

## Short Report

# Identification of 25 new mutations in 40 unrelated Spanish Niemann-Pick type C patients: genotype-phenotype correlations

Fernandez-Valero EM, Ballart A, Iturriaga C, Lluch M, Macias J, Vanier MT, Pineda M, Coll MJ. Identification of 25 new mutations in 40 unrelated Spanish Niemann-Pick type C patients: genotype-phenotype correlations

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To better characterize Niemann-Pick type C (NPC) in Spain and improve genetic counselling, molecular analyses were carried out in 40 unrelated Spanish patients. The search identified 70/80 alleles (88%) involving 38 different NPC1 mutations, 26 of which are described for the first time. No patient with NPC2 mutations was identified. The novel NPC1 mutations include 14 amino acid substitutions [R372W (c.1114C > T), P434L (c.1301C > T), C479Y (c.1436G > A), K576R (c.1727G > A), V727F (c.2179G > T), M754K (c.2261T > A), S865L (c.2594C > T), A926T (c.2776G > A), D948H (c.2842G > C), V959E (c.2876T > A), T1036K (c.3107C > A), T1066N (c.3197C > A), N1156I (c.3467A > T) and F1224L (c.3672C > G)], four stop codon [W260X (c.780G > A), S425X (c.1274C > A), C645X (c.1935T > A) and R1059X (c.3175C > T)], two donor splice-site mutations [IVS7+1G > A (g.31432G > A) and IVS21+2insG (g.51871insG)], one in-frame mutation [N961\_F966delinsS (c.2882del16bpins1bp)] and five frameshift mutations [V299fsX8 (c.895insT), A558fsX11 (c.1673insG), C778fsX10 (c.2334insT), G993fsX3 (c.2973\_78delG) and F1221fsX20 (c.3662delT)]. We also identified three novel changes [V562V (c.1686G > A), A580A (c.1740C > G) and A1187A (c.3561G > T)] in three independent NPC patients and five polymorphisms that have been described previously. The combination of these polymorphisms gave rise to the establishment of different haplotypes. Linkage disequilibrium was detected between mutations C177Y and G993fsX3 and specific haplotypes, suggesting a unique origin for these mutations. In contrast, I1061T mutation showed at least two different origins. The most prevalent mutations in Spanish patients were I1061T, Q775P, C177Y and P1007A (10, 7, 7 and 5% of alleles, respectively). Our data in homozygous patients indicate that the Q775P mutation correlates with a severe infantile neurological form and the C177Y mutation with a late infantile clinical phenotype.

Niemann-Pick type C (NPC) is an autosomal recessive lipid-storage disorder characterized by lysosomal/endosomal accumulation of unesterified cholesterol and glycolipids in many tissues as a consequence of defective intracellular sterol trafficking (reviewed in 1–3).

The clinical manifestations of the disorder are heterogeneous. Despite most patients exhibiting

progressive neurological disease, both age of onset and subsequent clinical course may vary (4).

Biochemically, variations in the severity of the cellular cholesterol lesion have been described, including both severe and mild alterations known as classic and variant biochemical phenotypes, respectively (5).

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More than 95% of the cases of NPC are known to be due to a defect in the NPC1 gene, which is located at 18q11-q12 (6), and the complete genomic sequence has recently been identified (7). Nearly 200 different mutations and more than 60 different polymorphisms have been described in this gene [for review, see (8, 9)]. Defects in the NPC2 gene, located at 14q24.3, are responsible for the remaining 5% of NPC patients (10). Only 14 different mutations have been described for this gene [for review, see (11)].

Although the exact defect in intracellular trafficking of cholesterol and glycolipids remains to be elucidated, recent data suggest that the products of both genes (the NPC1 and the NPC2 proteins) play important roles. The NPC1 protein has been ascribed to a permease located in the membrane of late endosomes/lysosomes that acts as a transmembrane efflux pump (12) and probably interacts with NPC2, a soluble lysosomal protein (13).

Here, we present a molecular analysis of the NPC1 and NPC2 genes in 40 unrelated Spanish patients diagnosed with NPC according to standard biochemical protocols.

## Materials and methods

### Patients

Samples were obtained from 41 patients belonging to 40 families with different forms of the disease. Diagnosis of NPC was determined by cytochemical demonstration of pathologically enriched cholesterol via filipin staining and low-density lipoprotein-induced cholesterol ester formation (5, 14). Patients were referred to our centre from various hospitals in Spain and can be considered as representative of the Spanish population.

### DNA preparation

DNA was prepared from cultured skin fibroblasts according to standard protocols (15).

### Polymerase chain reaction (PCR) amplification

Primers for amplification of each exon and the boundaries of the NPC1 gene were the same as those described previously (16), with the exception of the forward primer for exon 15: *F-aca tgc aca tga aca taa ga*.

Primers for amplification of each exon and the boundaries of HE1/NPC2 were the same as those described by Millat et al. (17).

PCR conditions were as described by Yamamoto et al. (16) except for amplification of exon 15, in which thermocycling conditions were as follows: initial denaturing step of 94 °C for 5 min, followed by 35 cycles of 94 °C (40 s), 55 °C (30 s) and final extension of 72 °C (3 min).

### Single-strand conformation polymorphism (SSCP) analysis and sequencing

Approximately 7 µl of the PCR product was mixed with formamide dye (96% formamide, 0.01% xylene cyanol and 0.01% bromophenol blue), heated to 96 °C for 3 min and rapidly cooled on ice. Electrophoresis conditions and detection of DNA fragments were as described elsewhere (18). Samples with abnormal band shifts were amplified by PCR, and products were purified (QIAquick PCR purification kit; Qiagen, Germany) and sequenced (dye terminator cycle sequencing ready reaction; Perkin Elmer Applied Biosystems Division, Foster city, CA).

### Restriction analysis

The prevalent mutations W942C, P1007A, A1035V and I1061T were detected using the restriction enzymes *Bst*NI(-), *Nhe*I (+), *Nla*IV(-) and *Rsa*I(+), respectively.

### Statistical analysis

The  $X^2$  test was used for analysis of the haplotypes.

## Results

### Clinical and biochemical phenotypes

The phenotype distribution of 40 unrelated Spanish NPC patients, according to the criteria of Vanier et al. (4, 5, 19), is summarized in Table 1. The group included 4/40 (10%) perinatal forms (PN). Other patients were classified according to their neurological presentation: 12/40 (30%) showed a severe infantile (SI) form (neurological onset at age >2 years); 8/40 (20%) a late infantile (LI) form (neurological onset at age 3–5 years); 12/40 (30%) a juvenile (J) form (neurological onset at age 5–16 years) and 3/40 (7.5%) an adult (A) form (neurological onset at age >16 years). In one patient (patient 2), the clinical phenotype could not be established due to the lack of data relating to disease progression. The only available clinical data were hepatosplenomegaly (HSM) and neonatal jaundice, after which biochemical diagnosis was made.

Table 1. Summary of clinical and biochemical characteristics of the 40 NPC Spanish patients

Patient	Biochemical phenotype	Clinical phenotype <sup>a</sup>	Age at biochemical diagnosis	Age at onset	Clinical signs at age at onset
1	Classical	SI	10 months	19 months	HSM
2	Classical	?	3 months	1 month	HSM, jaundice
3	Classical	LI	3 months	1 month	HSM, hypotonia, failure to thrive
4	Classical	SI	6 months	1 month	HSM, hypotonia, failure to thrive
5	Classical	J	12 years	8 years	Ataxia, progressive learning disability
6	Classical	J	11 years	2 months	HSM (seizures at 10 years)
7	Classical	SI	5 months	1 month	HSM, jaundice
8	Classical	LI	2 years	5 months	HSM
9	Classical	SI		Newborn	HSM
10	Classical	SI	2 years	1 years	HSM, hypotonia, psychomotor retardation
11	Classical	LI	5 years	2 years	HSM, psychomotor retardation
12	Classical	J	23 years	12 years	Cerebellum syndrome, supranuclear paralysis, learning disability
13	Classical	PN	Newborn	1 month	HSM, hepatic failure
14	Classical	SI	7 months	Newborn	HSM, jaundice, failure to thrive
15	Classical	LI	5 years	Newborn	HSM, hypotonia
16	Intermediate	A	36 years	?	Gait instability, dysarthria
17	Classical	SI	16 months	3 months	HSM
18	Classical	SI	8 years	Newborn	HSM, hypotonia
19	Classical	LI	5 years	Newborn	HSM
20	Classical	LI	18 months	3 months	HSM
21	Classical	LI		4 years	VSO, clumsiness, dysarthria
22	Classical	LI	5 years	1 month	?
23	Variant	J	16 years	8 years	Bradypsichia, ophthalmoplegia, clumsiness
24	Classical	J	17 months	17 months	HSM, irritability
25	Classical	SI	3 years	1 years	Motor impairment, spastic tetraparesis
26	Classical	J	13 years	6 years	Learning disability, behaviour problems
27	Variant	J	8 years	?	?
28	Classical	SI	2 years	Newborn	HSM, jaundice
29	Variant	J	11 years	9 years	Generalized seizures
30	Variant	J	13 years	5 years	HSM, progressive learning disability
31	Classical	PN	Newborn	Newborn	HSM, jaundice, hypotonia, psychomotor retardation
32	Variant	J	10 years	7 years	HSM, ophthalmoplegia, ataxia, dystonia, psychomotor retardation
33	Classical	A	28 years	?	?
33'	Classical	A	32 years	?	?
34	Classical	?	14 years	?	?
35	Classical	PN	1 year	Newborn	HSM, jaundice, hypotonia, psychomotor retardation
36	Classical	J	10 years	6 years	HSM, ophthalmoplegia, ataxia, mental retardation
37	Variant	A	32 years	20 years	Clumsiness
38	Variant	LI	6 years	2 years	HSM
39	Classical	PN	Newborn	Newborn	HSM, hepatic failure
40	Classical	SI	15 months	8 months	HSM, nistagmus

A, adult; HSM, hepatosplenomegaly; J, juvenile; LI, late infantile; PN, perinatal; SI, severe infantile; VSO, vertical supranuclear ophthalmoplegia.

Patients 33 and 33' are siblings.

?, data not available.

<sup>a</sup>Clinical phenotype attending to the first neurological symptoms.

In terms of biochemical phenotype, cultured fibroblasts from a majority of patients (80%) showed by filipin staining a typical massive accumulation of unesterified cholesterol in late endosomal/lysosomal vesicles (classical biochemical phenotype). The remaining 20% (8/40 patients) showed a moderate cholesterol accumulation (variant biochemical phenotype) (5). These

patients all presented a J/A phenotype, except for case 38 who presented a LI phenotype.

HSM appears to be important as a first symptom in many NPC patients, this disturbance functioning as a unique sign for a long period of time until neurological symptoms appear. Our series comprised three patients with an SI phenotype, in whom HSM was detected between the

newborn period and 3 months, and diagnosis was established between 1.5 and 2 years (patients 9, 17 and 28). We also had three patients with a LI phenotype (patients 8, 19 and 38), in whom HSM was detected between newborn period and 2 years, and the diagnosis was established between 2 and 6 years. Finally in one patient with a J phenotype (patient 6), HSM was detected at 2 months while the first neurological symptoms appeared at around 10 years of age.

#### Mutation analysis

We performed molecular analysis of the 25 exons and intron boundaries of the NPC1 gene. By screening for the four frequent mutations W942C, P1007A, A1035V and I1061T by restriction enzyme analysis, we covered 17/80 alleles (21%). In order to identify the remaining 63 alleles, we performed SSCP analysis and sequencing. This approach allowed identification of 70/80 alleles (88%) involving 38 different mutations, 25 of which are described here for the first time. All of the identified mutations and their locations are summarized in Table 2.

None of the 14 new missense mutations were found when 100 normal chromosomes were analysed. Mutation I1061T was the most frequently identified in the studied population, accounting for 8/80 (10%) of the mutant alleles. This mutation, together with another five mutations (C177Y, G993fsX3, Q775P, P1007A and A1035V) account for 31/80 (39%) alleles and 8/40 (20%) of the complete genotypes (Table 1); seven of which are in homozygosity: patients 6 (I1061T), 8 (I1061T), 9 (Q775P), 10 (A1035V), 15 (C177Y), 17 (G993fsX3) and 39 (G993fsX3).

Our analysis of the NPC1 gene based on study of genomic DNA allowed completion of only 31/40 genotypes. In eight patients (2, 3, 7, 21, 29, 36, 38 and 40), only one mutation was found. In all these cases, all the exons and intron boundaries have been sequenced, and further analysis of cDNA is currently underway. In patient 13, who displayed a classic biochemical phenotype without any identified mutation in the NPC1 gene, no alteration of the NPC2 gene was identified by the molecular study according to the manuscript of Millat et al. (17).

#### Polymorphism analysis

SSCP analysis of the PCR products of all the exons and intron boundaries of the NPC1 gene in patients and in control population led to the identification of five different previously

described polymorphisms (Y129Y, H215R, I858V and R1266Q described by Millat et al. 1999; Y642M described by Morris et al. 1999). Allele frequencies did not differ significantly between the patient group and a group of 50 Spanish control subjects (Table 3).

We also found three novel changes: V562V (c.1686G > A), A580A (c.1740C > G) and A1187A (c.3561G > T) found in three independent NPC patients (patients 29, 30 and 2, respectively). None of these rare variants change any amino acid in the NPC1 protein, and all were absent in 100 control chromosomes (Table 3).

#### Haplotype analysis

All 40 unrelated NPC patients were screened for the following five polymorphisms: Y129Y (c.387 T > C), H215R (c.644A > G), I642M (c.1926 C > G), I858V (c.2572A > G) and R1266Q (c.3797G > A). The results obtained are summarized in Table 4.

The C177Y mutation was always associated with haplotype [-,-,+,-,-] with respect to those five polymorphisms. In patients in whom this mutation was in heterozygosity with another mutation, phases could be established and/or confirmed by analysis of parental DNA. In the general population, the frequency of this haplotype was calculated to be 6.66% which is significantly different from its frequency when only C177Y alleles are considered ( $p > 0.001$ ).

In the case of the I1061T mutation, all chromosomes shared the same haplotype [-,-,-,-,-] when the same five polymorphisms were taken in account, and phases could also be established. The  $X^2$  test for the I1061T mutation and the [-,-,-,-,-] haplotype was also significantly different ( $p < 0.001$ ) when the frequency of this haplotype associated with alleles bearing this mutation was compared with the frequency estimated in the control population (10%).

Finally, in relation to the G993fsX3 (c.2973\_78delG) mutation, the two homozygous patients (patients 17 and 39) shared the same haplotype [-,-,-,-,-]. In the heterozygous patient, with a G993fsX3/I1061T genotype, phases could not be established.

## Discussion

#### Mutation and haplotype analysis

NPC is one of the most frequent lysosomal diseases found in Spain, with the identification of 2–6 new cases each year. This implies the search for heterozygous detection and prenatal

Table 2. Characteristics of mutations identified in the NPC1 gene of Spanish NPC patients

Mutation	Nucleotide change	Exon/intron	Location NPC1 protein <sup>a</sup>	Effect on NPC1 protein	Conserved residues <sup>b</sup>	Reference <sup>c</sup>
p.R116X	c.346C > T	E4	Lumen A	Truncated 115 aa	Yes	20
p.T137M	c.410C > T	E4	Lumen A	Thr > Met	No	21
p.C177Y	c.530G > A	E5	Lumen A	Cys > Tyr	No	22
<b>p.W260X</b>	<b>c.780G &gt; A</b>	<b>E6</b>	<b>Lumen A</b>	<b>Truncated 259 aa</b>	<b>Yes</b>	<b>This article</b>
<b>p.V299fsX8</b>	<b>c.895_896insT</b>	<b>E7</b>	<b>Cytoplasm B</b>	<b>Frameshift. Truncated by 307 aa</b>	<b>No</b>	<b>This article</b>
<b>IVS7 + 1G &gt; A</b>	<b>g.31432G &gt; A</b>	<b>I7</b>	<b>-</b>	<b>Variable splicing. Frameshift</b>	<b>-</b>	<b>This article</b>
<b>p.R372W</b>	<b>c.1114C &gt; T</b>	<b>E8</b>	<b>Lumen C</b>	<b>Arg &gt; Trp</b>	<b>Yes</b>	<b>This article</b>
<b>p.S425X</b>	<b>c.1274C &gt; A</b>	<b>E8</b>	<b>Lumen C</b>	<b>Truncated by 424 aa</b>	<b>Yes</b>	<b>This article</b>
<b>p.P434L</b>	<b>c.1301C &gt; T</b>	<b>E8</b>	<b>Lumen C</b>	<b>Pro &gt; Leu</b>	<b>Yes</b>	<b>This article</b>
p.P474L	c.1421C > T	E9	Lumen C	Pro > Leu	No	23
<b>p.C479Y</b>	<b>c.1436G &gt; A</b>	<b>E9</b>	<b>Lumen C</b>	<b>Cys &gt; Tyr</b>	<b>Yes</b>	<b>This article</b>
<b>p.A558fsX11</b>	<b>c.1673_1674insG</b>	<b>E11</b>	<b>Lumen C</b>	<b>Frameshift. Truncated by 569 aa</b>	<b>Yes</b>	<b>This article</b>
<b>p.K576R</b>	<b>c.1727A &gt; G</b>	<b>E11</b>	<b>Lumen C</b>	<b>Lys &gt; Arg</b>	<b>No</b>	<b>This article</b>
<b>p.C645X</b>	<b>c.1935T &gt; A</b>	<b>E12</b>	<b>Cytoplasm D, SSD</b>	<b>Truncated by 644 aa</b>	<b>No</b>	<b>This article</b>
p.V664M	c.1990G > A	E13	TM IV, SSD	Val > Met	No	8
<b>p.V727F</b>	<b>c.2179G &gt; T</b>	<b>E14</b>	<b>Cytoplasm F, SSD domain</b>	<b>Val &gt; Phe</b>	<b>Yes</b>	<b>This article</b>
<b>p.M754K</b>	<b>c.2261T &gt; A</b>	<b>E15</b>	<b>TM IV, SSD domain</b>	<b>Met &gt; Lys</b>	<b>Yes</b>	<b>This article</b>
p.Q775P	c.2324A > C	E15	TM IV, SSD	Gln > Pro	Yes	24
<b>p.C779fsX9</b>	<b>c.2336dupT</b>	<b>E15</b>	<b>TM IV, SSD</b>	<b>Frameshift. Truncated 788 aa</b>	<b>Yes</b>	<b>This article</b>
<b>p.S865L</b>	<b>c.2594C &gt; T</b>	<b>E17</b>	<b>Lumen I</b>	<b>Ser &gt; Leu</b>	<b>Yes</b>	<b>This article</b>
<b>p.A926T</b>	<b>c.2776G &gt; A</b>	<b>E18</b>	<b>Lumen I</b>	<b>Ala &gt; Thr</b>	<b>Yes</b>	<b>This article</b>
p.W942C	c.2819G > T	E19	Lumen I	Trp > Cys	Yes	22
p.D944N	c.2830C > A	E19	Lumen I	Asp > Asn	Yes	21
<b>p.D948H</b>	<b>c.2842G &gt; C</b>	<b>E19</b>	<b>Lumen I</b>	<b>Asp &gt; His</b>	<b>Yes</b>	<b>This article</b>
<b>p.V959E</b>	<b>c.2876T &gt; A</b>	<b>E19</b>	<b>Lumen I</b>	<b>Val &gt; Leu</b>	<b>Yes</b>	<b>This article</b>
<b>p.N961_F966 delinsS</b>	<b>c.2897delATATC ACTGACCAGTT and c.2897_2898insG</b>	<b>E19</b>	<b>Lumen I</b>	<b>In frame mutation</b>	<b>Yes</b>	<b>This article</b>
<b>p.G993fsX3</b>	<b>2973_78delG</b>	<b>E20</b>	<b>Lumen I</b>	<b>Loss of 5 aa and gain of 1 aa</b>	<b>Yes</b>	<b>This article</b>
p.P1007A	c.3019C > G	E20	Lumen I	Frameshift Truncated 995 aa	Yes	25
p.A1035V	c.3104C > T	E21	Lumen I	Pro > Ala	Yes	22
<b>p.T1036K</b>	<b>c.3107C &gt; A</b>	<b>E21</b>	<b>Lumen I</b>	<b>Ala &gt; Val</b>	<b>Yes</b>	<b>This article</b>
<b>p.R1059X</b>	<b>c.3175C &gt; T</b>	<b>E21</b>	<b>Lumen I</b>	<b>Thr &gt; Lys</b>	<b>Yes</b>	<b>This article</b>
p.I1061T	c.3182T > C	E21	Lumen I	Truncated 1058 aa	Yes	25
<b>p.T1066N</b>	<b>c.3197C &gt; A</b>	<b>E21</b>	<b>Lumen I</b>	<b>Ile &gt; Thr</b>	<b>Yes</b>	<b>This article</b>
<b>IVS21 + 2insG</b>	<b>g.5187_5188 insG</b>	<b>i21</b>	<b>-</b>	<b>Thr &gt; Asn</b>	<b>Yes</b>	<b>This article</b>
<b>p.N1156I</b>	<b>c.3467A &gt; T</b>	<b>E22</b>	<b>TM XI</b>	<b>Variable splicing. Frameshift</b>	<b>-</b>	<b>This article</b>
p.N1156S	c.3467A > G	E22	TM XI	Asn > Ile	Yes	6
p.F1221fsX20	c.3662delT	E24	Cytoplasm N	Asn > Ser	Yes	22
<b>p.F1224L</b>	<b>c.3672C &gt; G</b>	<b>E24</b>	<b>Cytoplasm N</b>	<b>Frameshift Truncated 1241 aa</b>	<b>Yes</b>	<b>This article</b>
				<b>Phe &gt; Leu</b>	<b>Yes</b>	<b>This article</b>

SSD, sterol-sensing domain.

Novel mutations are given in bold.

<sup>a</sup>Protein domains are indicated as determined by Carstee et al. (6).

<sup>b</sup>Species similarities of domains were determined using PolyPhen: prediction of functional effect of human SNPs for NPC1 sequences from human, cat, rabbit, pig, mouse, *Drosophila melanogaster*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*.

<sup>c</sup>For described mutations only the first citations are given.

Table 3. Polymorphism frequencies in NPC patients and in Spanish control population

Polymorphism	Exon	Number alleles in controls (frequency)	Number alleles in patients (frequency)	Reference
Y129Y (c.387T > C)	4	6/100 (6%)	2/80 (2.5%)	26
H215R (c.644A > G)	6	29/100 (29%)	11/80 (13.75%)	26
V562V (c.1686G > A)	11	0/100	1/80 (1.25%)	This article
A580A (c.1740C > G)	11	0/100	1/80 (1.25%)	This article
I642M (c.1926C > G)	12	25/100 (25%)	18/80 (22.5%)	27
I858V (c.2572A > G)	17	0/100	4/80 (5%)	26
A1187A (c.3561G > T)	23	0/100	1/80 (1.25%)	This article
R1266Q (c.3797G > A)	25	7/100 (7%)	1/80 (1.25%)	26

diagnosis in an increasing number of families with an NPC-affected member. It would also be desirable to better characterize the patients, in terms of prognosis and in view of future therapeutic trials. We therefore performed a search for

molecular defects in 40 unrelated patients, diagnosed between years 1988 and 2003, and thus representative of Niemann-Pick disease in Spain. This makes our study rather unique, as most other reports comprised a mixed population

Table 4. Genotypes and polymorphism analysis of Spanish NPC patients

Patient	Genotype	Y129Y	H215R	I642M	I858V	R1266Q
1	(p.Q775P) + (p.N1156I)	-/-	-/-	-/-	-/-	-/-
2	(IVS21 + 2insG) + ?	-/-	+/-	+/-	+/-	-/-
3	(p.C177Y) + ?	-/-	-/-	+/+	-/-	-/-
4	(p.W942C) + (p.N961_F966delinsS)	-/-	-/-	+/-	-/-	-/-
5	(p.I1061T) + (p.N1156S)	-/-	-/-	-/-	-/-	-/-
6	(p.I1061T) + (p.I1061T)	-/-	-/-	-/-	-/-	-/-
7	(p.V299fsX8) + ?	-/-	-/-	-/-	-/-	-/-
8	(p.I1061T) + (p.I1061T)	-/-	-/-	-/-	-/-	-/-
9	(p.Q775P) + (p.Q775P)	-/-	-/-	-/-	-/-	-/-
10	(p.A1035V) + (p.A1035V)	-/-	-/-	-/-	-/-	-/-
11	(p.S865L) + (p.R1059X)	-/-	-/-	-/-	-/-	-/-
12	(p.Q775P) + ?p.F1224L)	+/-	-/-	-/-	-/-	-/-
13	? + ?	-/-	-/-	-/-	-/-	-/-
14	(p.A558fsX11) + (p.A558fsX11)	-/-	-/-	-/-	-/-	-/-
15	(p.C177Y) + (p.C177Y)	-/-	-/-	+/+	-/-	-/-
16	(p.R372W) + (p.T1036K)	-/-	-/-	-/-	-/-	-/-
17	(p.G993fsX3) + (p.G993fsX3)	-/-	-/-	-/-	-/-	-/-
18	(p.C177Y) + (p.V959E)	-/-	-/-	+/+	-/-	-/-
19	(p.V727F) + (p.A1035V)	-/-	-/-	+/-	-/-	-/-
20	(p.G993fsX3) + (p.I1061T)	-/-	-/-	+/-	-/-	-/-
21	(p.S425X) + ?	-/-	+/-	-/-	-/-	-/-
22	(p.I1061T) + (p.W260X)	-/-	-/-	-/-	-/-	-/-
23	(p.P1007A) + (p.R1059X)	-/-	+/-	+/-	-/-	-/-
24	(p.P434L) + (p.M754K)	-/-	-/-	+/+	-/-	-/-
25	(IVS7+1G > A) + (p.W942C)	-/-	-/-	+/-	-/-	-/-
26	(p.S425X) + (p.P474L)	-/-	-/-	-/-	-/-	-/-
27	(p.D944N) + (p.P1007A)	-/-	-/-	+/-	-/-	-/-
28	(p.R116X) + (p.Q775P)	-/-	+/+	-/-	-/-	-/-
29	(p.C479Y) + ?	+/-	-/-	+/-	-/-	-/-
30	(p.P1007A) + (p.T1066N)	-/-	+/+	-/-	+/-	-/-
31	(p.A926T) + (p.D948H)	-/-	+/-	+/-	-/-	-/-
32	(p.C779fsX9) + (p.P1007A)	-/-	+/-	-/-	-/-	-/-
33	(p.C177Y) + (p.V664M)	-/-	-/-	+/-	-/-	-/-
33'	(p.C177Y) + (p.V664M)	-/-	-/-	+/-	-/-	-/-
34	(p.I1061T) + (p.T137M)	-/-	-/-	-/-	-/-	+/-
35	(p.R1059X) + (p.R1059X)	-/-	-/-	+/-	-/-	-/-
36	(p.N961_F966delinsS) + ?	-/-	+/-	-/-	-/-	-/-
37	(p.K576R) + (p.F1221fsX20)	-/-	-/-	-/-	-/-	-/-
38	(p.G1252R) + ?	-/-	-/-	+/-	+/-	-/-
39	(p.G993fsX3) + (p.G993fsX3)	-/-	-/-	-/-	-/-	-/-
40	(p.C645X) + ?	-/-	+/-	-/-	+/-	-/-

?, Unknown Mutation

(24) or a small number of patients (7, 20, 23) or did not provide clinical data (8). Using a strategy of SSCP plus sequencing of the abnormal patterns, and if necessary, sequencing all the exons and intron boundaries, we covered 70/80 mutant NPC1 alleles (86%), including 38 different mutations, 25 of which are novel and 12 previously described. The Spanish population, similarly to what has been reported for other countries, has significant allelic heterogeneity, in which only 10 mutations (C177Y, S425X, Q775P, W942C, N961\_F966delinsS, G993fsX3, P1007A, A1035V, R1059X and I1061T) have been found more than in one specific family. Moreover, the type and percentages of mutations in the study population do not differ significantly from those described for all mutations worldwide (16, 22, 24, 25). The majority of the molecular alterations in the patient series were missense 25/37 (68%), as occurs in the majority of genetic diseases (28). Additionally, we identified 5/37 (13.5%) frameshift mutations as a consequence of insertions and/or deletions, 4/37 nonsense mutations (11%), 2/37 (5%) splice site alterations and 1/37 (2.5%) in-frame deletion/insertions. Among the novel mutations, 56% (14/25) were amino acid substitutions, distributed throughout the gene. In this respect, four (R372W, P434L, C479Y and K576R) were located at the N-terminus of the protein, two (V727F and M754K) in the sterol-sensing domain and eight (S865L, A926T, D948H, V959E, T1036K, T1066N, N1156I and F1224L) at the C-Terminus of the protein, with a notable hot-spot in the cysteine-rich luminal loop (amino acids 855–1098), as described in other populations (8, 24, 25). Interestingly, the missense mutation, F1224L, and one frameshift mutation, F1221fsX20, represent the first mutant alleles described in the seventh luminal loop of the protein, which is largely a conserved domain. All these novel missense mutations seem to be functionally important, and although it should be confirmed by expression studies, several lines of evidence suggest that they could be pathogenic: (a) after exhaustive examination of the coding region of the NPC1 gene in the NPC1 patients, no other mutation was found in any of the cases; (b) none of the novel mutations were detected when 100 normal chromosomes were analysed; (c) all the amino acid residues were conserved in the NPC1 protein of different species, with the exception of K576R. The other 11/26 novel mutations are undoubtedly disease-causing mutations, as they produce a loss of five aminoacids and a gain of one aminoacid in the protein (N961\_F966delinsS), create a premature stop codon (W260X, S425X, C645X and

R1059X) or frameshifts (V299fsX8, A558fsX11, C778fsX10, G993fsX3 and F1221fsX20). For mutations IVS7 + 1G > A and IVS21 + 2insG, which affect the splice donor site in intron 7 and 21, respectively, it would be necessary to perform analysis at the mRNA level, to assure that these changes give rise to an abnormal splicing process.

In addition to the mutations found in the study population, the majority of patients showed one or more polymorphisms (Table 3). As occurs in Portuguese populations (22), there were no significant differences between the frequencies found in patients and in the control population. The existence of several polymorphisms and fixed haplotypes for specific mutations permitted us to infer their possible origins.

Mutation I1061T is the most prevalent mutation in the study population, accounting for 10% of the mutant alleles. While it appears to be the most frequent NPC1 mutant allele in all countries except Japan (16), its prevalence varies. In a study including patients originating from different countries with a majority from France, United Kingdom, Germany and North Africa an overall value of 18% was found (Millat et al. 1999). A lower prevalence has been reported in Portuguese (8%) (22), Italian (5%) (23) and German (7, 20) patients. Interestingly, while it is highly prevalent in Hispanic Americans from Colorado/New Mexico, due to a probable founder effect, its prevalence is much lower in other Hispanic Americans (26). Haplotype studies for this mutation in our Spanish patients population show that it is always associated with haplotype [–,–,–,–,–] with respect to the five polymorphic markers Y129Y (c.387T > C), H215R (c.644A > G), I642M (c.1926C > G), I858V (c.2572A > G) and R1266Q (c.3797G > A). Haplotype studies in the Portuguese population (22) show that the I1061T mutation is associated with haplotype [+,-,-,-] with respect to the polymorphisms Y129Y (c.387T > C), H215R (c.644A > G), I642M (c.1926C > G) and I858V (c.2572A > G). Taken together, these data suggest at least two different origins for this mutation.

In contrast, we have found the same haplotype association for the C177Y mutation, as observed in the Portuguese population: [–,–,+,-] with respect to the four polymorphic markers Y129Y (c.387T > C), H215R (c.644A > G), I642M (c.1926C > G) and I858V (c.2572A > G). Moreover, we extended the series including one more polymorphic marker, R1266Q (c.3797G > A), obtaining the haplotype [–,–,+,-,-]. We suggest a unique origin for this mutation, which on a worldwide scale has only been found in Portugal and Spain.

This situation is not unique for the C177Y mutation, because complete linkage disequilibrium was also observed for the novel mutation G992fsX4 and haplotype [-,-,-,-,-], suggesting a single Spanish origin for this mutation. Interestingly, although G992 is not a conserved domain, it seems to be a hot-spot, because two missense mutations have been found: G992W, which is known to be particularly prevalent in Nova Scotia (29) and is also reported in Portugal (22), and G992R, found in France (24). None of these mutations were found in Spain.

#### Genotype–phenotype correlation

As seen in all the NPC patient series described worldwide, the Spanish population exhibits considerable clinical and allelic heterogeneity. In our series, SI forms and J forms are the most frequent clinical presentations, both in equal percentages (30%). Interestingly, these values are similar to those obtained earlier in a compilation of 125 patients from mostly European and North African origin (4, 19). The Spanish population of NPC patients shows a high prevalence of SI forms. A similar percentage of this clinical form has also been found in Italy, in a small group of patients (23). This presentation with early neurological onset and rapid progression has been well documented in early European studies (30) but not in parallel studies from the USA (1). The frequency of the J form in Spain appears much lower than the 69% reported in Portuguese patients in a recent but possibly not comprehensive study (22).

Due to the high allelic heterogeneity of this disease, only a few genotype-biochemical/clinical phenotype correlations have been established. Among these correlations, we would like to introduce some considerations relating to mutations clearly correlated with the classical biochemical phenotype (I1061T, A1035V and Q775P). When the I1061T mutation presents in homozygosity, it has been suggested that it correlates with classical biochemical phenotype and juvenile clinical presentation (26). Our data confirms this observation (patient 6, Table 1) and extends the presentation of the first neurological symptom to before 5 years of age (patient 8, who began to display clumsiness at 3 years of age; Table 1). The A1035V mutation, identified for the first time in Portugal (22) and observed in patient 10, exhibits a correlation with the infantile presentation of the disease, from the phenotype observed in two homozygous patients. The Q775P mutation, affecting the sterol-sensing domain of the NPC1 protein, was previously

shown by studies in one homozygous patient to result in the complete absence of the protein, well in line with the severe neurological phenotype of the patient (24). Patient 9, homozygous for this mutation, displayed a similar clinical presentation of the disease, confirming the genotype/phenotype correlation. Q775P in combination with R116X or with N1156I (TM XI domain) also determined a severe disease. In combination with F1224L (7th luminal loop) (patient 12), however, it led to a juvenile onset neurological form.

Finally, P1007A and C177Y mutations were both claimed to induce a variant biochemical phenotype (22, 24), a finding challenged by Sun et al. (21) for P1007A. Our data (patients 23, 27, 30 and 32) support the conclusions of the French group regarding the P1007A mutation. Conversely, the C177Y mutation, found by us in homozygosity in one patient (case 15) and in heterozygosity in four patients (cases 1, 18, 33 and the sister of patient 33), presented in all our cases with the classical biochemical phenotype. These results clearly contradict those reported by Ribeiro et al. (22). What underlies the different results observed by Sun et al. or by Ribeiro et al. remains unclear. From our data (patient 15) as well as those in the Portuguese patient (22), C177Y indeed appears correlated with a LI neurological form of the disease. However, when in combination with the V664M mutation located in the sterol sensing domain, and somewhat unexpectedly as mutations in this domain are generally very deleterious, it led to an adult form (patients 33 and 33'). Valine-methionine substitutions generally induce less severe changes in the protein, and for example, homozygous NPC1 V950M was found to lead to a clear adult phenotype (24). This further demonstrates the limitations of predictions in compound heterozygotes, although genotype – phenotype correlations observed in NPC disease can be considered as relatively good compared with the situation in a number of other genetic metabolic disease.

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