

# Human $\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -NAGA) deficiency: new mutations and the paradox between genotype and phenotype

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## Abstract

Up to now eight patients with  $\alpha$ -NAGA deficiency have been described. This includes the newly identified patient reported here who died unexpectedly aged 1½ years of hypoxia during convulsions; necropsy was not performed.

Three patients have been genotyped previously and here we report the mutations in the other five patients, including two new mutations (S160C and E193X). The newly identified patient is consanguineous with the first patients reported with  $\alpha$ -NAGA deficiency and neuroaxonal dystrophy and they all had the  $\alpha$ -NAGA genotype E325K/E325K.

Clinical heterogeneity among patients with  $\alpha$ -NAGA deficiency is extreme. Two affected sibs, homozygotes for E325K, are severely affected and have the signs and symptoms of infantile neuroaxonal dystrophy, but prominent vacuolisation is lacking. The mildly affected patients (two families, three patients) at the opposite end of the clinical spectrum have clear vacuolisation and angiokeratoma but no overt neurological manifestations. Two of them are homozygous for the stop mutation E193X, leading to complete loss of  $\alpha$ -NAGA protein. These observations are difficult to reconcile with a simple genotype-phenotype correlation and we suggest that factors or genes other than  $\alpha$ -NAGA contribute to the clinical heterogeneity of the eight patients with  $\alpha$ -NAGA deficiency.

At the metabolic level, the patients with  $\alpha$ -NAGA deficiency are similar. The major abnormal urinary oligosaccharides are sialylglycopeptides of the O linked type. Our enzymatic studies indicated that these compounds are not the primary lysosomal storage products.

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Key words:  $\alpha$ -N-acetylgalactosaminidase;  $\alpha$ -NAGA deficiency; processing.

In 1987 two German infants were reported by Van Diggelen *et al*<sup>1</sup> with a profound deficiency of the lysosomal enzyme  $\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -NAGA). Schindler *et al*<sup>2</sup> reported that the patients had infantile neuroaxonal dystrophy without visceral involvement or dysmorphism. Two years later Kanzaki *et al*<sup>3-5</sup>

reported the second independent case of  $\alpha$ -NAGA deficiency with an entirely different clinical phenotype. This patient had a late onset disease with slight facial coarseness, disseminated angiokeratoma, and mild intellectual impairment (IQ=70), but without neurological symptoms. Unlike the infantile cases, this patient had prominent vacuolisation in all dermal cells, most prominently in vascular and lymphatic endothelial cells and eccrine sweat gland cells, but also in dermal neural cells and fibroblasts.<sup>5</sup> The glomerular endothelial cells but not the epithelial kidney cells are involved and also blood lymphocytes are vacuolised.<sup>6</sup>

These three patients shared, however, the abnormal urinary excretion of specific oligosaccharides. The major compounds are sialylglycopeptides of the O-glycosidic type with serine or threonine linked to the  $\alpha$ -N-acetylgalactosamine ( $\alpha$ GalNAc) moieties.<sup>7,8</sup> Using an  $\alpha$ GalNAc specific lectin, intralysosomal storage products were shown in lysosomes of cultured fibroblasts from the infantile patients.<sup>9</sup>

The  $\alpha$ -NAGA gene codes for 411 amino acids<sup>10</sup> and consists of nine exons.<sup>11</sup> Mutation analysis of the  $\alpha$ -NAGA gene showed the missense mutation E325K in the infantile cases<sup>13</sup>; both patients were homozygous for this mutation. The adult patient was found to be homozygous for the missense mutation R329W.<sup>14</sup>

Recently, four additional patients with  $\alpha$ -NAGA deficiency were reported in two independent families. The index case in the Dutch family, reported by de Jong *et al*,<sup>15</sup> had psychomotor retardation at the age of 4 years. Screening her sibs showed that a healthy sib with  $\alpha$ -NAGA deficiency has no overt clinical symptoms at the age of 3 years. Chabas *et al*<sup>16</sup> reported adult patients of Spanish origin with a mild phenotype consisting of angiokeratoma, slight dysmorphism, lymphoedema, and prominent vacuolisation in endothelial cells, resembling the case reported by Kanzaki *et al*.<sup>5</sup> In this paper we report the mutations of these four patients and describe a new German case with infantile  $\alpha$ -NAGA deficiency. The genotype and phenotype of the presently known eight cases of  $\alpha$ -NAGA deficiency are discussed.

## Patients and methods

### CASE REPORTS

The two German brothers, first reported by van Diggelen *et al*<sup>1</sup> and Schindler *et al*<sup>2</sup> with a severe infantile form of  $\alpha$ -NAGA deficiency, are

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Table 1 Oligonucleotide primers used to sequence and PCR amplify the coding regions of the  $\alpha$ -NAGA gene

Exon*	Primer sequences† and (location in gene*)	Length of fragment sequenced and (relative position to exon‡)
1	s: GCCTAAGGTTGAGGGCGG (1646–1663) as: GAAGGGCCAAAGCACTCCTT (1854–1873)	228 bp (–60§ to +31)
2	s: AGCTGTGGGGGCAACTGATA (3479–3498) as: GGCAAGGCTCATCAGGTGA (3683–3701)	223 bp (–11 to +2)
3	s: GAGGCAGGTTGTGGGTGA (4101–4118) as: GCCCTAAGCGAGGTAGGGTG (4389–4408)	308 bp (–29 to +32)
4	s: GTCCTGCTTGAGCCACTGT (4777–4796) as: CCTCCCCGTTTGCCTAC (5019–5036)	260 bp (–17 to +23)
5	s: CCAGACTGCCTCCTGCACCT (5244–5263) as: AGGTAAGGAGGCCTGGGTGT (5428–5447)	204 bp (–22 to +29)
6	s: CTGGTGCTGGACTCTGCCTT (6157–6176) as: GCAGGTCGTCACAGATGG (6386–6404)	248 bp (–7 to +18)
7	s: AGATAAGCTGGGGTGTGCTGGA (10832–10851) as: CTCAGCCCTCCCCTTCTT (9254–9272)	282 bp (–4 to +13)
8	s: CCCACAGAAATCTGAAGCCC (11163–11182) as: CTGTGCCCACTCTCATGG (11587–11605)	351 bp (–64 to +5)
9	s: CTGTGCCCACTCTCATGG (11587–11605) as: AGGCTCAGTGGTGCCACC (11795–11812)	226 bp (–12 to +9¶)

\* Exon number and position of nucleotide in  $\alpha$ -NAGA gene according to accession No M59199.

† s = sense strand; as = antisense. All primers shown in 5' to 3' direction.

‡ Relative position to exon; – = upstream and + = downstream of exon.

§ Position upstream of start codon in exon 1.

¶ Position downstream of stop codon in exon 9.

Table 2 The mutations in all patients with  $\alpha$ -NAGA deficiency

Patient	Nucleotide change* (exon/exon)	Protein change*	Reference
D1.1	G11005A (8/8)	E325K/E325K	13
D1.2	G11005A (8/8)	E325K/E325K	13
D2.1	G11005A (8/8)	E325K/E325K	Present paper
NL1.1	C4969G/G11005A (4/8)	S160C/E325K	Present paper
NL1.2	C4969G/G11005A (4/8)	S160C/E325K	Present paper
J1.1	C11017T (8/8)	R329W/R329W	14
E1.1	G5371T (5/5)	E193X/E193X	Present paper
E1.2	G5371T (5/5)	E193X/E193X	Present paper

\* Nucleotide and amino acid numbering is according to Yamauchi *et al.*<sup>10</sup> and accession No M59199.

The exons in which the mutation was found are shown in brackets.

designated D1.1 and D1.2. The patients have remained in a vegetative state for the last few years and are at present 12 and 11 years of age.

The newly diagnosed German patient D2.1 lived in the same village as the D1 family and is consanguineous with the German patients in family D1. The pregnancy and development up to the age of 6 months were normal. From 7 months, the patient had several convulsions, most of them during fever caused by upper respiratory tract infections. He died at the age of 18 months of hypoxia during a prolonged convulsion causing apnoea. During the last 3 months of his life, his development seemed to have stopped. He had never been admitted to a hospital and necropsy was not performed.

The two Dutch patients reported by de Jong *et al.*<sup>15</sup> are designated NL1.1 and NL1.2. Patient NL1.1 was last seen at the age of 3.8 years and was retarded. After the age of 2.4 years she had four convulsions, mostly associated with fever, despite treatment with antiepileptic drugs. In retrospect, the retardation began after a severe episode around the age of 1 year. The patient had a high fever, uncontrollable convulsions, and attacks of apnoea and bradycardia which required admission to the intensive care unit. The fever and convulsions responded to medication, but a few days after detubation she developed pneumonia with high fever and the clinical signs of sepsis with multiple organ failure, from which she recovered slowly without convulsions. Her brother (NL1.2) appeared healthy at the age of 3 years

on visual examination, but neurological tests have not been performed.

The Spanish patients reported by Chabas *et al.*<sup>16</sup> are designated E1.1 and E1.2.

#### CELL CULTURE AND ENZYME ASSAYS

Skin fibroblasts were cultured according to routine procedures in Ham's F10 medium supplemented with 10% fetal bovine serum and antibiotics. The cells were harvested with trypsin seven days after the last subculture and were stored at  $-70^{\circ}\text{C}$  until use.

$\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -NAGA) activity was assayed in homogenates prepared by sonication of cultured fibroblasts in water. Reaction mixtures consisted of 10  $\mu\text{l}$  homogenate (30  $\mu\text{g}$  protein for patient materials or 5  $\mu\text{g}$  for controls) and 20  $\mu\text{l}$  1 mmol/l MU- $\alpha$ GalNAc, Moscerdam Substrates, Rotterdam) in McIlvain's phosphate/citrate buffer, pH 4.7. After incubation for one hour at  $37^{\circ}\text{C}$ , the reactions were terminated by the addition of 200  $\mu\text{l}$  0.5 mol/l  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ , pH 10.7, and the fluorescence of 4-methylumbelliferone (MU) was measured with a Fluoroskan (Titer-tek) fluorimeter.  $\beta$ -galactosidase activity, using an MU substrate, and protein content of cell homogenates was determined as described previously.<sup>17</sup>  $\beta$ -galactosidase was also determined with the disaccharide Gal $\beta$ 1–3GalNAc (Oxford GlycoSystems) in a galactose dehydrogenase based assay.<sup>18</sup> Reaction mixtures (30  $\mu\text{l}$ ) contained 20  $\mu\text{g}$  protein from sonicated fibroblast homogenates, 2.7 mmol/l of the disaccharide, 67 mmol/l NaCl in 67 mmol/l sodium acetate buffer, pH 4.3, and were incubated for three hours at  $37^{\circ}\text{C}$ , after which 200  $\mu\text{l}$  0.1 mol/l Tris/HCl, pH 8.6 containing 0.6 mmol/l  $\text{NAD}^+$  and 0.13 U galactose dehydrogenase (Boehringer) was added. Free galactose was then determined by measuring the absorbance at 340 nm, after incubation for 25 minutes at room temperature.

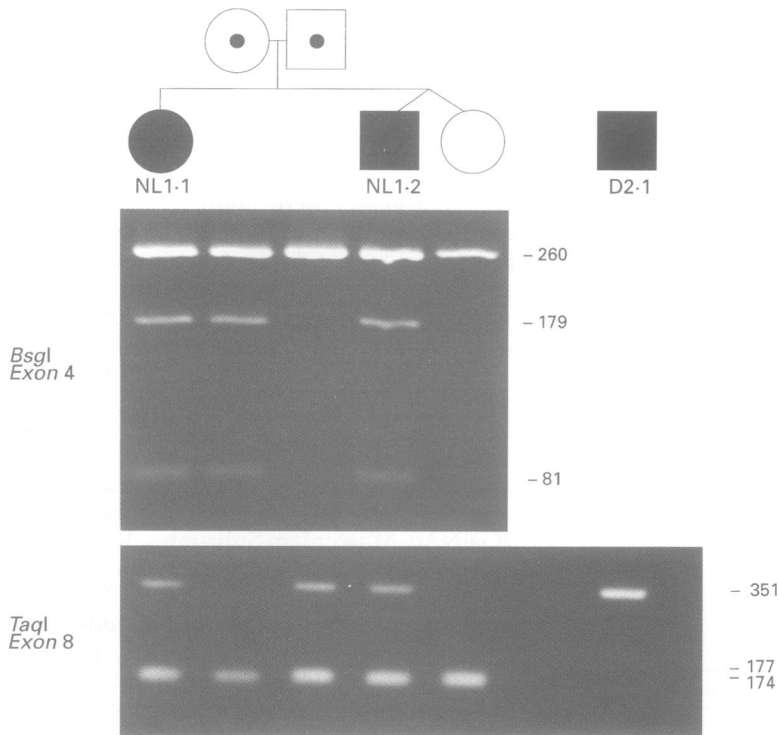
Neuraminidase activity was determined by the standard method using the MU substrate<sup>19</sup> as well as with a disialyltetraose substrate (DST, NeuAc $\alpha$ 2–3Gal $\beta$ 1–3[NeuAc $\alpha$ 2–6]GalNAc, Oxford GlycoSystems). Reaction mixtures (30  $\mu\text{l}$ ) contained 50  $\mu\text{g}$  protein from fibroblast homogenates (Potter homogenised), 1.3 mmol/l DST, 67 mmol/l NaCl in 67 mmol/l sodium acetate buffer, pH 4.3, and were incubated for three hours at  $37^{\circ}\text{C}$ . Then free N-acetylneuraminic acid (NANA) was purified as previously described<sup>20</sup> and determined with the thiobarbituric acid method as described by Denny *et al.*<sup>21</sup>

#### IMMUNOBLOTTING

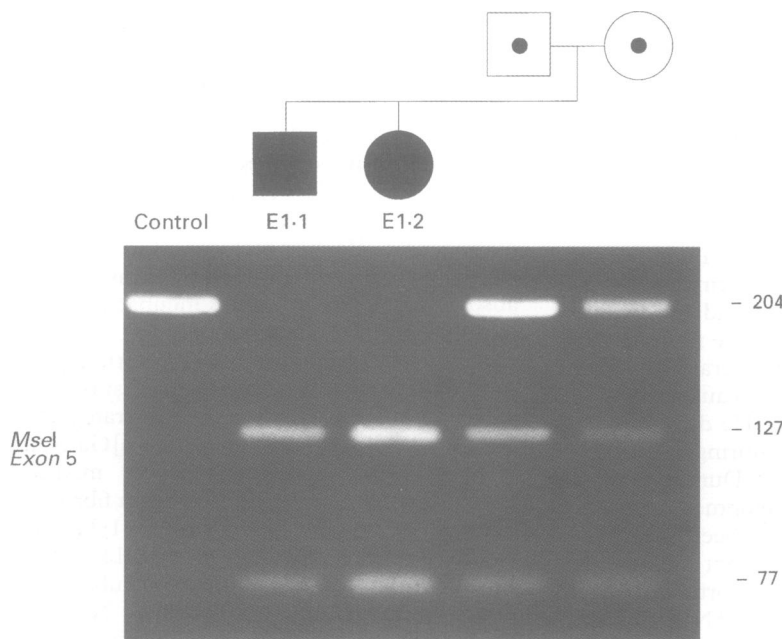
The polyclonal antibody against human  $\alpha$ -NAGA, raised in rabbits, was used at a 1000 fold dilution and immunoblotting was performed as previously described.<sup>12</sup>

#### LABELLING OF $\alpha$ -GALNAc MOIETIES BY LECTIN HISTOCHEMISTRY

The lectin from *Helix pomatia* (Sigma), which is specific for  $\alpha$ GalNAc, was used to stain



**Figure 1** Restriction enzyme analysis of PCR amplified  $\alpha$ -NAGA exons 8 and 4. Top panel: *BsgI* digests of exon 4 of NL1 patients, their parents, and a healthy sib. Bottom panel: *TaqI* digests of exon 8 of the NL1 patients, their parents, and patient D2.1.



**Figure 2** Restriction enzyme analysis of PCR amplified  $\alpha$ -NAGA exon 5. *MseI* digests of exon 5 of E1 patients and their parents.

$\alpha$ GalNAc containing material in cultured fibroblasts as described.<sup>9</sup>

#### METABOLIC LABELLING

Cultured fibroblasts were labelled with <sup>35</sup>S-methionine following the procedure described earlier.<sup>12</sup>

#### DNA AMPLIFICATION AND SEQUENCING

DNA was isolated from cultured fibroblasts by standard procedures.<sup>22</sup> PCR amplification and

sequencing was performed according to Hermans *et al.*<sup>23</sup> Restriction enzyme digestions (*TaqI*, Boehringer; *BsgI* and *MseI*, BioLabs) were performed according to the manufacturers' instructions.

## Results

### THE GENOTYPE OF $\alpha$ -NAGA DEFICIENCY

Using the intron primer sets shown in table 1, DNA fragments encompassing the entire open reading frame of the nine exons of the  $\alpha$ -NAGA gene were amplified and sequenced (except for patient D2.1); this included all flanking intron regions (table 1).

Since patient D2.1 is consanguineous with the D1 patients, only exon 8 was analysed, and the presence of the G11005A mutation (E325K) was confirmed (table 2). This mutation destroys a *TaqI* restriction site and its presence could be confirmed by *TaqI* digestion of the 351 bp long PCR products of exon 8. The digestion of the normal PCR product into fragments of 174 and 177 bp was not observed in the PCR products of the mutant alleles (fig 1). Sequence analysis of the sense and antisense strand as well as restriction enzyme analysis showed that patient D2.1 is homozygous for the E325K mutation (fig 1). We obtained the same results for the D1 patients (data not shown).

Sequence analysis of all  $\alpha$ -NAGA exons of the Dutch sibs (NL1) showed two different mutations. The C11017G substitution in exon 4 (S160C) could be confirmed with the restriction enzyme *BsgI* (the mutation creates a new restriction site). Fig 1 shows that the affected sibs are heterozygous for this mutation which is of maternal origin. The paternal mutation in these sibs is the previously described E325K mutation of the German patients. The father of the Dutch patients is not of German ancestry. The healthy sib of the NL1 patients showed the normal pattern in both exons 4 and 8, indicating that she is not a carrier of either mutation (fig 1). The S160C mutation was not detected in 80 Dutch white control alleles.

Sequence analysis showed that both Spanish (E1) patients were homozygous for the nonsense mutation E193X. Homozygosity was confirmed using the restriction enzyme *MseI* (the mutation creates a new restriction site). In agreement with this observation, both parents of the E1 sibs showed a heterozygous pattern after *MseI* digestion of their PCR product of exon 5 (fig 2).

The entire sequenced parts of the  $\alpha$ -NAGA gene (table 1) were identical in all patients and two controls (with the exception of the mutations). Differences, however, were found with the published sequence of the  $\alpha$ -NAGA gene (Wang and Desnick, accession No M59199). In intron 1, starting at position 3516, we detected CCCTTGCCCCC (discrepancies underlined, three Gs in sequence M59199). In exon 3 we detected three polymorphisms (no amino acid changes) in all samples analysed, which had previously been published by Yamauchi *et al.*<sup>10</sup> In all the DNA samples we tested, a C was found at position 4239 instead

Table 3  $\alpha$ -NAGA activity in fibroblasts from patients with infantile and adult  $\alpha$ -NAGA deficiency

	$\alpha$ -NAGA activity (nmol/h/mg)
Infantile cases	
D1.1	0.5
D1.2	1.0
D2.1	1.4
NL1.1	3.2
Adult patients	
E1.1	0.2
E1.2	0.2
Control range	
Range n = 34	40–130
(mean)	(81)

of an A and at position 4263 there was a T instead of a C. In both alleles of the two controls and in one allele of the NL1 patients, an A  $\rightarrow$  G substitution at position 4293 was found. In intron 3, starting at position 4348 we found four Cs instead of three Cs and starting at position 4364 we found GGCC instead of GCGC. In intron 5, at position 5404 we found a C  $\rightarrow$  G substitution.

#### THE BIOCHEMICAL PHENOTYPE OF $\alpha$ -NAGA DEFICIENCY

We studied the effect of these point mutations on the steady state level of  $\alpha$ -NAGA activity and observed differences among the various patients. The highest activity was measured in fibroblasts of patient NL1.1. About half this activity was present in cells from the D2.1 and D1 patients, whereas  $\alpha$ -NAGA activity in the E1 patients was barely detectable (table 3).

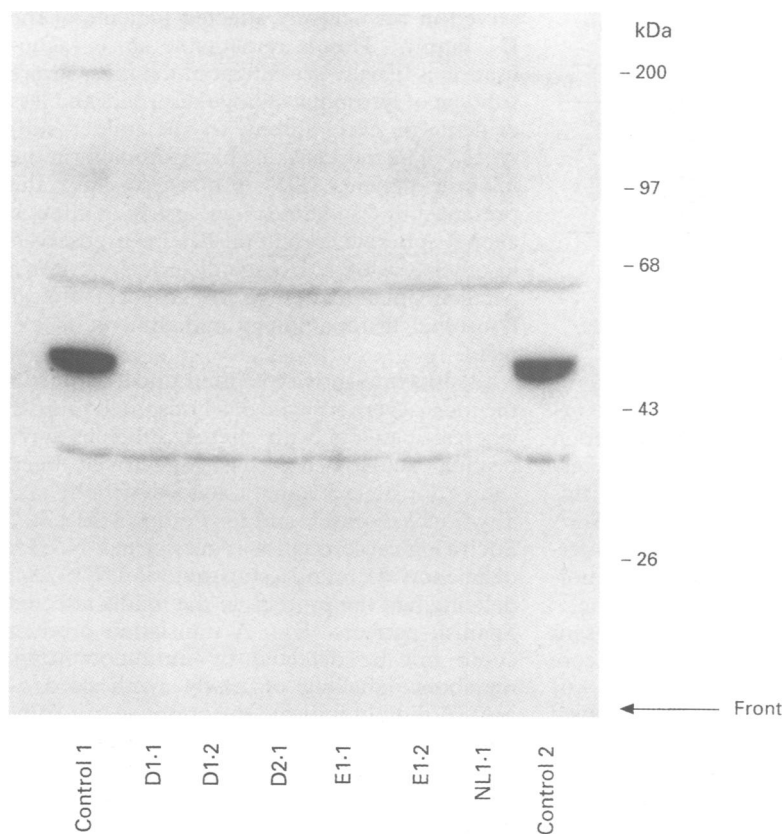


Figure 3 Immunoblot of  $\alpha$ -NAGA from patients with  $\alpha$ -NAGA deficiency. Fibroblast homogenates (150  $\mu$ g protein) were used. For patient codes see Patients and methods section.

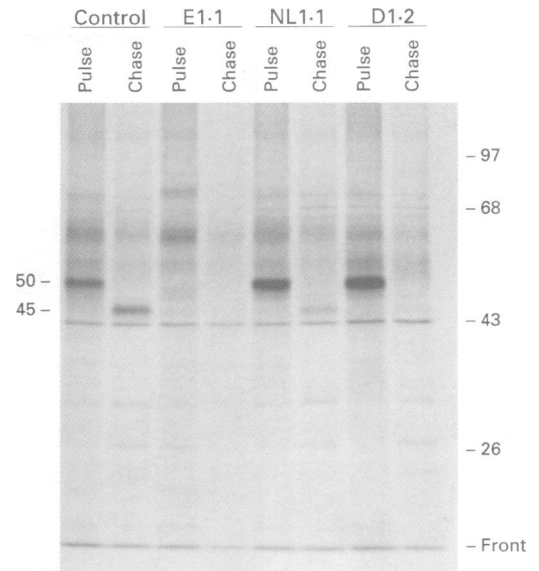


Figure 4 Pulse chase labelling of  $\alpha$ -NAGA in fibroblasts from patients with  $\alpha$ -NAGA deficiency. Cells were labelled with  $^{35}$ S-methionine for four hours (pulse), or they were labelled for four hours and subsequently maintained for 17 hours in label free medium.  $\alpha$ -NAGA was then immunoprecipitated and analysed by SDS-PAGE. Molecular weight markers are shown on the right hand side in kDa.

These low residual activities and their differences are genuine since the activities are linear with the amount of protein and incubation time and reproducible (data not shown). Using a polyclonal antibody against  $\alpha$ -NAGA, we showed that in all cases the deficiency of  $\alpha$ -NAGA enzyme activity was associated with undetectable levels of  $\alpha$ -NAGA protein (fig 3). Metabolic labelling of cultured fibroblasts for four hours, followed by immunoprecipitation of native  $\alpha$ -NAGA protein showed that the synthesis of precursor  $\alpha$ -NAGA (50 kDa) is normal in the NL1 patients and patient D1.2 (fig 4). The E1 patients, on the other hand, do not synthesise any  $\alpha$ -NAGA protein. Maturation of newly synthesised  $\alpha$ -NAGA was investigated after a 17 hour chase period and mature enzyme (45 kDa) was not detectable in the German patient D1.2, whereas a small amount was present in the NL1 patients (fig 4).

At the cellular level,  $\alpha$ -NAGA deficiency was reflected in the lysosomal storage of  $\alpha$ GalNAc containing compounds (fig 5), which were identified with the  $\alpha$ GalNAc specific lectin from *H pomatia*. On the other hand all patients with  $\alpha$ -NAGA deficiency excrete sialylglycopeptides, the new patient D2.1 included (data not shown). Unlike the unknown structure of the intralysosomal storage products, the abnormal urinary excretion products have been fully characterised. We have investigated whether fibroblast homogenates of the most severe patient with  $\alpha$ -NAGA deficiency (D1.2) can hydrolyse the core structures of these urinary compounds: NeuAc $\alpha$ 2-3Gal $\beta$ 1-3 [NeuAc $\alpha$ 2-6]GalNAc and Gal $\beta$ 1-3GalNAc. The results show that fibroblasts with  $\alpha$ -NAGA deficiency are fully normal in their sialidase and  $\beta$ -galactosidase activity towards these oligosaccharides (table 4).

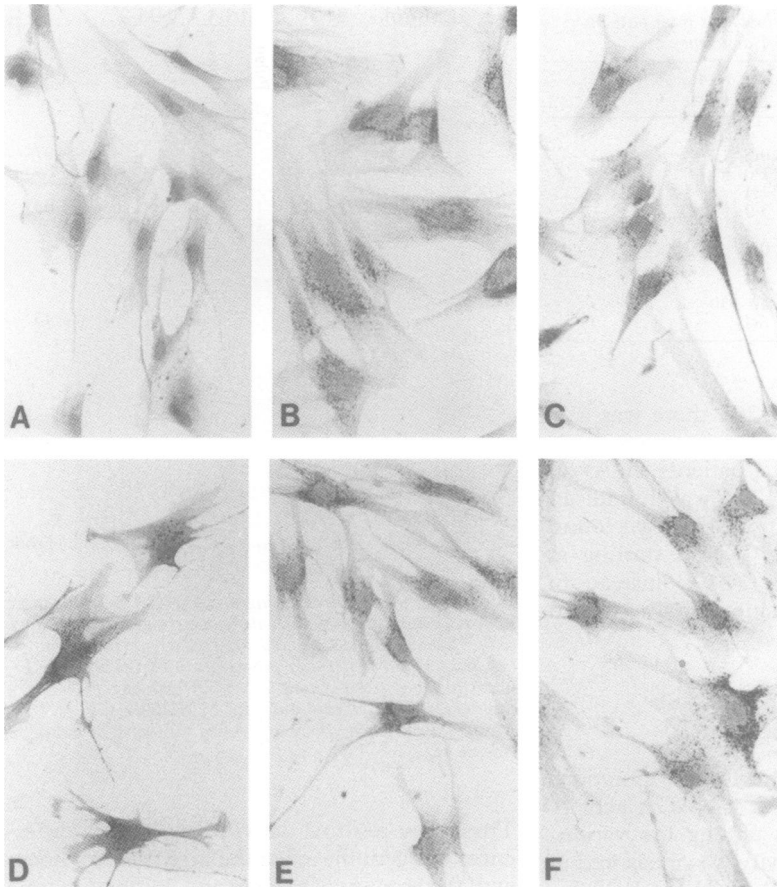


Figure 5 Intracellular labelling of  $\alpha$ -N-acetylgalactosamine containing compounds in fibroblasts from patients with  $\alpha$ -NAGA deficiency. Lectin histochemistry, using the  $\alpha$ GalNAc specific lectin from *Helix pomatia*, was carried out on cultured fibroblasts from a control (A), the patients D1.2 (B), D2.1 (C), NL1.1 (D), E1.1 (E), and E1.2 (F).

Table 4 Degradation of core structures of O-glycosidic glycoproteins by neuraminidase and  $\beta$ -galactosidase

	Neuraminidase activity*		$\beta$ -galactosidase activity*	
	MU-NANA†	DST‡	MU- $\beta$ Gal§	Gal $\beta$ 1-3GalNAc
$\alpha$ -NAGA deficiency (D1.2)	78	28	750	650
Control	45	18	560	480
G <sub>M1</sub> -gangliosidosis	35	13	2	Not detectable
Sialidosis	0.3	1	430	450

\* Enzyme activities: nmol/h/mg.

† 4-methylumbelliferyl- $\alpha$ -D-N-acetylneuraminic acid.

‡ DST = disialyltetraose: NeuAc $\alpha$ 2-3Gal $\beta$ 1-3[NeuAc $\alpha$ 2-6]GalNAc.

§ 4-methylumbelliferyl- $\beta$ -D-galactoside.

## Discussion

Deficiency of  $\alpha$ -N-acetylgalactosaminidase ( $\alpha$ -NAGA) would imply major urinary excretion products containing terminal  $\alpha$ GalNAc residues at the non-reducing end; however, this is not observed. All patients with  $\alpha$ -NAGA deficiency excrete sialylglycopeptides with terminal neuraminic acid and the  $\alpha$ -GalNAc moiety is internal,  $\alpha$  linked to serine or threonine.<sup>7,8</sup> The structures are identical to those present in O linked glycoproteins. Using their core structure (NeuAc $\alpha$ 2-3Gal $\beta$ 1-3[NeuAc $\alpha$ 2-6]GalNAc and Gal $\beta$ 1-3GalNAc) we showed that fibroblasts from the severe D1.2 patient (table 5) can hydrolyse sialic acid and galactose from these compounds. This indicates that the sialylglycopeptides from urine are not the primary lysosomal storage products, which will probably be GalNAc $\alpha$ 1-Ser/Thr. Minor amounts of these compounds have been de-

tected in urine of the D1 patients.<sup>24</sup> These putative primary storage products could subsequently serve as acceptor for the synthesis of the O linked type oligosaccharides. Addition of glycosides to primary lysosomal storage products (resynthesis) has been reported for aspartylglucosaminuria<sup>25,26</sup> and  $\beta$ -mannosidosis.<sup>27</sup> Although we have shown an  $\alpha$ GalNAc containing lysosomal storage product in fibroblasts of all patients (we did not have permission to investigate fibroblasts from the Japanese patient), the structure of these compounds is still unknown. Studies are in progress to identify these primary lysosomal storage products.

$\alpha$ -NAGA deficiency is one of the rarest and probably most heterogeneous lysosomal storage disorders. At present, only eight patients are known from five families of German (D), Japanese (J), Dutch (NL), and Spanish (E) descent, including the new case reported here (table 5). Clinical variability is common in lysosomal storage disorders but overlapping signs and symptoms of subtypes is the rule. In  $\alpha$ -NAGA deficiency, however, infantile and adult patients have no obvious overlap of signs and symptoms. This led us to consider the possibility that the phenotype of the eight patients is not solely determined by the  $\alpha$ -NAGA deficiency. Three observations are difficult to reconcile with a simple genotype-phenotype correlation.

(1) At the histological level, prominent vacuolisation, the hallmark of lysosomal storage disorders, was observed in dermal endothelial cells of the late onset patients (patient J1.1,<sup>3,5</sup> patients of the E1 family<sup>16</sup>) but was not observed in the severely affected patients of the D1 family.<sup>2</sup> This is remarkable since vacuolisation is usually prominent in the most severe subtype of lysosomal storage disorders and less evident, or even absent, in the milder subtypes.<sup>28</sup> The most striking histopathology in the infantile patients (D1) is non-lysosomal, the presence of "spheroids" in axons. Although axonal spheroid formation has been observed in other lysosomal storage disorders,<sup>29,30</sup> it is a peculiar phenomenon in the absence of clear lysosomal histopathology and difficult to explain.

(2) Enzyme activity is often undetectable in the most severe subtype of a lysosomal disorder whereas patients with milder subtypes have residual activity. This has conclusively been shown for metachromatic leucodystrophy and Tay-Sachs disease<sup>31</sup> and for Pompe's disease.<sup>32</sup> Such a logical correlation is missing in  $\alpha$ -NAGA deficiency. We found a stop mutation (E193X), deleting half the protein, in the mildly affected Spanish patients (E1). A translation product could not be detected by immunoblotting, metabolic labelling of newly synthesised  $\alpha$ -NAGA did not indicate the presence of a truncated protein, and enzyme activity was virtually zero. This indicates that these patients have, in essence, null alleles and one would expect the most severe phenotype. In contrast, these patients presented with a mild, late onset disease, whereas the most severely affected patients (D1) have significant, albeit very low,

Table 5 Summary of all patients with  $\alpha$ -NAGA deficiency

	Infantile $\alpha$ -NAGA deficiency					Adult $\alpha$ -NAGA deficiency		
	D1.1*	D1.2*	D2.1†	NL1.1‡	NL1.2‡	J1.1§	E1.1	E1.2
Age								
Age at onset	1 y	6 mth	7 mth	1 y	No symptoms?	28 y	14 y	NI¶
Present age (age at death)	13 y	12 y	(1.5 y)	5 y	3.6 y	51 y	42 y	38 y
Neurological signs								
Convulsions during fever	+	+	+	+	-	-	-	-
Epilepsy	+++	+++	+	-	-	-	-	-
Psychomotor retardation	+++	+++	?	+	?	-	-	-
Hypotonia	+++	+++	?	-	-	-	-	-
Physical signs								
Skeletal abnormalities	-	-	-	-	-	-	-	-
Dysmorphism	-	-	-	-	-	±	±	?
Organomegaly	-	-	-	-	-	-	-	-
Angiokeratoma	-	-	-	-	-	+++	++	+
Lymphoedema	-	-	-	-	-	-	+++	+++
Histology								
Vacuolisation	-	-	NI¶	-	-	++	++	++
Neuroaxonal dystrophy	+++	+++	NI¶	-	-	-	-	-
$\alpha$ -GalNAc lectin staining	++++	++++	+++	+++	NA**	NA**	+++	+++
Mutations	Homozygous E325K			S160C/E325K		Homozygous R329W††		Homozygous E193X

\*D1: van Diggelen *et al.*<sup>1</sup>, Schindler *et al.*<sup>2</sup>

†D2.1: present paper.

‡NL1: de Jong *et al.*<sup>15</sup>§J1.1: Kanski *et al.*<sup>3</sup>||E1: Chabas *et al.*<sup>16</sup>

¶NI = not investigated.

\*\*NA = material not available.

††Wang *et al.*<sup>14</sup>

residual enzyme activity. A logical correlation was similarly lacking in studies of Wang *et al.*<sup>13</sup> comparing the severe German patients (D1) and the Japanese patient J1.1 with adult  $\alpha$ -NAGA deficiency.<sup>14</sup> The authors could not detect significant  $\alpha$ -NAGA enzyme activity in either case.

(3) After the clinical description of the first patients with  $\alpha$ -NAGA deficiency (D1), many laboratories, including ours, have screened Seitelberger patients (infantile neuroaxonal dystrophy) for  $\alpha$ -NAGA deficiency, but a second patient with combined  $\alpha$ -NAGA deficiency and neuroaxonal dystrophy has not been found.

These three observations indicate that factors other than  $\alpha$ -NAGA contribute to the phenotypic variation of the patients. The simplest explanation would be that the severe infantile patients (D1) have a "double disease", neuroaxonal dystrophy in addition to  $\alpha$ -NAGA deficiency, without a causal relationship. Accidental occurrence of two independent monogenic diseases in one patient can be observed, particularly in consanguineous families (the parents of the patients D1.1 and D1.2 are consanguineous). For example, in one of the contributing laboratories (Rotterdam) four cases are known: glycogenosis II combined with methylmalonic aciduria, neurofibromatosis I with multiple sulphatase deficiency, Niemann-Pick type C with Hurler disease, and combined Zellweger syndrome and sulphite oxidase deficiency. One of the first patients with  $\beta$ -mannosidosis<sup>33</sup> had mucopolysaccharidosis type III A as a second disease.<sup>33,34</sup>

If the severely affected German patients (D1) did have a double disease, this phenotype is not representative of "true"  $\alpha$ -NAGA deficiency. The phenotype associated with complete  $\alpha$ -NAGA deficiency would be a mild, late onset disease with angiokeratoma manifested in patient J1.1 and the Spanish patients (E1). The Dutch patient NL1.2, aged 3.3 years without overt signs or symptoms, could be a pre-clinical case of  $\alpha$ -NAGA deficiency detected

through screening. The newly identified missense mutation in the Dutch patients (S160C) results in 4% residual activity, the highest level among all the patients with  $\alpha$ -NAGA deficiency; this may contribute to the mild phenotype. Only in these patients a small amount of mature  $\alpha$ -NAGA protein was found 17 hours after metabolic labelling of the enzyme in cultured fibroblasts, which is in agreement with the significant residual enzyme activity. The newly identified patient D2.1, who had identical  $\alpha$ -NAGA mutations as the patients with neuroaxonal dystrophy (D1), could in time have provided important information as to the relation of  $\alpha$ -NAGA deficiency and neuroaxonal dystrophy, but the patient died at the age of 1½ years of hypoxia during a convulsion without being examined histologically and neurologically.

In summary, all the genetic, biochemical, histological, and clinical data of all patients with  $\alpha$ -NAGA deficiency strongly suggest that  $\alpha$ -NAGA deficiency is not a single disease entity. The possibility that factors other than  $\alpha$ -NAGA play a major role in determining the phenotypic variation of the eight known patients with  $\alpha$ -NAGA deficiency deserves serious consideration. The future discovery of new patients with  $\alpha$ -NAGA deficiency will eventually establish which phenotypes are solely caused by  $\alpha$ -NAGA deficiency and if other factors/genes play a role in other phenotypes. This will probably take a long time since the discovery of the first eight patients took almost a decade. It will therefore pay to study the association of  $\alpha$ -NAGA deficiency and neuroaxonal dystrophy in an  $\alpha$ -NAGA knock-out mouse.

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