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# Niemann-Pick Disease Type A and B are Clinically but also Enzymatically Heterogeneous: Pitfall in the Laboratory Diagnosis of Sphingomyelinase Deficiency Associated with the Mutation Q292 K

## Abstract

This study describes a diagnostic pitfall in the laboratory diagnosis of patients with sphingomyelinase deficiency (SMD; Niemann-Pick disease types A and B; NPA and NPB), in cases where sphingomyelinase activity was not determined with sphingomyelin as the natural enzymic substrate. Four of 24 SMD patients studied had falsely normal or enhanced activity, when a so-called artificial sphingomyelinase substrate, 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine (HNP), was used, whereas SMD was clear with the sphingomyelin substrate. Those four patients had the Q292 K mutation of the acid sphingomyelinase gene (*SMPD1*) on at least one allele. Three of the four patients (no data available from one) experienced only late-infantile or juvenile, though distinct, neurological involvement, where learning disabilities, hypo- or areflexia or mild ataxia were initial signs. The laboratory pitfall with HNP substrate, which is used in many laboratories, raises the risk that some SMD patients are overlooked, and it prevents the consideration of a late-manifesting neurological course in some patients as well as the planning of enzyme substitution therapy in non-neurological SMD (NPB) patients. Since classical NPB is very rare, it is suggested that SMD patients with late- or mild-manifesting neurological symptoms should better be assigned to additional SMD subgroups than grouped with NPB.

## Key words

Niemann-Pick disease · classification · mutation · sphingomyelinase · synthetic substrate

## Abbreviations

HNP	2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine
NPA, NPB, NPC	Niemann-Pick disease, types A, B, C, respectively
SMD	sphingomyelinase deficiency
SMD1, SMD2, SMD3, SMD4	proposed subgroups of SMD
<i>SMPD1</i>	acid sphingomyelinase gene

## Introduction

Genetic neuropilipidoses have not rarely to be considered in children (but also older patients) presenting with retardation of cognitive and motor development, muscle hypotonia, other neurological symptoms and/or spleno(hepato)megaly. Some main neuropilipidoses are Niemann-Pick disease types A (NPA), B (NPB), and C (NPC). NPA (OMIM 257200) and NPB (OMIM 607616) are known as sphingomyelinase deficiencies (SMD) [13], but NPC includes two types (NPC1, OMIM 257220; NPC2, OMIM 607625) of so-called cholesterol trafficking defects [11].

If in suspected neuropilipidosis patients the spleen or liver are enlarged, a bone marrow cytologic screening for histiocytic storage phenomena including Niemann-Pick cells can be helpful. A detection of storage cells has to be followed by specific diagnostic laboratory investigations. In cases of suspected Niemann-Pick disease, the first step is the determination of sphingomyelinase

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activity in white blood cells or cultured skin fibroblasts in order to diagnose or exclude SMD (caused by acid sphingomyelinase gene [*SMPD1*] mutations [13]). This should be done before the complex laboratory testing for the abnormal cellular cholesterol processing characteristic of the genetically distinct NPC condition [11] or other tests are started.

Measuring sphingomyelinase activity is not a trivial task. A number of assay methods for sphingomyelinase activity have been described that include methods using the natural, usually radio-labelled, sphingomyelin as the enzymic substrate but also methods with synthetic [4], i.e., artificial substrates which have slightly or distinctly different chemical properties as compared to the natural sphingomyelin [3,7]. Artificial substrates have been recommended for use in simpler sphingomyelinase assay procedures as compared to those using radioactive sphingomyelin as the natural substrate. This has induced many laboratories to rely on sphingomyelinase assays with artificial substrates. The present study describes a diagnostic pitfall in SMD, when sphingomyelinase activity was determined with the mostly used artificial substrate, 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine (HNP). Four late-infantile or juvenile patients are reported whose SMD diagnoses would have been missed if the natural sphingomyelin substrate had not been used in addition. These patients had the Q292 K (874 C>A) mutation [12] of the *SMPD1* gene on at least one allele. Clinically, the total of 24 studied SMD patients suggested that the two classical SMD groups, NPA (neuropathic SMD) and NPB (non-neuropathic SMD), were inadequate for the grouping of a number of patients. These patients could better be assigned to additional SMD subgroups.

## Patients and Methods

### Patients

When diagnosed as having SMD, the youngest of the present 24 patients was 5 months old and the oldest 73 years old. A number of patients could not easily be grouped with NPA or NPB (see newly proposed additional SMD subgroups; Table 1). Learning difficulties and partial areflexia in the juvenile patients nos. 1, 2, and 4 (Table 1), and strabismus convergens and thumb muscle atrophy in the adult patient no. 9, were suggestive of some late neurological involvement. In some of the NPA patients, neurological symptoms, though severe, became evident only some months or even years after their SMD diagnosis had been made. All patients had hepatosplenomegaly or splenomegaly of different degrees and vacuolated cells ("Niemann-Pick cells") and/or sea blue histiocytes in the bone marrow smears. Retinal degeneration marked by cherry red macular spots was found in all but one of the infantile NPA patients. Also in some of the juvenile or adolescent patients the maculae were slightly red but with a surrounding greyish granular halo [5,13]. Patient no. 3, who had a too short clinical follow-up for being assessed neurologically, was remarkable for bone involvement with thickened lower ends of the femura. Lung infiltration was present in most of the patients. Lung fibrosis was severe in the 73-year-old NPB patient no. 20, who had had long known hypoxemia without secondary elevation of red blood cell counts, and his frequent infections never led to distinct leukocytosis, suggesting a substantial bone marrow insufficiency.

The four SMD patients with the diagnostic pitfall in the sphingomyelinase determination were clinically reported as follows.

*Patient no. 6:* As a baby this patient was difficult to feed and his spleen and liver were enlarged. Only when aged 18 months, he was able to stand but lost this ability at 24 months and developed spasticity and pes equinovarus. His speech was restricted to a few words. At age 4 years he had muscle weakness and atrophy, reduced tendon reflexes, and trunk ataxia, and he was severely mentally retarded. Body weight and size were below the 3rd percentile. Chest X-ray showed an enlarged thorax, thickened ribs, and lung infiltration. The eye fundi were obviously free of cherry red macular spots. Eye movements were impaired in the vertical saccade reactions. Laboratory tests revealed a mild anemia, increased chitotriosidase and angiotensin-converting enzyme activities. A biochemical test sensitive for certain types of lysosomal storage in cultured fibroblasts (methylamine test [6]) was strongly positive. White blood cell sphingomyelinase activity was determined in two external laboratories with contradictory results; only the one laboratory suggested a diagnosis of SMD (courtesy of Prof. G.F. Hoffmann, University of Marburg, now Heidelberg, Germany).

*Patient no. 8:* When 4 years old this girl had massive hepatosplenomegaly and distinct lung infiltration on chest X-ray, but was free of neurological symptoms. Numbers of vacuolated lymphocytes and Niemann-Pick cells were seen in the blood and bone marrow smears, respectively. No additional clinical information was available.

*Patient no. 21* (courtesy of Prof. T. Voit, University of Essen, Germany): At the age of 6 years, this boy showed clumsy movements and had a spastic gait. He had highly increased cerebrospinal fluid protein content (18.5 mg/L; highest normal for age: 4.5 mg/L) and strongly reduced motor nerve conduction velocity (19 m/s; lowest normal for age and investigated nerve: about 30 m/s). The acoustic evoked potentials were pathologic.

*Patient no. 22:* When 2.5 years old, this boy had muscle hypotonia and mild limb ataxia. At age 8 years, he was severely mentally retarded and had some gross motor problems, but his overall condition was relatively stable.

### Methods

The method for measuring *sphingomyelinase* activity towards *natural* sphingomyelin substrate was adapted from different methods known from the literature. A vial was prepared to contain 0.75 nmol (radioactivity, 1200 dps) of [<sup>14</sup>C-methyl]sphingomyelin (no. CFA 566, Amersham Pharmacia Biotech, Buckinghamshire, England) dissolved in 40 µL buffer (sodium acetate, pH 4.8, 0.25 M, containing 0.1% [v/v] Triton X-100). Then 10 µL fibroblast cell lysate ( $2 \times 10^4$  trypsinised cells [about 8 µg protein] lysed in 0.25% [v/v] aqueous Triton X-100 with freezing, thawing, and sonication) were added. The assay mixture was incubated for 30 min at 37°C and the reaction stopped by adding 0.8 mL chloroform/methanol (2/1, by vol.) and 0.25 mL water and vigorously shaking the mixture. Then the mixture with two solvent phases was centrifuged for 1 min at 3000 rpm, and 0.2 mL of the

**Table 1** Fibroblast enzyme activities as compared to controls, and *SMPD1* mutations, in sphingomyelinase-deficient (SMD) patients and controls. Bold typed enzyme activities indicate misleading results with the artificial HNP substrate for sphingomyelinase

Identification	SMD patient age at appearance of first symptoms (years)	Assignment of patient to Niemann-Pick disease type A or B	Assignment of patient to one of the newly proposed SMD sub-groups: SMD1 to 4 <sup>a</sup>	Sphingomyelinase activity towards the synthetic HNP substrate [pmol HNP cleaved per hour by 10 <sup>4</sup> cells]	Sphingomyelinase activity towards <sup>14</sup> C-sphingomyelin [pmol sphingomyelin cleaved per hour by 10 <sup>4</sup> cells]	SMPD1 mutations
Normal controls (1- to 35-year-old), n = 15						
Mean value				697	58.0	
Standard deviation				249	26.1	
Patient no.						
1	1	B	SMD3 <sup>b</sup>	30	1.1	
2	5	B	SMD3 <sup>b</sup>	170	11.9	R289 H; R441 X
3	1	not assigned	not assigned	50	0.8	
4	1.5	B	SMD3 <sup>b</sup>	87	1.3	V36 A <sup>d</sup> ; G245 S
5	at birth	A	SMD1	3	0.9	C89 H; C89 H
6	<2	A	SMD2	<b>1625</b>	1.0	G254 S; Q292 K
7	<1	A	SMD1	5	1.1	
8	3	not assigned	not assigned	<b>1150</b>	1.3	S248 R; Q292 K
9	<30	B	SMD3 <sup>c</sup>	44	2.4	
10	<1	B?	SMD3	6	0.8	
11	<1	A	SMD1	<2	0.9	R496 L; A595fs602 X
12	<1	A	SMD1	6	0.9	
13	<1	A	SMD1	<2	1.0	
14	<1	A	SMD1	2	1.1	
15	<1	A	SMD1	39	1.1	
16	<1	A	SMD1	<2	0.9	C594 Y; C594 Y
17	<4	B	SMD3	<2	1.1	
18	about 30	B	SMD4	24	1.0	
19	<1	A	SMD1	86	0.8	S190fs254 X; S190fs254 X
20	<55	B	SMD4	197	16.8	
21	1	A	SMD2	<b>1690</b>	1.1	Q292 K; Q292 K
22	<1	A	SMD2	<b>441</b>	0.8	S190fs254 X; Q292 K
23	<1	A	SMD1	<2	0.9	
24	9	B	SMD4	138	7.9	G166 R; I176 N

<sup>a</sup> See Discussion. <sup>b</sup> Not grouped with SMD4 because of reported learning disabilities. <sup>c</sup> Not grouped with SMD4 for the neurological symptoms mentioned in the Patients section. <sup>d</sup> Another laboratory has additionally found R608del.

clear hydrophilic upper phase containing 36% of the enzymatically released radioactive phosphorylcholine was  $\beta$ -counted by liquid scintillation.

The determination of the activity of *sphingomyelinase* towards the *artificial* substrate, 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine (briefly: HNP) [4] was also adapted from literature methods [3,7]. A solution of this substrate (no. 376581, Merck Biosciences/Calbiochem, Schwalbach, Germany) was always freshly prepared by dissolving 13.4 mg HNP in 1 mL sodium acetate buffer (pH 5.3, 0.1 M, containing 0.2% [w/v] sodium azide) at 0°C (important) during about 30 min. A fibroblast cell lysate was prepared to contain  $4 \times 10^6$  trypsinised cells/mL water and processed by freezing, thawing, and sonication. A detergent solution was prepared to contain 1.5% (w/v) sodium taurocholate (no. T-4009, Sigma, Deisenhofen, Germany) and 0.02% (w/v) sodium azide in water. Two volumes of the fibroblast lysate were mixed with one volume detergent solution and kept at 0°C. The enzyme assay was started by mixing 20  $\mu$ L of this lysate-detergent mixture ( $5.3 \times 10^4$  cells; about 21  $\mu$ g protein) with 20  $\mu$ L substrate solution and warming up to 37°C, at which temperature the assay was incubated for 3 h. The reaction was stopped by adding 0.2 mL glycine/sodium hydroxide buffer (pH 10.3, 0.067 M, dissolved in 32% [v/v] ethanol in water). The assay was centrifuged for 5 min at 10 000 rpm. In the supernatant, absorption of the enzymatically released 2-N-(hexadecanoyl)-amino-4-nitrophenol was read at 405 nm. Blanks were run by separately incubating both of the lysate-detergent mixture and the substrate solution and mixing the glycine buffer with both components immediately before reading absorption.

For the *mutational analysis* of the *SMPD1* gene, DNA extraction and sequencing were performed as described previously [1]. Briefly, DNA was prepared within 24 hours after blood sampling. Genomic DNA was isolated from 5 to 10 mL anticoagulated blood using a standard salting out procedure [9]. DNA from cultured fibroblasts was extracted by means of HighPure™ extraction kits (Roche Diagnostics, Mannheim, Germany). *SMPD1* gene DNA-specific fragments covering the entire open reading frame were amplified by PCR in 25  $\mu$ L reaction volumes containing 100 ng DNA as template, 10 pmol of each primer (Table 2), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 100  $\mu$ M deoxynucleoside triphosphates, 1.0 U Taq polymerase (Amersham Pharmacia Biotech, Freiburg, Germany) using standard thermocycling. PCR fragments were purified with "Qiaquick PCR purification" filter centrifugation (Qiagen, Hilden, Germany) according to the manufacturer's instructions, sequenced by BigDye™ chemistry (Qiagen), and analysed by an ABI 377 automated DNA sequencer.

## Results

The sphingomyelinase activities towards *natural*, radiolabelled sphingomyelin substrate and *artificial*, chromogenic HNP substrate, respectively, in skin fibroblast strains from the present 24 SMD patients, in comparison with controls, are listed in Table 1. Also given are the acid sphingomyelinase (*SMPD1*) mutations analysed in some of the patients. In patients nos. 21 and 22 the mutational results were confirmed by the findings of corresponding heterozygous mutations in both patients' parents.

**Table 2** PCR primer sequences and positions within *SMPD1* (acid sphingomyelinase) DNA<sup>a</sup>. PCR setup and cycling profile were identical for all primer pairs (see Methods)

Exon	DNA-position	Sequence (5' > 3')	Length
01	FP	revm13-gagggctggctagggtccag	
	RP	-21 m13-ccagccccagcactcctttc	434
02	FP	revm13-cctctgctctgcctctgatt	
	RP	-21 m13- ttcccttctgggtttccac	853
03	FP	revM13-ggaggaccaggattggaaca	
	RP	-21M13-cagagggtgccagctcaac	295
04	FP	revM13-gattcagctcatggctactg	
	RP	-21M13-ggatggtgagatgctcaagg	266
05	FP	revM13-cccctcctagaactctctg	
	RP	-21M13-ccaccaactccaggataagg	231
06	FP	revM13-cagtccagccccacatccttg	
	RP	-21M13-gcctggtgaaccacagcag	514

<sup>a</sup> GeneBank accession number X63600

In patients nos. 6, 8, and 21, there was a remarkable discrepancy between the profoundly deficient activity towards [<sup>14</sup>C]sphingomyelin (natural substrate) and the increased activity towards HNP, as compared to the control range. Also in patient no. 22, the activity found towards HNP was not suggestive of SMD, as it was in the lower control range. The remaining 20 patients had a roughly parallel, profound reduction of the activities towards both the natural and the artificial substrate. In some but not all of the NPB patients, especially in patients no. 2 (juvenile), no. 20 (late adult), and no. 24 (adult), the residual activities were higher than in the NPA patients.

In Table 1, a panel is given where SMD1 to SMD4 appear as newly proposed clinical subgroups of SMD patients, to account for the high clinical, in particular neurological disease variability, which is often inconsistent with the classical two disease subgroups, NPA and NPB.

## Discussion

The present study aimed at demonstrating a possible pitfall in the laboratory diagnosis of SMD, when using the artificial HNP substrate for assaying acid sphingomyelinase activity in cultured fibroblasts (Table 1) and white blood cells (similar results, not shown). The diagnoses of SMD in the present patients nos. 6, 8, 21, and 22 would not have been made, unless sphingomyelinase activity had been tested using the natural (radio-labelled) sphingomyelin substrate. The test with the HNP substrate was false-negative, i.e., revealed almost normal or increased instead of clearly reduced or deficient enzyme activities in these patients.

The mutated *SMPD1* genes in these four patients shared the Q292 K mutation at least on one allele. Others of the present patients were also studied for their *SMPD1* mutations (Table 1, or not shown), but none had the Q292 K mutation which was originally described in two SMD patients with an unusual clinical

phenotype [12]. For Q292 K it was hypothesised that the substitution of lysine (charged positively) for glutamine (uncharged) at sphingomyelinase amino acid position 292 yielded a, though defective, enzyme protein with a paradoxically high affinity for, and enzymic hydrolysis of, the artificial so-called sphingomyelinase substrate HNP with its phenyl-linked phosphorylcholine moiety. For the S248 R mutation, which was associated with Q292 K in patient no. 8, the positively charged arginine was similarly substituted for the uncharged serine.

In conclusion, the laboratory diagnosis or exclusion of SMD should not rely on the use of an artificial so-called sphingomyelinase substrate such as HNP, but assays for acid sphingomyelinase activity should always include a method using radio-labelled sphingomyelin as the natural substrate. The described pitfall can lead to inappropriate measures in patients with an overlooked SMD diagnosis. The pitfall is fatal, if a still present SMD condition manifests after some months or years with neurological symptoms but SMD is not re-considered. Moreover, a missed SMD diagnosis will prevent the planning of sphingomyelinase enzyme substitution therapy, which is expected in the near future [10] to prove beneficial for non-neurological SMD (NPB) patients or patients with mild neurological but severe visceral involvement.

The broad spectrum of clinical, in particular neurological, phenotypes in these 24 SMD patients who were difficult to divide into the classical groups, NPA (neuropathic sphingomyelinosis) and NPB (non-neuropathic sphingomyelinosis) [13], was remarkable. The majority of these patients initially grouped with NPB developed discrete or distinct neurological symptoms in their late-infantile, adolescent or adult age. This suggested that permanently non-neurological SMD, i.e., classical NPB, was very rare. Therefore, an exact neurological assessment at diagnosis and during the disease course is necessary. Our experience with the present SMD patient sample induced us to make an addendum to this laboratory diagnostic study for the delineation of clinical SMD subtypes in addition to the classical subtypes NPA and NPB, in particular to account for the observed variability in the neurological disease [13]. The following four SMD groups are proposed.

- SMD1, acute infantile neuropathic sphingomyelinosis with visceral signs (liver, spleen, lungs), failure to thrive, floppiness, massive neurological involvement, often cherry-red macular spots and death within the first three years (classical NPA) [13].
- SMD2, late infantile and childhood neuropathic sphingomyelinosis with subacute clinical course [2,8], neurovisceral involvement almost as in SMD1, often retinal involvement with grey (“macular halo”) macular degeneration instead of cherry red macular spots; death within the first two decades. The slower neurodegeneration as compared to SMD1 may allow for a detailed assessment of growth and mental retardation, muscle hypotonia, ataxia, areflexia, sometimes vertical gaze paresis, spasticity, and/or peripheral neuropathy.
- SMD3, near to non-neuropathic sphingomyelinosis [12,13,15] manifested in childhood or adolescence with visceral involvement and mostly minor neurological and cerebral signs such as clumsiness, ocular symptoms, possible retinal involvement as in SMD2, learning disabilities. Visceral decompensation can lead to death in adolescence or adulthood, even though

spleen and liver size may decrease during the childhood, when different skills and mental abilities may slightly improve as compared to their earlier impairment.

- SMD4, late onset non-neuropathic sphingomyelinosis, where very mild neurological symptoms may occur but are probably secondary. Visceral (possible liver cirrhosis, lung infiltration) and hematological (bone marrow; hypersplenism) involvement essentially characterize the chronic clinical course with survival up to several decades (classical NPB) [14].

The proposed classification was based on the disease severity or acuteness (decreasing from SMD1 to SMD4) being determined by the degree of neurological involvement. The pure neurological basis may not always be valid in the also highly visceropathic SMD disease, but at least in the present patient sample any acute or subacute course in a patient was dominated by neurology.

The clinical phenotype in three of the present Q292 K mutated patients (the fourth could not be followed up) was close to the phenotype introduced as SMD2. Yet, it may not always be easy to distinguish the proposed SMD phenotypes. The use of the proposed classification will have to be confirmed by the investigation of additional patients. Although it is long known that there are phenotypes intermediate between NPA (SMD1) and NPB (SMD4), the present patients assigned to SMD2 and SMD3, respectively, were clinically too different for being included in only one “NPI” (I for intermediate between A and B) phenotype.

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