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MUTATION IN BRIEF

HUMAN MUTATION

Mucopolysaccharidosis Type IIID: 12 New Patients and 15 Novel Mutations

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ABSTRACT: Mucopolysaccharidosis III D (Sanfilippo disease type D, MPS IIID) is a rare autosomal recessive lysosomal storage disorder previously described in only 20 patients. MPS IIID is caused by a deficiency of N-acetylglucosamine-6-sulphate sulphatase (GNS), one of the enzymes required for the degradation of heparan sulphate. So far only seven mutations in the *GNS* gene have been reported. The clinical phenotype of 12 new MPS IIID patients from 10 families was studied. Mutation analysis of *GNS* was performed in 16 patients (14 index cases). Clinical signs and symptoms of the MPS IIID patients appeared to be similar to previously described patients with MPS III. Early development was normal with onset of behavioral problems around the age of 4 years, followed by developmental stagnation, deterioration of verbal communication and subsequent deterioration of motor functions. Sequence analysis of the coding regions of the gene encoding GNS (*GNS*) resulted in the identification of 15 novel mutations: 3 missense mutations, 1 nonsense mutation, 4 splice site mutations, 3 frame shift mutations, 3 large deletions and 1 in-frame small deletion. They include the first missense mutations and a relatively high proportion of large rearrangements, which warrants the inclusion of quantitative techniques in routine mutation screening of the *GNS* gene. ©2010 Wiley-Liss, Inc.

KEY WORDS: Sanfilippo syndrome, mucopolysaccharidosis IIID, α-N-acetylglucosamine-6-sulphate sulphatase, α-N-acetylglucosamine-6-sulphatase, *GNS*, mutations

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INTRODUCTION

Mucopolysaccharidosis type III (MPS III, Sanfilippo disease) is an autosomal recessive neurodegenerative disorder characterized by progressive mental deterioration, only moderate physical abnormalities and death in the third decade of life. The clinical symptoms are caused by accumulation of excessive amounts of the glycosaminoglycan (GAG) heparan sulphate in various tissues. Defects of four different lysosomal enzymes involved in degradation of heparan sulphate are associated with the 4 known subtypes of Sanfilippo disease: types A, B, C and D. MPS IIIA is caused by deficiency of sulphamidase (SGSH), MPS IIIB by deficiency of α-N-acetylglucosaminidase (NAGLU), whereas deficient acetyl–CoA:α-glucosaminide N-acetyltransferase (HGSNAT) activity leads to MPS IIIC.

MPS IIID (*GNS* gene; MIM# 252940) is caused by a deficiency of α-N-acetylglucosamine-6-sulphate sulphatase (GNS, EC 3.1.6.14). GNS specifically cleaves a sulphate, bound at the 6-hydroxyl position moiety of N-acetylglucosamine from heparan and keratan sulphate (Kresse et al., 1980). However, keratan sulphate does not accumulate in MPS IIID, as the β-linked N-acetylglucosamine 6-sulphate can be cleaved off by β-hexosaminidase A (Kaplan and Wolfe, 1987; Kresse et al., 1980).

The different types of MPS III appear to be clinically indistinguishable, although suggestions have been made that MPS IIIA might have a more rapidly progressive course (van de Kamp et al., 1981).

Development during the first year of life is generally normal, after which behavioural problems and cognitive impairment lead to severe dementia (Valstar et al., 2008). Death usually supervenes before the 3rd decade of life and is often due to respiratory complications, although longer survival has been reported (van de Kamp et al., 1981).

Sanfilippo syndrome type D (Mucopolysaccharidosis type IIID, MPS IIID) was first reported in 1980 by Kresse et al. in a patient from East India (Kresse et al., 1980). Since then, only 19 MPS IIID patients have been reported (Beesley et al., 2003; Beesley et al., 2007; Coppa et al., 1983; del Canho H. et al., 1993; Gatti et al., 1982; Jansen et al., 2007; Jones et al., 1997; Kaplan and Wolfe, 1987; Ozand et al., 1994; Siciliano et al., 1991; Tylki-Szymanska et al., 1998). MPS IIID is the rarest subtype of MPS III in the Netherlands (Poorthuis et al., 1999).

The α-N-acetylglucosamine-6-sulphate sulphatase gene (*GNS*; MIM# 607664) was identified by Robertson et al. (Robertson et al., 1988). It consists of 14 exons (Mok et al., 2003) dispersed over approximately 43 kb and is located on chromosome 12 (Robertson et al., 1988). The predicted protein has 552 amino acids including a leader peptide of 36 amino acids (Robertson et al., 1988), a sulphatase domain (45-492, Swiss-Prot database, entry P15586), and contains 13 potential *N*-glycosylation sites (Robertson et al., 1988).

Only seven disease-causing mutations in MPS IIID patients have been reported until now, whereas large numbers of disease-causing mutations have been described for MPS IIIA, MPS IIIB and MPS IIIC (Valstar et al., 2008). In 2003, Beesley et al. and Mok et al. (Beesley et al., 2003; Mok et al., 2003) identified the first 2 disease-causing mutations in *GNS*. In 2007, another five mutations were reported (Beesley et al., 2007; Jansen et al., 2007). Here we report 12 new patients with MPS IIID, 15 novel *GNS* mutations in 16 patients and a literature review of MPS IIID.

MATERIALS AND METHODS

Patients

MPS IIID patients were diagnosed by demonstration of an isolated deficiency of GNS activity in fibroblasts and leukocytes, when available. An overview of all reported patient is shown in Table 1. Clinical data on the 12 newly reported patients are summarised in Table 2, including patient 12, which was originally reported in Dutch (del Canho H. et al., 1993). Only partial clinical data could be obtained from patient 6 and no data from patient 8. Three Dutch patients (patients 1, 2 and 12) and their parents or legal representatives were seen and interviewed by a clinical geneticist (MWW). For all other patients, physicians who had requested the diagnostic studies were approached to provide medical records of their patients.

GAG determination and enzyme analysis

GAG concentration (mg/mmol creatinine) in urine samples was determined using dimethylmethyleneblue as described by De Jong et al (de Jong et al., 1992). Precipitation of GAG for two-dimensional electrophoresis was performed according to Whiteman and Henderson (1977) and GAG electrophoresis according to Abeling et al (Abeling et al., 1974). The enzyme activity of α-N-acetylglucosamine-6-sulphatase was determined with the fluorogenic substrate (Moscerdam Substrates) as described (He et al., 1993).

Molecular studies

We report mutation analysis of 16 biochemically proven MPS IIID patients (14 index cases) of various ethnic origins. They include all 12 new patients as well as patients 13, 14, 15 and 16, which were previously reported as MPS IIID patients, but without a *GNS* genotype, except a heterozygous mutation in patient 16.

Genomic DNA from patients and first-degree relatives, when available, was isolated from peripheral blood using the Chemagic DNA Blood kit (Chemagen) or from cultured fibroblasts using PureGene® Cell & Tissue kit (Gentra systems). Mutation analysis was performed in 16 patients.

RNA isolation was performed from cultured fibroblasts of four individuals (RNAzol^{TM} B, CAMPRO Scientific) to obtain cDNA (Superscript II RT, Invitrogen).

For genomic and cDNA sequencing reactions, intronic and exonic primers were designed, (details available on request).

Direct sequencing of the 14 exons and exon/intron boundaries of *GNS* (NM_002076.3 http://www.ncbi.nlm.nih.gov/nuccore/NM_002076.3 and NP_002067.1 http://www.ncbi.nlm.nih.gov/protein/NP_002067.1) was performed using the BigDye terminator chemistry (Applied Biosystems). PCR products were loaded on an ABI 3730 XL automatic DNA sequencer and analyzed with SeqScape software version 2.6 (Applied Biosystems). For notation of the mutations the Reference sequence NM_002076.3
http://www.ncbi.nlm.nih.gov/nuccore/NM_002076.3 (available at the NCBI
database, _build 37.1_) was used. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The frequency of the novel variants was assessed in a panel of 188 alleles from unrelated control individuals (Italian origin) for the c.1019A>G mutation detected in an Italian patient and in 188 alleles (Dutch origin) for the mutations detected in patients of Dutch and Polish origin (c.281G>T and c.1253G>A).

We used four computer programs to predict the possible consequences on splicing of any non-truncation sequence change within exons and in introns within the area analysed (at least 30 nucleotides of the intron/exon boundaries)**:** Splice Site Prediction by Neural Network http://www.fruitfly.org/seq_tools/splice.html, Splice Site Finder http://www.genet.sickkids.on.ca/bioinfo_resources/software.html, NetGene2, http://www.cbs.dtu.dk/services/NetGene2/ and SpliceView program, http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html. Furthermore, three prediction programs: PolyPhen (http://genetics.bwh.harvard.edu/pph/index.html), Pmut (http://mmb2.pcb.ub.es:8080/PMut/) and SIFT (http://sift.jcvi.org/) were used to evaluate the possible effect of amino acid changes on the protein function.

For the detection of large rearrangements “real-time” quantitative PCR (Q-PCR) was applied using the TaqMan technology on an ABI 7500 instrument followed by (long range) PCR and characterisation of the breakpoints by direct sequencing (details available on request).

Table 1. Overview of all reported MPS IIID patients

Patient	M/F	Ancestry	Age (years)#	Consanguinity	Relatives	Literature references: clinical [mutations]
1	M	Dutch/African/Indonesian	23	Yes	Brother of patient 2	new patient [Table 4]
2	M	Dutch/African/Indonesian	[†24]	Yes	Brother of patient 1	new patient [Table 4]
3	M	French	[†18]	No	Brother of patient 4	new patient [Table 4]
4	M	French	[†29]	No	Brother of patient 3	new patient [Table 4]
5	F	Turkish	14	Yes	-	new patient [Table 4]
6	F	Turkish	15	Yes	-	new patient [Table 4]
7	M	Iranian	15	Yes	Cousin of patient 8	new patient [Table 4]
8	M	Iranian	NA	Yes	Cousin of patient 7	new patient [Table 4]
9	M	Turkish	7	NA	-	new patient [Table 4]
10	F	Bulgarian	9	No	-	new patient [Table 4]
11	M	Turkish	13	Yes	-	new patient [Table 4]
12	M	Dutch	[†21]	No	-	Del Canho et al, 1993 [Table 4]
13	F	Italian	4	No	-	Gatti et al,1982 [Table 4]
14	F	Italian	10	Yes	-	Gatti et al,1982 [Table 4]
15	M	Polish	11	Yes	-	Tylki-Szymanska et al, 1998 [Table 4]
16	M	Belgian	15	No	-	Jansen et al, 2007 [*ibid* and Table 4]
17	M	Pakistanian	3	Yes	-	Beesley et al, 2003 [*ibid*]
18	M	Caucasian	[†14]	NA	-	Jones et al, 1997 [not reported]
19	F	Caucasian	[†17]	NA	-	Jones et al, 1997 [not reported]
20	F	Saudi Arabian	7	Yes	-	Ozand et al,1994 [not reported]
21	M	Italian	9	No	-	Coppa et al, 1983 [not reported]
22	F	Italian	5	No	-	Coppa et al, 1983 [not reported]
23	F	Italian	19	Yes	Sister of patient 24	Siciliano et al, 1991 [not reported]
24	F	Italian	14	Yes	Sister of patient 23	Siciliano et al, 1991 [not reported]
25	M	Italian	11 (Kaplan), 31 (Jansen)	No	Brother of patient 26	Kaplan and Wolfe, 1987 [Jansen et al, 2007]
26	M	Italian	3 (Kaplan), 23 (Jansen)	No	Brother of patient 25	Kaplan and Wolfe, 1987 [Jansen et al, 2007]
27	M	Indian	7	NA	-	Kresse et al, 1980 [Mok at al 2003]
28	M	Italian/Brazilian	10	No	Brother of patient 29	Beesley et al, 2007[*ibid*]
29	F	Italian/Brazilian	7	No	Sister of patient 28	Beesley et al, 2007[*ibid*]
30	M	Italian	7	Yes	-	Beesley et al, 2007[*ibid*]
31	F	Turkish/English	15	No	-	Jansen et al, 2007[*ibid*]

#[†age] indicates patient is deceased .

RESULTS

Patients

The early clinical features of the 12 new patients are listed in Table 2.

Early development was normal in most patients with onset of behavioral problems around the age of 4 years, followed by developmental stagnation, deterioration of verbal communication and subsequent deterioration of motor functions. Patients showed extensive behavioral problems and mild somatic disease. Within this general pattern of Sanfilippo syndrome, however, considerable variation was found in the progress of deterioration of speech and motor functions. For example: patient 5 was almost completely bed-ridden at the age of 14, while patient 1, at the age of 23 years, was still able to walk considerable distances.

Four patients were deceased at the time of the study (patients 2, 3, 4 and 12) at the age of 24, 18, 29 and 21 years respectively. The cause of death could only be retrieved in patient 2 and 12 who both died of pneumonia at the age of 24 and 21 years.

GAG screening and Enzyme analysis

All patients had clinical symptoms suggesting MPS III and/or had abnormal urinary mucopolysaccharides (GAGs). In 2 centres (Erasmus MC and UMC Nijmegen), GAG excretion was analysed in 7 of the 16 patients and found to be comparable to that in patients with MPS III types A, B and C (Fig. 1).

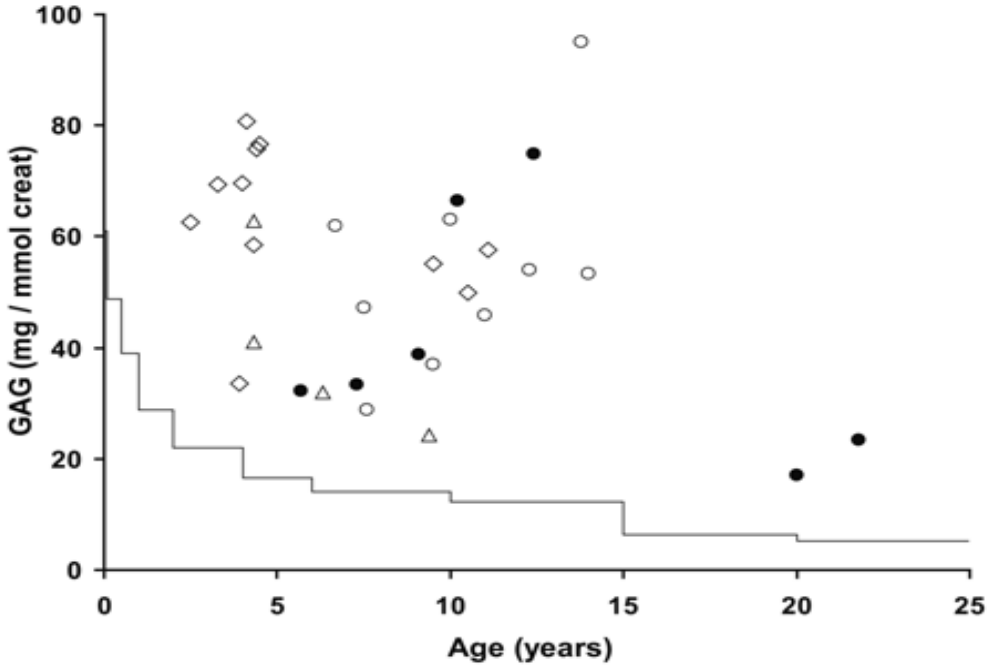


Figure 1. GAG concentration in urine samples of 7 MPS IIID patients (●), compared to MPS IIIA (◇; n=11), B (○; n=9) and C (Δ; n=4). Broken line marks the upper limit of normal reference values. MPS IIID patient identification (age; patient No.): (5.7 y; patient 7) (7.3 y; patient 12) (9.1 y; patient 10) (10.2 y; patient 5) (12.4 y; patient 8) (20 y; patient 1) (21.8 y; patient 2).

Two-dimensional electrophoresis showed increased heparan sulphate concentrations, typical of MPS III (data not shown). In all the 16 MPS IIID patients studied, an isolated deficiency of GNS activity was found (residual activity <2.5 %; Table 3). The enzyme activities of SGHS (MPS IIIA), NAGLU (MPS IIIB) and HGSNAT (MPS IIIC) were all within the control ranges for those enzymes.

Molecular studies

A total of 15 novel *GNS* mutations were detected, in homozygous (n=12) or compound heterozygous state (n=4) in 16 patients (Table 4). Each family had private mutations and the majority of exons as well as introns were involved. Various types were observed: 3 missense mutations (c.281G>T [p.S94I], c.1019A>G [p.K340R], c.1253G>A [p.G418E]), 1 nonsense mutation (c.1162A>T [p.K388X]), 4 splice site mutations (c.875+2delT, c.1097_1098+1delAGGinsGGT, c.1309-2A>G, c.1420-2A>G), 3 frame shift mutations (c.59_66del8, c.109dupG, c.1231dupG), 3 large deletions (Del EX1+, Del EX6,7 (c.625-637_875+6del3346ins8), delEX9-14 (c.1046_1659+16210del36529ins9)) and 1 in-frame small deletion (c.911_919del9 [p.E304_L306del]).

In the 2 French patients (patients 3 and 4) 3 heterozygous nucleotide changes were detected. Two of these were c.1097A>G and c.1098+1G>T at the exon 9 - intron 9 boundary. The other change was c.1420-2A>G. DNA from the father was heterozygous only for the c.1097A>G + c.1098+1G>T sequence changes, indicating their presence in *cis* in patients 3 and 4. Hence, we describe these changes as a single sequence variant, c.1097_1098+1delAGG insGGT. This mutation is predicted to shift the donor splice site by one nucleotide (Fig. 2) and to create a frame shift starting at codon 366, with the new frame ending in a stop codon 7 positions downstream. PCR reactions using cDNA from these patients as template and a primer pair covering exon 9, revealed two bands of 473 bp (normal) and approximately 430 bp.

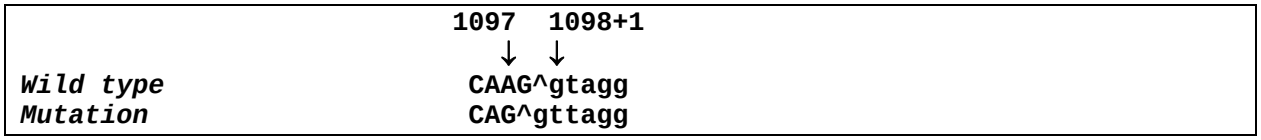


Figure 2. The splice site at the border of exon 9 and intron 9 and the effect of c.1097_1098+1delAGGinsGGT. The wild-type nucleotide numbers are shown above the sequences. This del-ins creates a frame shift starting at codon 366 with the new frame ending in a stop codon 7 positions downstream.

The other mutation in patients 3 and 4, c.1420-2A>G, is at a 100% conserved splice site position. The 4 computer programs that apply different algorithms to detect splicing sites in human genes, predicted the abolition of the normal splice site on both alleles of the French patients and the introduction of new cryptic splice sites.

For the c.1420-2A>G variant, cDNA amplified with a primer pair spanning the region from the middle of exon 12 to the 3' UTR region, showed two extra products of 450 and 320 bp, besides the normal band of 495 bp. This indicated that c.1420-2A>G alters normal splicing of exon 13. Direct sequencing of the 320 bp PCR product revealed a double sequence starting at the first base of exon 13. The extra sequence corresponded to exon 14, indicating that exon 13 was missing in one of the alleles. According to the splicing prediction programs, the 450 bp fragment is lacking the first part of exon 13 due to an activation of a cryptic splice site at the beginning of exon 13.

The newly detected missense mutations c.281G>T, c.1019A>G and c.1253G>A, were not found in 188 control alleles from unrelated individuals.

Table 2. Main clinical features 12 new patients

Patient	Age at study#	Birth weight (grams)	Early psychomotor development	Age at onset	First signs	Age at diagnosis (years)
1	23	3200	Normal	4 years	Delay in speech development	7
2	[†24]	3250	Normal	4 years	Developmental delay, behavioral problems	10
3	[†18]	4070	Normal	2.5 years	Delay in speech development, behavioral problems	14
4	[†29]	4080	Normal	4 years	Behavioral problems	16
5	14	no data	no data	7 years	Developmental delay, walking problems, coarse facial features	9
6	15	no data	Normal	no data	Growth failure, walking problems, coarse facial features	14
7	15	3100	Normal	5 months	Hearing problems	6
8	no data					
9	7	4350	Normal	4 years	Developmental delay (speech), dysmorphic features	4
10	9	3550	Normal	3 years	Developmental delay (mainly speech), behavioral problems	9
11	13	3300	Normal	2.5 years	Developmental delay (mainly speech)	10
12	[†21]	4000	Normal	5 years	Delay in speech development, learning difficulties with visual problems and dysmorphic features	7

#[†age] indicates patient is deceased

Table 3. α-N-acetylglucosamine-6-sulphate sulphatase (GNS) activity in leukocytes and fibroblasts.

	Leukocytes (nmol/24h/mg)	Fibroblasts (nmol/17h/mg)
Normal range	10–32 (n=106)	36–129 (n=122)
MPS IIID patients	0 (n=4)	0–2.2 (n=16)

DNA of patients 5 and 9 consistently failed to yield PCR products of one or more exons and homozygous deletions were suspected. Subsequent Q-PCR analysis suggested homozygous deletions encompassing exon 1 (patient 9) and exons 6 and 7 (patient 5). The (intronic) boundaries of del EX6,7 were determined by direct sequencing and this gene defect was designated c.625-637_875+6del3346ins8. The breakpoints of the suspected deletion involving exon 1 remain to be determined. In one of the patients described by others [Jansen et al., 2007], only one mutant *GNS* allele had been defined (patient 16). The application of Q-PCR and direct sequencing revealed a large (> 36 kb) heterozygous deletion starting in exon 9 and extending beyond the last exon accompanied by a 9 nucleotide insertion (c.1046_1659+16209del36535ins9) showing that this patient is a compound heterozygote for two deleterious gene defects. Including the homozygous deletion previously found by Beesley et al., del EX2,3 (c.192+2488_296del8723), and assuming that the observations on exon 1 in patient 9 will be shown to reflect a deletion, in this cohort the allele frequency of large rearrangements in the *GNS* gene is as high as 25%.

DISCUSSION

Clinically, the new MPS IIID patients described here seem to follow the general pattern of Sanfilippo syndrome types A, B and C as previously described (Meyer et al., 2007; Ruijter et al., 2008; Valstar et al., 2008; van de Kamp et al., 1981). Development during the first year of life is normal, after which behavioral problems and a progressive mental retardation leading to severe dementia become apparent. The variation in the progressive deterioration of speech and motor functions, as indicated in patients 5 and 1, was also mentioned by van de Kamp et al. (van de Kamp et al., 1981) for MPS types A, B, and C and by Siciliano et al. (Siciliano et al., 1991), reporting two sisters with MPS IIID. MPS IIID is the rarest subtype of MPS III with only 31 patients from 26 families reported worldwide, including the 12 new patients described here. Overall, from 26 MPS IIID families reported until now, 31 % (8 of 26) are from Italian origin and 19 % (5 of 26) from Turkish origin (Beesley et al., 2007; Coppa et al., 1983; Gatti et al., 1982; Jansen et al., 2007; Kaplan and Wolfe, 1987; Siciliano et al., 1991), however, no founder mutations have been identified.

GNS, the gene encoding α-N-acetylglucosamine-6-sulphate sulphatase, was identified as early as 1988, but it took 15 years until the first *GNS* mutations were reported: a homozygous deletion of a single nucleotide (c.1169delA) in exon 9 (Beesley et al., 2003) and a homozygous stop codon mutation c. 1063C>T [p.R355X] in exon 10 (Mok et al., 2003). In 2007, another five mutations were reported (Beesley et al., 2007; Jansen et al., 2007). Here we identified 15 novel mutations (Table 4).

Overall, the 22 different human *GNS* mutations include 3 missense mutations (c.281G>T, c.1019A>G, c.1253G>A), 4 nonsense mutations (c.814C>T, c.1063C>T, c.1162A>T, c.1168C>T), 5 splice site mutations (c.876-2A>G, c.875+2delT, c.1097_1098+1delAGG insGGT, c.1309-2A>G, c.1420-2A>G), 5 frame shift mutations (c.59_66del8, c.109dupG, c.1138_1139insGTCCT, c.1169delA, c.1231dupG), 4 large deletions (Del EX1+, Del EX2,3 (c.192+2488_296del8723), Del EX6,7 (c.625-637_875+6del3346ins8) Del EX9-14 (c.1046_1659+16210del36529ins9) and 1 deletion of 3 amino acids (c.911_919del9).

Table 4. Mutations in MPS IIID patients

Patient	Mutation (1)	Mutation (2)	Effect (1)	Effect (2)	exon or intron	Literature Ref. mutations
1	**c.281G>T**	**c.281G>T**	**p.S94I**	**p.S94I**	exon 3	novel
2	**c.281G>T**	**c.281G>T**	**p.S94I**	**p.S94I**	exon 3	novel
3	**c.1097_1098+1delAGGinsGGT**	**c.1420-2A>G**	**Splicing/ p.K366fs**	**Splicing**	exon 9 + intron 9, intron 12	novel
4	**c.1097_1098+1delAGGinsGGT**	**c.1420-2A>G**	**Splicing/ p.K366fs**	**Splicing**	exon 9+ intron 9, intron 12	novel
5	**c.625-637_875+6del3346ins8**	**c.625-637_875+6del3346ins8**	**Large intragenic deletion**	**Large intragenic deletion**	exons 6 +7; introns 5-7	novel
6	**c.109dupG**	**c.109dupG**	**p.V37fs**	**p.V37fs**	exon 1	novel
7	**c.1162A>T**	**c.1162A>T**	**p.K388X**	**p.K388X**	exon 10	novel
8	**c.1162A>T**	**c.1162A>T**	**p.K388X**	**p.K388X**	exon 10	novel
9	**del ex1 +**	**del ex1 +**	**Deletion of exon 1, with unknown involvement of upstream region[a]**	**Deletion of exon 1, with unknown involvement of upstream region[a]**	exon 1 +	novel
10	**c.1309-2A>G**	**c.1309-2A>G**	**Splicing**	**Splicing**	intron 11	novel
11	**c.1231dupG**	**c.1231dupG**	**p.D411fs**	**p.D411fs**	exon 11	novel
12	**c.59_66del8**	**c.875+2delT**	**p.P20fs**	**Splicing**	exon 1 / intron 7	novel
13	**c.911_919del9**	**c.911_919del9**	**p.E304_L306del**	**p.E304_L306del**	exon 8	novel
14	**c.1019A>G**	**c.1019A>G**	**p.K340R**	**p.K340R**	exon 9	novel
15	**c.1253G>A**	**c.1253G>A**	**p.G418E**	**p.G418E**	exon 11	novel
16	c.876-2A>G	**c.1046_1659+16210del36529ins9**	Splicing	**Large intragenic deletion**	intron 7,exons 9-14, introns 9-13, 3'UTR	Jansen et al. 2007 / novel
17	c.1169delA	c.1169delA	p.Q390fs	p.Q390fs	exon 10	Beesley et al. 2003
25	c.1168C>T	c.1168C>T	p.Q390X	p.Q390X	exon 10	Jansen et al. 2007
26	c.1168C>T	c.1168C>T	p.Q390X	p.Q390X	exon 10	Jansen et al. 2007
27	c.1063C>T	c.1063C>T	p.R355X	p.R355X	exon 9	Mok et al 2003
28	c.192+2488_296del8723	c.192+2488_296del8723	Large intragenic deletion	Large intragenic deletion	exons 2 +3	Beesley et al. 2007
29	c.192+2488_296del8723	c.192+2488_296del8723	Large intragenic deletion	Large intragenic deletion	exons 2 +3	Beesley et al. 2007
30	c.814 C>T	c.814 C>T	p.Q272X	p.Q272X	exon 7	Beesley et al. 2007
31	c.1138_1139insGTCCT	c.1138_1139insGTCCT	p.D380fs	p.D380fs	exon 10	Jansen et al. 2007

[a] Characterisation in progress



Figure 3. Alignment of three regions of the GNS protein and the three highly conserved amino acids of the missense mutations found in MPS IIID patients (shown above the sequences).

All nonsense mutations and insertions or deletions causing frame-shifts were considered to be pathogenic. They result in truncated or degraded protein products or may be subject to nonsense-mediated messenger RNA decay (Kuzmiak and Maquat, 2006; Lualdi et al., 2006).

All 3 novel missense mutations affect highly conserved amino acids (Fig. 3) within the sulphatase domain (amino acids 45–492; Swiss Prot, PFam databases), suggesting their pathogenicity. The serine of p.S94I is part of the tri-peptide Pro-Ser-Arg, that is identical in four sulphatases (GNS, ARSA, ARSB, and ARSC), indicating the importance of this domain for sulphatase activity (Robertson et al., 1992). The novel in-frame deletion of three amino acids, c.911_919del9, is also located within the sulphatase domain of the protein. These four sequence changes are likely to be pathogenic, although this has not been corroborated by in vitro studies on their effects on GNS activity.

All four novel splice site mutations were predicted by computer analysis and two of them were confirmed by cDNA studies.

It is remarkable that 18% (4/22) of the mutations known to date in this cohort are large deletions. This high proportion is unprecedented among genes involved in lysosomal enzymopathies (Human Gene Mutation Database; www.hgmd.cf.ac.uk). The relative paucity of large rearrangements observed in other genes may also reflect a lack of routine application of quantitative techniques in the recent past. Obviously, heterozygous large deletions and insertions are missed during sequence analysis. When a heterozygous small sequence change is found in an exon that is part of a large rearrangement, the resulting pattern is apparently homozygous, which will only be noticed if one of the parents "fails" to be heterozygous. A correct and diagnostically reliable interpretation of mutation analysis in the index patient relies on the availability of parental DNA samples, as was also demonstrated by the observed segregation of c.1097_1098+1delAGG insGGT and c.1420-2A>G (patients 3 and 4).

Several 'homozygous patterns' for novel small sequence changes were found in our study (Table 4). They are believed to reflect true homozygosity in those cases where consanguinity was reported, although this has not been rigorously proven (Table 1). The apparent homozygosity of patients 6, 10 and 13, on the other hand, may represent regional founder mutations, but it is advisable to test their parents to exclude large heterozygous gene defects before any diagnostic application of mutation analysis is done.

In conclusion, we here describe the largest cohort of Sanfilippo IIID patients to date and report 15 novel mutations in *GNS*. The relatively high proportion of large rearrangements warrants the inclusion of quantitative techniques in routine mutation screening of the *GNS* gene.

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