Elevated CSF *N*-acetylaspartylglutamate in patients with free sialic acid storage diseases

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ABSTRACT

Objective: To investigate body fluids of patients with undiagnosed leukodystrophies using in vitro ^1H -NMR spectroscopy (H-NMRS).

Methods: We conducted a cross-sectional study using high-resolution in vitro H-NMRS on CSF and urine samples.

Results: We found a significant increase of free sialic acid in CSF or urine in 6 of 41 patients presenting with hypomyelination of unknown etiology. Molecular genetic testing revealed pathogenic mutations in the *SLC17A5* gene in all 6 patients. H-NMRS revealed an increase of *N*-acetylaspartylglutamate in the CSF of all patients with *SLC17A5* mutation (range 13–114 $\mu\text{mol/L}$, reference $<12 \mu\text{mol/L}$).

Conclusion: In patients with undiagnosed leukodystrophies, increased free sialic acid in CSF or urine is a marker for free sialic acid storage disorder and facilitates the identification of the underlying genetic defect. Because increase of *N*-acetylaspartylglutamate in CSF has been observed in other hypomyelinating disorders, it can be viewed as a marker of a subgroup of hypomyelinating disorders. **Neurology® 2010;74:302–305**

GLOSSARY

COSY = correlation spectroscopy; **GCP II** = glutamate carboxypeptidase II; **H-NMRS** = ^1H -NMR spectroscopy; **ISSD** = infantile free sialic acid storage disease; **NAA** = *N*-acetylaspartate; **NAAG** = *N*-acetylaspartylglutamate; **SASD** = free sialic acid storage disease.

Mutations in the *SLC17A5* gene encoding the lysosomal transporter sialin are associated with the free sialic acid storage diseases (SASD): Salla disease (or the Finnish type of sialuria), the more severe infantile free sialic acid storage disease (ISSD), and intermediate phenotypes with clinical findings of both Salla disease and ISSD.¹ SASD are characterized by the abnormal retention of free sialic acid in the lysosome (Online Mendelian Inheritance in Man 604369 and 269920). Patients with SASD usually present with nystagmus, progressive cerebellar ataxia, spasticity, and severe psychomotor delay. These symptoms are associated with diffuse supratentorial hypomyelination, thin corpus callosum, and cortical and cerebellar atrophy. The increase of free sialic acid in urine has been considered the biochemical hallmark of the disease.¹ However, we have recently reported that patients with SASD can present without sialuria.² The mechanisms leading to hypomyelination in SASD remain largely unknown. Here, we report the increase of *N*-acetylaspartylglutamate (NAAG) in the CSF of patients with SASD, which may provide new insight into the pathophysiology of the disease.

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Table Clinical and biochemical characteristics of the patients with free sialic acid storage diseases

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5 ²	Patient 6 ²
Sex	Female	Female	Female	Male	Male	Female
Age at examination	15 mo	4 y	6 y	9 y	19 y	12 y
Symptoms at examination						
Cognition	Severe global delay	Severe global delay	Mental retardation	Mental retardation	Mental retardation	Mental retardation
Epilepsy	Yes	Yes	No	No	No	No
Cerebellar ataxia	Yes	No	Yes	Yes	No	No
Spasticity	Yes	Mild pyramidal signs	Yes	No	No	No
Cerebral MRI						
Hypomyelination	+++	+++	+++	++	+	+
Thin CC	+++	+++	+++	++	++	++
Vermian atrophy	–	++	++	+	–	–
Phenotype	Salla disease	Severe Salla disease (intermediate)	Salla disease	Salla disease	Mild Salla disease	Mild Salla disease
FSA CSF, $\mu\text{M/L}$ (reference <17) ⁷	40 ^a	28 ^a	37 ^a	15	29 ^a	28 ^a
FSA urine, $\mu\text{M/mM creatinine}$ (reference range)	182 ^a (<100)	ND	95 ^a (20–46)	86 ^a (15–36)	43 (11–46)	32 (8–35)
FSA fibroblasts, nmol/mg protein (reference range 0.1–1.8)	35.1 ^a	3.8 ^a	2.5 ^a	ND	3.1 ^a	2.6 ^a
NAAG CSF, $\mu\text{M/L}$ (reference <12) ^{3,4}	114 ^a	13 ^a	85 ^a	25 ^a	73 ^a	69 ^a
<i>SLC17A5</i> mutations	115 C>T, exon 2; 553 delC, exon 4	291 G>A, exon 2 (homozygous)	115 C>T, exon 2 (homozygous)	115 C>T, exon 2 (homozygous)	406 A>G, exon 3 (homozygous)	406 A>G, exon 3 (homozygous)
Predicted polypeptide consequence	R39C/frameshift, 33 novel aa, premature stop	Splicing defect, partial deletion of exon 2, frameshift, premature stop	R39C/R39C	R39C/R39C	K136E/K136E	K136E/K136E

Superscript numbers refer to references.

Abbreviations: aa = amino acids; CC = corpus callosum; FSA = free sialic acid; NAAG = N-acetylaspartylglutamate; ND = not determined.

– indicates absence; +, mild; ++, moderate; +++, severe.

^aAbnormal biochemical value.

METHODS **Standard protocol approvals, registrations, and patient consents.** Eighty-three children and adults from 2 referral centers for neurogenetics and neurometabolism were enrolled in clinical protocols approved by the ethics committees of the National Institutes of Neurological Disorders and Stroke, Bethesda, Maryland, and the Radboud University Nijmegen Medical Center, The Netherlands. Written informed consent was obtained from all patients or their legal guardians.

Patients. Patients with leukodystrophies of unknown etiologies ($n = 41$) were classified as hypomyelinating ($n = 27$) or demyelinating ($n = 10$) leukodystrophies or delayed myelination ($n = 4$) according to the pattern on brain MRI. The disease control group consisted of 42 patients with leukodystrophies caused by known genes (*PLP*, *EIF2B5*, *GFAP*, *GBE1*, *ABCD1*, *GALC*, *ASPA*, *ALDH3A2*, *ARSA*, *D2HGDH*, *DARS2*) or defined leukodystrophy syndromes of unknown etiologies (hypomyelination hypogonadotropic hypogonadism and hypodontia syndrome, leukodystrophy with calcification and cysts, cerebellar ataxia with increased cerebrospinal free sialic acid).

Methods. To identify new metabolic abnormalities, frozen urine and CSF (stored at -80°C) were prepared for ^1H -NMR spectroscopy (^1H -NMRS) with minimal handling² as

part of a cross-sectional study. In addition to ^1H -NMRS, urinary sialic acid levels were determined by quantitative colorimetric assay² and compared with urine samples from age-matched controls. Sialic acid was also measured in fibroblasts as described.² All exons of the *SLC17A5* gene (GenBank NM_012434) were amplified from the patient's genomic DNA using specific intronic primers.

RESULTS **Increase of free sialic acid in the CSF or urine of 6 patients leading to the diagnosis of SASD.** Among the cohort of 41 patients with leukodystrophies of unknown etiology, high-resolution in vitro ^1H -NMR spectroscopy of CSF revealed an increased concentration of free sialic acid in 5 patients, including 2 siblings previously reported (table). Free sialic acid presents as a prominent singlet resonance at 2.05 ppm and other resonances at 1.85 and 2.26 ppm in 1-dimensional NMRS (figure) with characteristic cross peaks in 2-dimensional correlation spectroscopy (COSY) NMR spectroscopy.² Increase of free sialic acid was also found in the urine of 3 of the 5 patients, as well as in the urine of a sixth patient for which the level of CSF free sialic acid level was consid-

Figure Identification of *N*-acetylaspartylglutamate by ^1H -NMR spectroscopy of CSF

One-dimensional 500-MHz spectrum of the CSF of patient 1, showing the increase of both free sialic acid (NANA) and *N*-acetylaspartylglutamate (NAAG).

ered to be within the normal range (table). Free sialic acid in fibroblasts was found to be increased in the 4 patients who were tested. The increase of free sialic acid in the CSF or urine or both suggested the diagnosis of SASD. *SLC17A5* sequencing revealed known homozygous or compound heterozygous mutations in all 6 patients (table). The clinical and brain MRI characteristics of the patients are summarized in the table. The prevalence of patients with SASD in our cohort of patients with hypomyelination was 22% (6/27).

Increase of NAAG in the CSF of patients with SASD. In vitro ^1H -NMR spectroscopy of CSF revealed a wide range of NAAG increase in the CSF of all 6 patients with SASD (table). NAAG presents as a prominent singlet resonance at 2.04 ppm and smaller resonances at 2.85 (AB system), 2.01, 2.23, 2.48, 4.44, and 4.72 ppm (multiplet resonances) (figure) with characteristic cross peaks in 2-dimensional COSY NMR spectroscopy.³ We confirmed the increase of NAAG in patients with *PLP* mutations ($n = 2$). However, increased levels of CSF NAAG were not seen in patients with other hypomyelinating disorders, such as *eIF2B5* mutations, in patients with a demyelinating brain pattern or delayed myelination.

DISCUSSION We report 6 patients with SASD presenting with an increase of NAAG in their CSF. We previously identified 2 patients with SASD with nor-

mal urinary excretion of free sialic acid,² suggesting that *SLC17A5* mutations should be considered in patients with hypomyelination despite the absence of sialuria. The current report reveals that patients with SASD can also present with normal free sialic acid levels in their CSF, emphasizing that the analysis of both urine and CSF is helpful in the screening of patients with free sialic acid-related diseases. Constant clinical features in our patients with SASD were cognitive impairment and thin corpus callosum (table). Thus, in the appropriate clinical and neuroimaging setting, the isolated increase of NAAG in CSF should also suggest SASD and lead to sequencing of *SLC17A5*. Of interest, we found a high prevalence of SASD in our cohort of patients with hypomyelination (22%).

Increased CSF NAAG has been reported in 3 other hypomyelinating disorders: Pelizaeus-Merzbacher disease due to *PLP1* mutations,⁴ Pelizaeus-Merzbacher-like disease 1 due to *GJA12* mutations,⁵ and a yet unidentified hypomyelinating disorder.³ A mildly increased concentration of NAAG in CSF has been described in combination with highly increased concentrations of *N*-acetylaspartate (NAA) in a case of Canavan disease.⁶ However, CSF NAAG was normal in a large cohort of patients with nonneurodegenerative neurologic conditions or white matter disease of other origin.⁷ Despite what has been suggested in Pelizaeus-Merzbacher disease,⁴ there seems to be no correlation between the levels of NAAG in patients' CSF and the severity of the SASD phenotype in patients' CSF and the severity of the SASD phenotype. Interestingly, levels of free sialic acid do not correlate with clinical severity of SASD either.

NAAG is the most abundant neuropeptide in the CNS and is present in a number of cells, including oligodendrocytes.⁸ It is synthesized by the enzyme NAAG synthase from NAA and glutamate and is degraded by peptidases, especially glutamate carboxypeptidase II (GCPII) that is present in the membrane of neurons, astrocytes, and Schwann cells and possibly oligodendrocytes. NAAG is an endogenous agonist of metabotropic glutamate receptor 3 and acts as a weak antagonist at the NMDA receptor. The mechanism of its increase in patients with hypomyelinating leukodystrophy is not known, but one can hypothesize that it results from a decrease in GCPII activity secondary to the glial cell abnormality present in these leukodystrophies.³ This is supported by our observation that the increase of NAAG in the CSF of patients with SASD is not associated with an increase of NAA. However, despite a marked loss of oligodendrocytes, the levels of NAAG were normal in the CSF of our patients with Krabbe disease. Furthermore, it is unlikely that NAAG contributes to

the hypomyelination phenotype observed in SASD. NAAG was indeed recently reported to have a negligible action on oligodendrocytes' NMDA receptors.⁹ The increase of NAAG in the extracellular space resulting from inhibition of GCPII has also been shown to be neuroprotective.¹⁰

In patients with undiagnosed leukodystrophies, increased free sialic acid in CSF or urine suggests the diagnosis of SASD. The frequent increase of CSF NAAG in SASD and in other hypomyelinating disorders makes it a marker of a subgroup of hypomyelinating disorders. NAAG is readily detectable by NMRS, a technique confirmed to be useful for the identification of biomarkers associated with white matter disease.

DISCLOSURE

Dr. Mochel may accrue revenue on patent EP 06291873.5 (issued: 12/4/2006): anaplerotic therapy of Huntington disease and other polyglutamine diseases (BIO06353). Dr. Engelke, Ms. Barritault, Dr. Yang, and Mr. McNeill report no disclosures. Dr. Thompson has received speaker honoraria from BioMarin; and serves as an ACMG Foundation committee member. Dr. Vanderver has received travel expenses and/or honoraria for lectures or educational activities not funded by industry; may accrue revenue on a pending patent re: sialic acid measurement in CSF in the diagnosis of vanishing white matter disease (filed: 9/30/05); and receives research support from the NIH/NINDS [K08 (PI)] and the American Academy of Neurology. Dr. Wolf has received travel expenses and/or honoraria for lectures or educational activities not funded by industry. Dr. Willemsen, Dr. Verheijen, Dr. Seguin, and Dr. Wevers report no disclosures. Dr. Schiffmann serves on scientific advisory boards for and has received funding for travel from Shire Human Genetic Therapies and Amicus Therapeutics; may accrue revenue on a pending patent re: tetrahydrobiopterin in Fabry disease, 61/168,150 (filed: 4/9/2009); serves as a consultant for Zacharon Pharmaceuticals; and receives research support from Shire Human Genetic Therapies, Amicus Therapeutics, and Baylor Research Foundation.

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