(3):235-6.
60

1
2
3
4
5
6 He X, Miranda SRP, Xiong X, Dagan A, Gatt S, Schuchman EH. 1999.
7
8 Characterization of human acid shingomyelinase purified from the media
9
10 overexpressing Chinese hamster ovary cells. *Biochim Biophys Acta*
11
12 1432:251-264
13
14

15
16
17 Jones I, He X, Katouzian F, Darroch PI, Schuchman EH. 2008. Characterization
18
19 of common SMPD1 mutations causing types A and B Niemann-Pick
20
21 disease and generation of mutation-specific mouse models. *Mol Genet*
22
23 *Metab* 95(3):152-162
24
25

26
27
28
29 Levran O, Desnick RJ, Schuchman EH. 1991. Niemann-Pick type B disease.
30
31 Identification of a single codon deletion in the acid sphingomyelinase
32
33 gene and genotype/phenotype correlations in type A and B patients. *J*
34
35 *Clin Invest* 88(3):806-10.
36
37

38
39
40
41 McGovern MM, Aron A, Brodie SE, Desnick RJ, Wasserstein MP. 2006. Natural
42
43 history of Type A Niemann-Pick disease: possible endpoints for
44
45 therapeutic trials. *Neurology* 66(2):228-32.
46
47
48

49
50
51 Pavlu-Pereira H, Asfaw B, Poupctova H, Ledvinova J, Sikora J, Vanier MT,
52
53 Sandhoff K, Zeman J, Novotna Z, Chudoba D and others. 2005. Acid
54
55 sphingomyelinase deficiency. Phenotype variability with prevalence of
56
57 intermediate phenotype in a series of twenty-five Czech and Slovak
58
59 patients. A multi-approach study. *J Inherit Metab Dis* 28(2):203-27.
60

1
2
3
4
5
6 Pittis MG, Ricci V, Guerci VI, Marcais C, Ciana G, Dardis A, Gerin F, Stroppiano
7
8 M, Vanier MT, Filocamo M and others. 2004. Acid sphingomyelinase:
9
10 identification of nine novel mutations among Italian Niemann Pick type B
11
12 patients and characterization of in vivo functional in-frame start codon.
13
14 Hum Mutat 24(2):186-7.
15
16
17

18
19
20 Ricci V, Stroppiano M, Corsolini F, Di Rocco M, Parenti G, Regis S, Grossi S,
21
22 Biancheri R, Mazzotti R, Filocamo M. 2004. Screening of 25 Italian
23
24 patients with Niemann-Pick A reveals fourteen new mutations, one
25
26 common and thirteen private, in SMPD1. Hum Mutat 24(1):105.
27
28
29

30
31
32 Schuchman EH. 1995. Two new mutations in the acid sphingomyelinase gene
33
34 causing type a Niemann-pick disease: N389T and R441X. Hum Mutat
35
36 6(4):352-4.
37
38
39

40
41 Schuchman EH, Desnick RJ. 2001. Niemann-Pick disease types A and B: acid
42
43 sphingomyelinase deficiencies. In: Scriver CR, Beaudet AL, Sly WS,
44
45 Valle D, editors. The Metabolic and Molecular Bases of Inherited
46
47 Disease. New York: McGraw-Hill. p 3589-3610.
48
49

50
51
52
53 Schuchman EH, Suchi M, Takahashi T, Sandhoff K, Desnick RJ. 1991. Human
54
55 acid sphingomyelinase. Isolation, nucleotide sequence and expression of
56
57 the full-length and alternatively spliced cDNAs. J Biol Chem
58
59 266(13):8531-9.
60

1
2
3
4
5
6 Simonaro CM, Desnick RJ, McGovern MM, Wasserstein MP, Schuchman EH.

7
8 2002. The demographics and distribution of type B Niemann-Pick
9
10 disease: novel mutations lead to new genotype/phenotype correlations.
11
12 Am J Hum Genet 71(6):1413-9.
13
14
15

16
17 Vanier MT, Ferlinz K, Rousson R, Duthel S, Louisot P, Sandhoff K, Suzuki K.

18
19 1993. Deletion of arginine (608) in acid sphingomyelinase is the
20
21 prevalent mutation among Niemann-Pick disease type B patients from
22
23 northern Africa. Hum Genet 92(4):325-30.
24
25
26

27
28
29 Wan Q, Schuchman EH. 1995. A novel polymorphism in the human acid

30
31 sphingomyelinase gene due to size variation of the signal peptide region.
32
33 Biochim Biophys Acta 1270(2-3):207-10.
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

FIGURE LEGENDS

1
2
3
4
5
6
7 **Figure 1.** Residual acid sphingomyelinase activity of the proteins expressed in
8 COS-7 cells from wild-type and mutant alleles, expressed as the percentage of
9 wild-type (WT) activity. The data are shown as mean \pm SD of two different
10 experiments, each performed in triplicate. Error bars correspond to standard
11 deviation. WT: wild-type.
12
13
14
15
16
17

18
19
20
21 **Figure 2.** Western blot analysis of wild-type and mutant acid sphingomyelinase
22 proteins expressed in COS-7 cells. The "COS" lane corresponds to cells
23 transfected with an empty pcDNA3.1 plasmid. A total of 35 μ g of protein extract
24 was loaded in each lane. ASM: acid sphingomyelinase; TUB: tubulin; WT: wild-
25 type.
26
27
28
29
30
31
32
33

34
35 **Figure 3. A:** RT-PCR amplification of RNA extracted from patient 14 (NPB14)
36 or WT fibroblasts, untreated (NT) or treated (CHX) with cycloheximide, using
37 non-specific primers (see Methods). **B:** specific RT-PCR amplification of alleles
38 that do not bear the p.R608del mutation (the c.1257+4_1257+7delAGGG allele
39 of patient 14 or wild-type alleles), on the same samples described in part A of
40 the Figure. **C:** digestion with *HinfI* of the same RT-PCR products shown in B.
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
The expected patterns after *HinfI* digestion for each transcript are: 394, 282 and
147 bp (transcript 1); 282, 262 and 147 bp (transcript 2); 282, 222 and 147 bp
(transcript 3). The 222-bp band (arrowhead), specific for transcript 3, is only
observed after CHX treatment. M: molecular weight marker.

Table 1. Mutations found in patients analysed in the present study

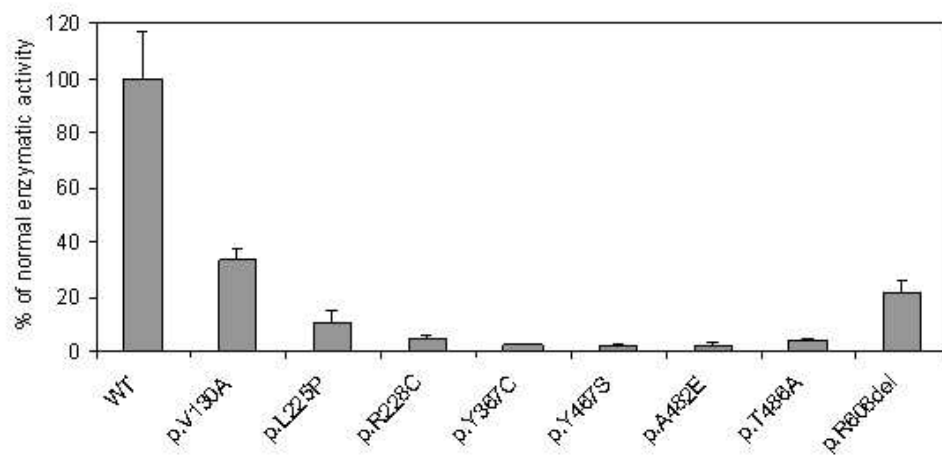
Exon / intron	Mutation	Coding effect	Number of alleles	Reference
2	c.503G>A	p.W168X	2	This report
2	c.682C>T	p.R228C	2	Simonaro, et al., 2002
2	c.733G>A	p.G245S	2	Simonaro, et al., 2002
2	c.939C>A	p.Y313X	2	This report
3	c.1100A>G	p.Y367C	1	This report
3	c.1128G>A	p.R376H	1	Simonaro, et al., 2002
3	c.1159delC	p.R387VfsX7	1	This report
3	c.1169_1171delTCT	p.F390del	2	This report
in3	c.1257+4_1257+7delAGGG	Splicing	1	This report
4	c.1262A>G	p.H421R	1	McGovern, et al., 2006
4	c.1321C>T	p.R441X	1	Schuchman, 1995
5	c.1400A>C	p.Y467S	2	This report
5	c.1420C>T	p.R474W	1	Simonaro, et al., 2002
5	c.1445C>A	p.A482E	4 (9.5%)	This report
5	c.1456A>G	p.T486A	1	This report
6	c.1774_1776delACT	p.T592del	2	This report
6	c.1823_1825delGCC	p.R608del	16 (38.1%)	Levrán, et al., 1991

Gene nucleotides were numbered according to sequence RefSeq NM_000543.3, with +1 as A of the ATG start codon. The ATG codon represents +1 for amino acid numbering, according to the preprotein sequence NP_000534.3.

Table 2. Significance of the differences in pair-wise comparisons between mutant enzymes with detectable levels of activity and the wild-type enzyme

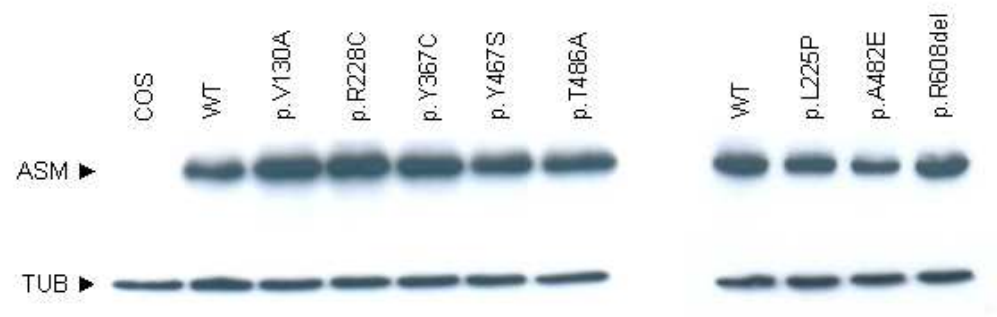
V130A	L225P	R228C	Y367C	Y467S	A482E	T486A	R608del	Alleles	n
P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	Wild-type	18
	p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.004	V130A	6
		p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.009	L225P	6
			p=0.002	p=0.002	p=0.015	NS	p=0.004	R228C	6
				NS	NS	p=0.002	p=0.004	Y367C	6
					NS	p=0.002	p=0.004	Y467S	6
						p=0.041	p=0.004	A482E	6
							p=0.004	T486A	6
								R608del	5

N: number of replicates; NS: not significant.

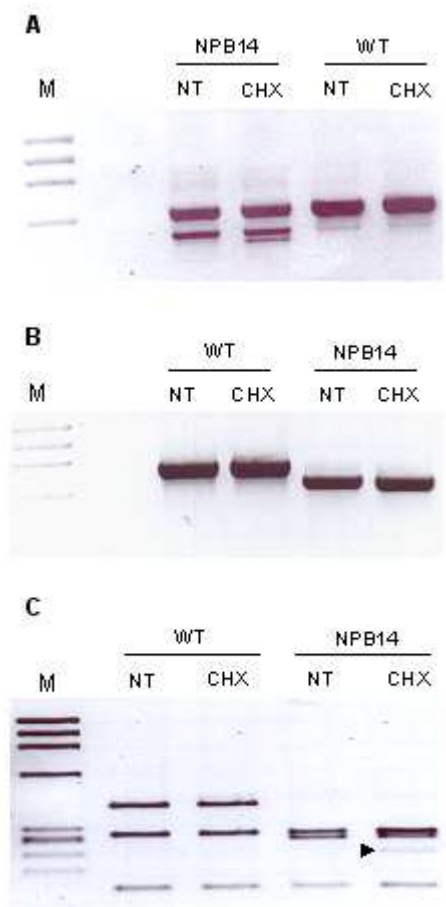


Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



Or Peer Review



Supp. Table S1. Primers used for site-directed mutagenesis

Mutation	Primer sequence 5'3'
p.V130A	sense-GATAGCACACCTGCC G GTGCCAATCCATTGTCC
(c.389T>C)	antisense-GGACAATGGATTGGCAC g CGGCAGGTGGTGTCTATC
p.L225P	sense-CTGTGCAGACCCAC C GTGCTGCCGCCGGG
(c.674T>C)	antisense-CCCGGCGGCAGCAC g GTGGGTCTGCACAG
p.R228C	sense-CCACTGTGCTG C iGCCGGGGTTCTGGC
(c.682C>T)	antisense-GCCAGAACCCCGGC a GCAGCACAGTGG
p.Y367C	sense-CCTCAGAATTGGGGGGTTCT g TGCTCTTTCCCC
(c.1100A>G)	antisense-GGGGAAAGAGCA c AGAACCCCCCAATTCTGAGG
p.Y467S	sense-GAGGTCTTCT c TGATGAAGAGACTCTGAGCCG
(c.1400A>C)	antisense-CGGCTCAGAGTCTCTTCATCA g AGAAGACCTC
p.A482E	sense-GCTGTAGCCTTCCTGG a ACCCAGTGCAACTACC
(c.1445C>A)	antisense-GGTAGTTGCACTGGGT t CCAGGAAGGCTACAGC
p.T486A	sense-GGCACCCAGTGC g CTACCTACATCGGC
(c.1456A>G)	antisense-GCCGATGTAGGTAG c TGCACTGGGTGCC
p.R608del	sense-GCTGACAGCCCTGCTCTGTG --- CCACCTGATGCCAGATGGG
(c.1823_1825delGCC)	antisense-CCCATCTGGCATCAGGTGG --- CACAGAGCAGGGCTGTACAGC

RefSeq cDNA NM_000543.3, with +1 corresponding to the A of the ATG translation initiation codon.

The nucleotide change corresponding to the mutation is indicated in bold.

Supp. Table S2. Genotypes and main clinical findings of the NPD patients

Patient	Sex	Type	Genotype	Age of onset	Age of diagnosis	Clinical signs	ASM activity (% control) ^a
2 ^b	M	A	p.[Y313X]+[Y313X]	8 mo	1y 4mo	HSM, osteopenia, cherry-red spot in macula, bone marrow foam cells, decreased body weight	ND
8	F	A	p.[Y467S]+[Y467S]	NA	1y 2mo	HSM, low weight and height, mental retardation, cherry-red spot macula, muscular weakness, anemia, leucopenia, thrombocytopenia	ND
9	F	A	p.[F390del]+[F390del]	4 mo	4 mo	HSM, severe neurological involvement, low weight and height, joint stiffness, muscular atrophy	3.5
11	M	A	p.[A482E]+[A482E]	4 mo	4 mo	HSM, hypotonia, developmental delay, craneofacial dysmorphism	0.2
12	M	A	p.[W168X]+[W168X]	4 mo	8 mo	HSM, horizontal nystagmus, cherry-red spot macula, moderate brain atrophy, foam cells	ND
16	F	A	p.[G245S]+[Y367C]	6 mo	10 mo	HSM, cherry-red spot macula, respiratory difficulties	ND

1								
2								
3								
4								
5	28	M	A	p.[G245S]+[H421R]	4 mo	1y	HSM, moderate demyelination, coarse facies, bone marrow foam cells	ND
6								
7								
8								
9								
10	32	F	A	p.[T592del]+[T592del]	5 mo	1y 2mo	HSM, hypotonia, decreased growth	1.0
11								
12								
13	1	M	B	p.[R376H]+[R387VfsX7]	adult	adult	SM	ND
14								
15								
16	3	M	B	p.[R608del]+[R608del]	50 y	54 y	HSM, dysphagia, spleen foam cells	5.7
17								
18								
19	4	F	B	p.[R441X]+[R474W]	2y 6mo	5 y	HSM, thrombocytopenia, low weight and height	1.0
20								
21								
22	5	F	B	p.[R228C]+[R228C]	1y 5mo	1y 5mo	HSM, hypotonia, motor delay ^c	ND
23								
24								
25	6	M	B	p.[R608del]+[A482E]	52y	52y	HSM, cardiorespiratory difficulties, thrombocytopenia, bone marrow sea blue histiocytosis	3.6
26								
27								
28								
29								
30	7	M	B	p.[R608del]+[R608del]	6y	7y	HSM, bone marrow foam cells	3.6
31								
32								
33	13	F	B	p.[R608del]+[R608del]	60y	63y	HSM, bone marrow sea blue histiocytosis	8.2
34								
35								
36	14	M	B	[p.R608del]+	1y 9mo	3y 6mo	HSM, growth delay, bone marrow foam cells, anemia, respiratory difficulties	5.9
37								
38				[c.1257+4_1257+7delAGGG]				
39								
40								
41								
42								
43								
44								
45								
46								
47								

15	F	B	p.[R608del]+[T486A]	NA	2y	HSM, pulmonary difficulties	6.2
17 ^b	M	B	p.[R608del]+[R608del]	6mo	4y	SM, sea-blue histiocytosis	NA
18	F	B	p.[R608del]+[R608del]	1y 6mo	3y	SM, bone marrow foam cells, moderate motor polyneuropathy, pectum excavatum	7.7
19	F	B	p.[R608del]+[R608del]	1y 11mo	3y 9mo	SM, bone marrow foam cells,	10.1
20	M	B	p.[R608del]+[A482E]	6y	17y	HSM, growth delay	1.3

^a ASM activity in the patients' fibroblasts referred to the mean value of several controls analyzed in the same experiment. Range for normal values: 72-256 nmols/h x mg protein. ND: Not detectable. NA: Not available.

^b Maghreb origin; y: year; m: month, HSM: hepatosplenomegaly; SM: splenomegaly

^c Follow-up data: at 6y of age, the patient presented a mild restrictive respiratory insufficiency, normal psychomotor development and school performance, hepatosplenomegaly:11.5 cm, splenomegaly: 15 cm, reduced muscle tone. Neither pyramidal symptoms nor abnormal movements were observed. These data are consistent with the classification of the patient as type B.