

# Glycosphingolipid Expression in Cerebrospinal Fluid of Infants with Neurological Abnormalities: Report of Three Cases

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## ABSTRACT

*The aim of this study was to analyse glycosphingolipid expression in cerebrospinal fluid (CSF) from one idiopathic West syndrome (IWS) infant, one with Reye like syndrome, and one with congenital hydrocephalus, in comparison to control group (n=7) using highly sensitive thin-layer chromatography-immunostaining methods. Gangliotetraose-series gangliosides (acidic glycosphingolipids) were not detected in CSF of infant with idiopathic West syndrome and infant with congenital hydrocephalus. CSF of infant with IWS showed traces of neolacto-tetraose ganglioside fractions, which were absent in all other CSF examined. In addition, lactosylceramide fraction, and one ceramide fraction were highly expressed only in IWS CSF. These results confirmed previously described lack of gangliotetraose-series gangliosides in IWS patient and for the first time is described increased expression of neolacto-series glycosphingolipids in IWS patient. Since follow up until the age of five years showed almost normal IWS patient psychomotor development, the described shift of glycosphingolipid expression may implicate on transient inhibition of specific glycosyl transferases in the age of seven months.*

**Key words:** idiopathic West syndrome, Reye like syndrome, congenital hydrocephalus, cerebrospinal fluid, glycosphingolipids

## Introduction

A disturbance of brain maturation may be considered to be a major contributing factor to the neuropathogenesis of central nervous system. Although the basic mechanism is still unknown, infantile spasms become apparent during a phase of very active brain development when neurites, dendrites, and synapses are maturing<sup>1,2</sup>.

A neurochemical understanding of the cell surface events that accompany the development of a neuroblast into a functionally mature neuron is important for determining the pathogenesis of age-dependent diseases. Because they are abundant in the outer surface of neuronal plasma membrane, glycosphingolipids (GSLs) may play an important role in these events<sup>3,4</sup>.

Izumi et al. found earlier low levels of cerebrospinal fluid (CSF) gangliotetraose-series gangliosides in patients

with symptomatic or IWS<sup>5</sup>. In contrast to neutral GSLs which don't contain neuraminic acid, gangliosides are acid class of GSLs. In addition to the gangliotetraose-series gangliosides analyses, for the first time we performed determination of neolactoseries gangliosides and neutral GSLs in an infant with IWS, one with Reye like syndrome, and in one with congenital hydrocephalus, in comparison to control group (n=7).

## Materials and Methods

### Subjects

Of the 3 infants, ages 1–12 months, with neurological abnormalities one was diagnosed with idiopathic West

syndrome (IWS), one with Reye like syndrome, and one with congenital hydrocephalus. We analysed those samples in comparison to CSF samples obtained from 7 infants admitted to our Clinic who required lumbar puncture due to seizures, to exclude CNS infections or metabolic disease. All of 7 control patients had febrile seizures, ages 1–12 months, and all of them were without psychomotor retardation or growth failure.

IWS patient had uneventful history: previous development until the onset of seizures at six months of age was normal, spasms were typical. EEG showed hypsarrhythmia. Cranial magnetic resonance imaging (MRI) showed no demonstrable underlying cerebral lesion. Metabolic findings were normal. CSF fluid was obtained be-



Fig. 1. Cranial ultrasound finding in infant with congenital hydrocephalus – frontal section.



Fig. 2. Cranial ultrasound finding in infant with congenital hydrocephalus – parasagittal section.

**TABLE 1**  
ROUTINE CEREBROSPINAL FLUID FINDINGS IN PATIENTS WITH NEUROLOGICAL DISTURBANCES AND CONTROL PATIENTS

Patient/age (months)	Cell	Count	Proteins	Glucose	Lactate
	WBC	RBC	g/L	mmol/L	mmol/L
IWS / 7	1/3	12/3	0.23	2.8	0.9
RLS / 12	1/3	0/3	0.37	1.3	–
CH / 1	6/3	15/3	0.83	3.4	4.0
control 1/5	12/3	12/3	0.45	3.1	–
control 2/6	18/3	0/3	0.43	4.8	–
control 3/1	20/3	208/3	0.72	2.0	1.5
control 4/8	3/3	15/3	0.52	2.8	–
control 5/7	7/3	19/3	0.39	3.1	1.3
control 6/10	2/3	6/3	0.29	3.2	1.0
control 7/12	1/3	15/3	0.33	4.4	–

IWS – idiopathic West syndrome, RLS – Reye like syndrome, CH – congenital hydrocephalus.

fore starting tetracoside therapy and showed no abnormalities (Table 1). Patient responded well on therapy and was seizure free after seven days. At follow up until the age of five years patient showed almost normal psychomotor development.

The patient with congenital hydrocephalus was admitted due to myoclonic seizures at age of 34 days. Patient was severely neurologically impaired and hypotonic, and cranial ultrasound was performed<sup>6</sup>. Patient's head circumference was appropriate for age, but cranial ultrasound showed extreme hydrocephalus (Figure 1 and 2). MRI revealed lissencephaly, hydrocephalus, callosal agenesis, brainstem, cerebellar, and especially vermian hypoplasia. Metabolic findings showed lactacidosis and further investigation proved pyruvate dehydrogenase deficiency. CSF was obtained before medication and showed increased lactate level (Table 1).

The Reye-like syndrome patient was previously healthy child. Patient was admitted at age of 12 months due to lethargic state after urinary tract infection. Laboratory findings showed abnormalities of hepatic function: marked elevation of aminotransferase and lactic dehydrogenase activity, hyperammoniaemia and prolongation of prothrombin time. CSF was normal. Cranial ultrasound showed no cerebral oedema. Screening for metabolic disease and further investigation showed ornithine transcarbamylase deficiency (OTC def), proved by DNA analysis.

Additional seven patients had only febrile seizures with normal developmental milestones and no neurological disturbances. In all of the control patients (n=7) with typical febrile seizures lumbar puncture was performed to exclude CNS infection (Table 1).

In all patients (n=10) CSF specimens were obtained before any therapy.

### Biochemical analyses of CSF glycosphingolipids

After routine analyses for clinical purpose we used these specimens for analyses of glycosphingolipids. CSF samples were stored at  $-80^{\circ}\text{C}$  until use. GSLs were extracted from defrosted samples with chloroform/methanol (2/1, v/v), chloroform/methanol (1/1, v/v), and chloroform/methanol (1/2, v/v) (11-fold volumes of the CSF volume) for 30 min with sonication<sup>7</sup>. The combined extracts were evaporated, resuspended in chloroform/methanol/water (30/60/8, by vol.), and gangliosides were separated from neutral GSLs by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany). The ganglioside fraction was incubated for 1 h at  $37^{\circ}\text{C}$  in aqueous 1 N NaOH to saponify phospholipids, followed by neutralization with acetic acid and dialysis. After final column chromatography purifications (Iatrobeads column 6RS-8060 for gangliosides, Macherey-Nagel, Düren, Germany; silica gel 60 for neutral GSLs, Merck, Darmstadt, Germany) final GSL fractions were adjusted to defined volumes of chloroform/methanol (2/1) corresponding to half of the original CSF volumes<sup>8</sup>.

Gangliosides were separated on silica gel 60 precoated high-performance thin-layer chromatography plates (HPTLC-plates, size 10 x 10 cm, thickness 0.2 mm, Merck) using chloroform/methanol/water (120/85/20, each by vol.) with 2 mM  $\text{CaCl}_2$ .

The HPTLC immunobinding assay using cholerae ganglioside specifically detect GM1a ganglioside. To reveal the presence of GD1a, GD1b, and GT1b, the silica gel plates fixed with polyisobutylmethacrylate were incubated with 5 mU/mL *V. cholerae* neuraminidase (Behring Werke AG) in 0.05 M NaAc, 9 mM  $\text{CaCl}_2$ , pH5.5 (2 h,  $37^{\circ}\text{C}$ ) prior to combined cholerae ganglioside-immunostaining with the aim to convert GD1a, GD1b, GT1b and GQ1b gangliosides into GM1. Unspecific protein binding was blocked by a 15 min incubation of the plate with solution A (phosphate buffer saline, PBS supplemented with 1% bovine serum albumin and 0.02%  $\text{NaN}_3$ ) and then for 2 h with 250 ng/mL cholerae ganglioside (Sigma, Deisenhofen, Germany) diluted in solution A. Unbound cholerae ganglioside was removed by washing of plates five times with solution B (0.05% Tween 21, 0.02%  $\text{NaN}_3$  in PBS) followed by goat anti-cholerae ganglioside (Calbiochem, Frankfurt, Germany) and secondary rabbit anti-goat IgG antibody incubation (both diluted 1:2000 in solution A). After 1 h incubation with secondary antibody, the plates were washed again, followed by twofold rinsing with glycine buffer (0.1 M glycine, 1 mM  $\text{ZnCl}_2$ , 1 mM  $\text{MgCl}_2$ , pH 10.4), to remove phosphate. Bound antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) in glycine buffer. Each antibody analysis was performed twice, with identical results.

The anti-nLc4Cer antibody recognizes the Gal $\beta$ 1-4GlcNAc-residue and it shows some cross reactivity with Gal $\beta$ 1-4Glc $\beta$ 1-1Cer (lactosylceramide). Neuraminidase treatment of neolacto-series gangliosides with ( $\alpha$ 2-3)-substituted sialic acid is necessary prior to immunostaining

with anti-nLc4Cer antibody, whereas ( $\alpha$ 2-6)-sialylated neolacto-type gangliosides can be detected without enzyme treatment, because sialylation at the position 6 of the terminal galactose does not hinder recognition. Fixed silica gel plates were incubated with 2.5 mU/mL *V. cholerae* neuraminidase (Behring Werke AG) for 2 h at  $37^{\circ}\text{C}$  in the buffer described above, followed by immunostaining with anti-nLc4Cer antibody. Secondary rabbit anti-chicken IgG, affinity chromatography-purified and labeled with alkaline phosphatase (0.6 mg/mL), was purchased from Dianova and used in 1:2000 dilution. Bound antibodies were visualized as described above.

Neutral GSLs were separated on silica gel 60 precoated HPTLC-plates by solvent system chloroform/methanol/water (120:70:12) containing 2 mM  $\text{CaCl}_2$  (ratios are v/v/v) and visualized with orcinol.

The HPTLC immunostaining of neolacto-series neutral GSLs was performed with anti-nLc4Cer antibody but without neuraminidase pretreatment.

### Results and Discussion

Using HPTLC-immunostaining chromatography, it was possible to us to detect CSF GSLs in as little as 10–30  $\mu\text{L}$  of CSF<sup>5</sup>. Three or more fractions of gangliotetraose-series gangliosides, including GM1a, GD1a, GD1b, GT1b, and GQ1b, were detected in all samples except in the IWS CSF and hydrocephalus CSF (Figure 3).

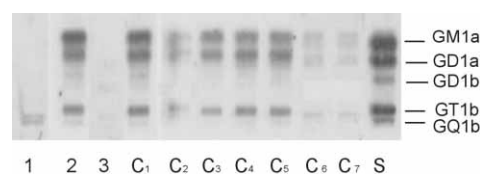


Fig. 3. HPTLC immunostain with cholera toxin B subunit after *V. cholerae* neuraminidase treatment of ganglioside fractions corresponding to 10  $\mu\text{L}$  of the CSF of IWS (line 1), Reye's syndrome (line 2) and congenital hydrocephalus (line 3). Control CSFs from neurological healthy infants were applied at lines C<sub>1</sub>–C<sub>7</sub>. As a reference was applied 10 ng of mouse brain gangliosides (lane S).

No expression of gangliotetraose-series gangliosides in IWS CSF in this study corresponds to the previous finding by Izumi et al. who described low levels of CSF gangliotetraose-series gangliosides both in symptomatic and idiopathic West syndrome<sup>5</sup>. Due to more sensitive method, we applied ten fold less amount of CSF at chromatograms than Izumi et al.<sup>5</sup>. In such small sample it was possible to detect GSL fractions in the most CSF samples examined, but not in IWS and congenital hydrocephalus CSF which obviously contained low levels of gangliotetraose-series gangliosides. It could be suspected a problem in the glycosphingolipid biosynthesis at the SIAT9 level, although our patients' normal development contrasts to the severe disease present in children with SIAT9 deficiency (Figure 4: deficient gangliosides GM1a, GD1a, GD1b, GT1b and GQ1b are marked in yellow colour). Ab-

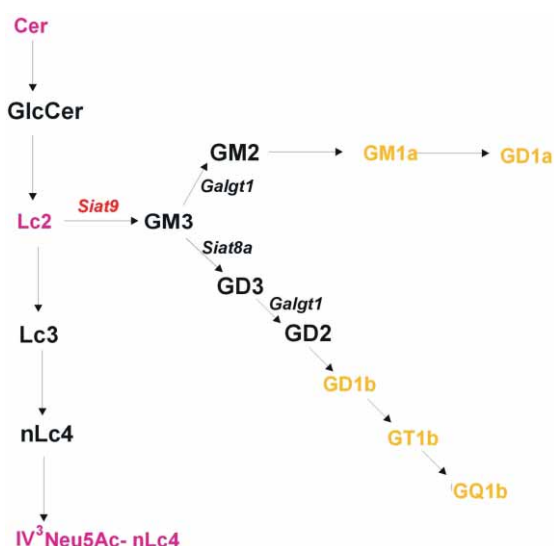


Fig. 4. A schematic overview of GSL biosynthesis. GlcCer, Lc2, Lc3 and nLc4 are neutral GSLs synthesized by step-wise addition of sugar residues by specific glycosyl transferases. All other molecules shown, except ceramide (Cer), are gangliosides synthesized by addition of sugar residues by distinct glycosyl transferases including sialyltransferases. Mutations of glycosyl transferase genes encoding Galgt1, Siat8a and Siat9 cause seizures in affected individuals<sup>13,14</sup>.

sence of gangliotetraose-series gangliosides in the CSF fluid of the subject with congenital hydrocephalus is in the discrepancy with previous finding by others<sup>9,10</sup>. The method used in this study is more sensitive and advantageous for analysis of GSLs in CSF. Ten fold diluted sample compared to previous assays allows optimal recognition and interaction between GM1a glyco- antigen and cholera toxin. Congenital hydrocephalus patient had in the same time pyruvate dehydrogenase (PDH) deficiency. Anaerobic glycolysis is slower due to the decreased regeneration of NADH coenzyme in respiratory chain. The precursor of sialic acid is glycolytic intermediate, phosphoenolpyruvate. It is tempting to speculate that congenital hydrocephalus patient with PDH deficiency has lower content of oligosialogangliosides due to decreased level of phosphoenolpyruvate and sialic acid.

Immunostaining with antibody against neolacto-series glycosphingolipids, after neuraminidase treatment, showed traces of neolacto-tetraose (IV<sup>3</sup>nLc4) ganglioside in CWS CSF (Figure 5). All other samples examined were negative (data not shown). Immunostaining of chromatogram of neutral glycosphingolipid extract, with antibody against neolacto-series glycosphingolipids, without neuraminidase treatment, revealed high level of one neutral glycosphingolipid lactosylceramide (Figure 6, lane 1, Lc2), and one ceramide fraction (Figure 6, lane 1, Cer). All other samples examined were negative (data not shown).

Using anti-neolacto-series GSL antibody, for the first time we detected traces of neolacto-tetraose gangliosides (IV<sup>3</sup>Neu5Ac-nLc4), high level of one fraction of lactosylceramide (Lc2), and one ceramide (Cer) fraction in IWS

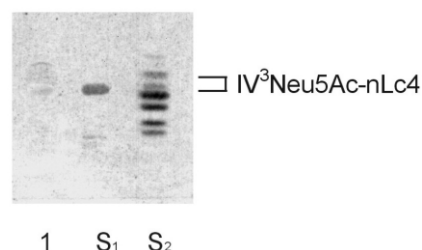


Fig. 5. HPTLC immunostain of neolacto-series gangliosides in 18 µL of the ISF of IWS (line 1). As a reference were applied 250 ng of mouse brain gangliosides (lane S<sub>1</sub>) and 2 µg of human granulocytes gangliosides (lane S<sub>2</sub>).

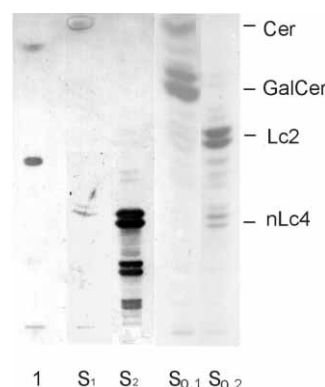


Fig. 6. HPTLC immunostain of neolacto-series neutral GSLs. Neutral GSL amount corresponding to 30 µL of IWS CSF (line 1), was chromatographed with 2 µg of mouse brain neutral GSLs (lane S<sub>1</sub>) and 5 µg of human granulocytes neutral GSLs (lane S<sub>2</sub>). Orcinol stain of neutral GSL references applied in double amount is shown at lines S<sub>0,1</sub> and S<sub>0,2</sub>.

CSF (Figure 4: IV<sup>3</sup>Neu5Ac-nLc4, Lc2 and Cer are marked in pink colour). Only scarce data were available about neolacto-type gangliosides and neutral GSLs in the brain. Vukelić et al.<sup>11</sup> described enhanced level of neolacto-tetraose ganglioside in both cerebral and anencephalic tissues compared to normal tissue. Mouse enzyme Lc3 synthase (see overview of GSL synthesis in Figure 4) is the key regulator of lacto-series GSL biosynthesis<sup>12</sup>. It is broadly expressed during embryonic development, whereas its expression postnatally is restricted to splenic B lymphocytes, placenta, and cerebellar Purkinje cells.

Idiopathic epilepsies have been linked to genes controlling ion channel function and receptor signalling on neurons, whereas symptomatic epilepsies have been linked to genetic defects causing neurodegeneration and disordered brain development<sup>13</sup>. Recently, Simpson et al. described that defect in GM3 ganglioside synthase gene underlied a severe early-onset symptomatic epilepsy syndrome<sup>14</sup>. Mice lacking correct GD3, GM2, and GD2 synthase genes suffer seizures and they are unable to extend Lc2 through GM2, GD3, and GD2 pathway to crucial complex brain gangliosides<sup>13</sup>. Our finding fits in that re-

cent observation. The block of complex ganglioside synthesis obviously caused accumulation of gangliosides precursors and increased built in of Lc2 in neolacto-series GSLs instead in GM3 and consequently in complex brain gangliosides (see overview of GSL synthesis in Figure 4). Follow up until the age of five years showed almost normal IWS patient psychomotor development. Therefore, the described shift of glycosphingolipid expression may implicate on transient inhibition of specific glycosyl transferases in the age of seven months. The discovery by Simpson et al. that a human epilepsy syndrome is caused by a defect in ganglioside synthesis was the beginning of this important story<sup>14</sup>. This case report is just one contribution to confirm it. Further unravelling of the mechanism underlying seizures will not only increase

our understanding of epilepsies but also illuminate the crucial functions of gangliosides in the brain.

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### REFERENCES

- HAMANO S, TANAKA M, KAWASAKI S, NARA T, HORITA H, ETO Y, KOHNO, *Pediatr Neurol*, 23 (2000) 219. — 2. RENER-PRIMEC Z, LOZAR-KRIVEC J, KRIVEC U, NEUBAUER D, *Pediatr Neurol*, 35 (2006) 197. — 3. COLOMBAIONI L, GARCIA-GIL M, *Brain Res Brain Res Rev*, 46 (2004) 328. — 4. BARIŠIĆ N, HORVATH R, GRKOVIĆ L, MIHELČIĆ D, LUETIĆ T, *Coll Antropol*, 30 (2006) 945. — 5. IZUMI T, OGAWA T, KOIZUMI H, FUKUYAMA Y, *Pediatr Neurol*, 9 (1993) 293. — 6. BOŠNJAK V, BESENSKI N, MARUŠIĆ-DELLA MARINA B, KOGLER A, *Dev Med Child Neurol*, 31 (1989) 66. — 7. MARKOTIĆ A, ČIKEŠ ČU-LIĆ V, TIČINOVIĆ KURIR T, MEISEN I, BÜNTEMEYER H, BORASKA V, ZEMUNIK T, PETRI N, MESARIĆ M, PETER-KATALINIĆ J, MÜTHING J. *Biochem Biophys Res Commun*. 330 (2005) 131. — 8. MAR-
- KOTIĆ A, MARUŠIĆ A, *Immunol Invest*, 33 (2004) 335. — 9. SHAPIRO K, GINNS E, BRADEN K, *Z Kinderchir*, 34 (1981) 419. — 10. GINNS E, FRENCH J, FLEISCHMAN A, COHEN S, *Pediatr Res*, 14 (1980) 1276. — 11. VUKELIĆ Z, METELMANN W, MÜTHING J, KOS M, PETER-KATALINIĆ J, *Biol Chem*, 382 (2001) 259. — 12. HENION TR, ZHOU D, WOLFER DP, JUNGALWALA FB, HENNET T, *J Biol Chem*, 276 (2001) 30261. — 13. PROIA RL, *Nat Genet*, 36 (2004) 1147. — 14. SIMPSON MA, CROSS H, PROUKAKIS C, PRIESTMAN DA, NEVILLE DC, REINKENMEIER G, WANG H, WIZNITZER M, GURTZ K, VERGANELAKI A, PRYDE A, PATTON MA, DWEK RA, BUTTERS TD, PLATT FM, CROSBY AH, *Nat Genet*, 36 (2004) 1225.

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### IZRAŽAJ GLIKOSFINGOLIPIDA U CEREBROSPINALNOM LIKVORU DOJENČADI S NEUROLOŠKIM ODSUPANJIMA: PRIKAZ TRIJU SLUČAJEVA

#### SAŽETAK

Cilj ovog rada bila je analiza izražaja glikosfingolipida u cerebrospinalnom likvoru tri dojenčeta s tri različita neurološka odstupanja: idiopatskim Westovim sindromom (IWS), Reyevim sindromom i kongenitalnim hidrocefalusom, u usporedbi s kontrolnom skupinom (n=7) upotrebom tankoslojne kromatografije visokog razlučivanja u kombinaciji s imunobojeanjem. U dojenčeta s idiopatskim Westovim sindromom i u dojenčeta s kongenitalnim hidrocefalusom nisu dokazani gangliozidi gangliotetraozne serije (kiseli glikosfingolipidi). U likvoru dojenčeta s IWS gangliozidi neolaktotetraozne frakcije nađeni su u tragovima, a u svim ostalim uzorcima nisu dokazani. Pored toga samo u uzroku likvora dojenčeta s IWS bila je izražena frakcija laktozilceramida i jedna ceramidna frakcija. Ovi rezultati potvrđuju ranije opisani nedostatak gangliozida gangliotetraozne serije u pacijenata s IWS. Prvi put opisan je povećan izražaj glikosfingolipida neolacto-serije u pacijenata s IWS. Obzirom da je IWS pacijent pokazao normalan psihomotorni razvoj tijekom praćenja do pete godine života, opisano skretanje izražaja GSL ukazuje na moguću prolaznu inhibiciju specifičnih glikozil transferaza u dobi od sedam mjeseci.