Sulfatide levels correlate with severity of neuropathy in metachromatic leukodystrophy

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Abstract

Objective: Metachromatic leukodystrophy (MLD) is an autosomal recessive lysosomal storage disorder due to deficient activity of arylsulfatase A (ASA) that causes accumulation of sulfatide and lysosulfatide. The disorder is associated with demyelination and axonal loss in the central and peripheral nervous systems. The late infantile form has an early-onset, rapidly progressive course with severe sensorimotor dysfunction. The relationship between the degree of nerve damage and (lyso)sulfatide accumulation is, however, not established.

Methods: In 13 children aged 2–5 years with severe motor impairment, markedly elevated cerebrospinal fluid (CSF) and sural nerve sulfatide and lysosulfatide levels, genotype, ASA mRNA levels, residual ASA, and protein cross-reactive immunological material (CRIM) confirmed the diagnosis. We studied the relationship between (lyso)sulfatide levels and (1) the clinical deficit in gross motor function (GMFM-88), (2) median and peroneal nerve motor and median and sural nerve sensory conduction studies (NCS), (3) median and tibial nerve somatosensory evoked potentials (SSEPs), (4) sural nerve histopathology, and (5) brain MR spectroscopy.

Results: Eleven patients had a sensory-motor demyelinating neuropathy on electrophysiological testing, whereas two patients had normal studies. Sural nerve and CSF (lyso)sulfatide levels strongly correlated with abnormalities in electrophysiological parameters and large myelinated fiber loss in the sural nerve, but there were no associations between (lyso)sulfatide levels and measures of central nervous system (CNS) involvement (GMFM-88 score, SSEP, and MR spectroscopy).

Interpretation: Nerve and CSF sulfatide and lysosulfatide accumulation provides a marker of disease severity in the PNS only; it does not reflect the extent of CNS involvement by the disease process. The magnitude of the biochemical disturbance produces a continuously graded spectrum of impairments in neurophysiological function and sural nerve histopathology.

Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive disorder of lipid metabolism characterized by deficient activity of the lysosomal enzyme arylsulfatase A (ASA) with inability to degrade galactosylceramide-3-O-sulfate (sulfatide) and galactosylsphingosine-3-O-sulfate (lysosulfatide). Progressive accumulation of (lyso)sulfatide within oligodendrocytes and Schwann cells is associated with demyelination and axonal loss in the central (CNS) and peripheral nervous systems (PNS). Of the three phenotypes of MLD (late infantile, juvenile, and adult–onset),
the late infantile form is most frequent and is usually diagnosed in the second year of life. The major presenting symptom is progressive gait abnormality with areflexic spasticity; death occurs during childhood. In late infantile MLD, peripheral neuropathy often precedes the CNS manifestations of the disease and is characterized by severe slowing of motor and sensory conduction. Studies of sural nerve histopathology in MLD have shown reduced large myelinated fiber density and accumulation of sulfatide within macrophages, Remak cells, and Schwann cells.

Since therapeutic approaches to MLD are being explored, we evaluated a wide range of potential markers of disease severity including biochemical and molecular analyses (ASA activity, genotype, ASA mRNA levels, and residual ASA CRIM), measurements of motor function (GMFM-88, gross motor function measure-88), brain MRI and MR spectroscopy, neurophysiological assessments and sural nerve biopsy. Particular emphasis was placed on cerebrospinal fluid (CSF) and sural nerve (lyso)sulfatide levels, as the relationship between severity of PNS and CNS impairment and extent of accumulation of sulfatide and lysosulfatide remains uncertain. We found strong correlations between (lyso)sulfatide levels, motor and sensory nerve function, and sural nerve histopathology and morphometry. There were no associations between these variables and measures of CNS dysfunction implying that disease burden in CNS and PNS are separable and discrete. We suggest that the severity of PNS axonal loss and demyelination is related to the toxic effects of tissue accumulation of sulfatide and lysosulfatide.

**Material and Methods**

**Study design and patient selections**

The study presents exploratory analyses of the baseline data collected for a single center, open-label, nonrandomized, uncontrolled, Phase I clinical trial of intravenous enzyme replacement therapy in late infantile MLD (EudraCT 2006-005341-11, ClinicalTrials.gov identifier number NCT00418561). Thirteen children (Table 1), eight girls, and five boys, age range 25–59 months were enrolled. There were no screen failures. All of the children were referred between January and August 2007 from European clinics. Inclusion criteria were: (1) established diagnosis of MLD, (2) age between one and 5 years with onset of symptoms before age four, and (3) presence of residual voluntary motor function. Baseline evaluations provided the data for this report. They included clinical and motor function assessments, neuropsychological testing, nerve conduction studies (NCS) of the median, peroneal and sural nerves, median and tibial somatosensory evoked potentials (SSEPs), sural nerve biopsy with light and electron microscopic analyses, measurement of CSF sulfatide and sural nerve sulfatide and lysosulfatide concentrations, determination of relative ASA mRNA expression level, estimation of ASA CRIM (cross-reactive immunological material), and MR spectroscopy studies with measurement of N-acetylaspartate levels (NAA) in the centrum semiovale. Since there was a broad spectrum of motor dysfunction, the children were representative of a cross-sectional sample of the late infantile MLD population in the specified age range.

**Table 1.** Demographic data, clinical features, and mutations in 13 children with late infantile MLD.

| Patient ID | Gender | Age (months) | Mutation 1 | Mutation 2 | Clinical features | GMFM-88 score |
|------------|--------|--------------|------------|------------|-------------------|---------------|
| 1          | Female | 30           | c.459+1G>A | c.459+1G>A | Able to walk, unsteady | 180           |
| 2          | Female | 51           | c.392_403del | c.1171A>G;p.5391G | Able to sit, grabs toys | 36            |
| 3          | Male   | 31           | c.459+1G>A | c.1304C>G;p.435R | Unable to sit, moves head | 13            |
| 4          | Male   | 34           | c.287C>T;p.596F | c.459+1G>A | Unable to sit, moves head | 15            |
| 5          | Male   | 44           | c.296dupG | c.1217_1225del | Unable to sit, moves head | 25            |
| 6          | Female | 36           | c.459+1G>A | c.730C>T;p.R244C | Unable to sit, moves head | 21            |
| 7          | Female | 41           | c.635C>T;p.A212V | c.635C>T;p.A212V | Able to walk, slight spasticity, slightly impaired motor function | 131           |
| 8          | Male   | 59           | c.459+1G>A | c.602A>G;p.Y201C | Able to sit, grabs toys | 38            |
| 9          | Female | 26           | c.459+1G>A | c.1240T>C;p.C414R | Unable to sit, moves head | 47            |
| 10         | Female | 40           | c.731G>A;p.R244H | c.919G>A;p.E307K | Unable to sit, moves head | 18            |
| 11         | Male   | 34           | c.459+1G>A | c.459+1G>A | Unable to sit, moves head | 21            |
| 12         | Female | 25           | c.443C>T;p.P148L | c.443C>T;p.P148L | Able to walk and run | 141           |
| 13         | Female | 37           | c.459+1G>A | c.973+1G>A | Unable to sit, moves head | 34            |

MLD, metachromatic leukodystrophy.
Standard protocol approval, registration and patient consent

The clinical study was approved by the Ethical Committee of Copenhagen. Written informed consent was obtained. Control sural nerve samples for biochemical (eight adult) and morphometric (three children) analyses were obtained from patients who underwent diagnostic evaluation for suspected neuromuscular disorders at Johns Hopkins Medical Institutions. Patients consented to use of portions of the biopsy specimens for research purposes. The amplitudes of sensory nerve action potentials (SNAP) and detailed histological analyses were normal for all control patients.

Motor function evaluation (GMFM-88)

A physiotherapist specialized in child development examined motor function using the GMFM-88, which records motor skills that are typical of normal development (Table 1). The test is validated to evaluate children with neurological disorders.6,7

Molecular analyses

Genomic DNA was isolated from whole blood using a standard salting out method,8 and the entire coding and intronic sequence of ASA were amplified by PCR and sequenced directly in a diagnostic setting (primers and PCR conditions are available upon request, Table 1).

Quantitation of ASA mRNA

Total RNA was isolated from cultured fibroblasts from the patients and normal donor controls using RNeasy (Qiagen, Copenhagen, Denmark), and cDNA was generated using Superscript II™ (Invitrogen, Life Technologies Europe BV, Naerum, Denmark) in combination with random hexamer primers, both according to the manufacturer’s description. Quantitative real-time PCR (qPCR) was performed with cDNA-specific primers for ASA (exon 4–5) and the human 18S locus in combination with fluorescent-marked TaqMan probes. The relative amount of target ASA mRNA was normalized to 18S rRNA and expressed as a percentage of healthy control values using the ΔΔCT method9 (primer and probe sequences are available upon request Table 2).

ASA CRIM in fibroblasts

Fibroblasts from skin biopsies of MLD patients enrolled in the clinical trial were harvested and expanded at the clinical site. Control fibroblast cell lines from normal donors were obtained from Coriell Cell Repositories (Camden, NJ, USA) they were expanded in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and harvested by trypsinization. Cell lysates were prepared by homogenizing cells in lysis buffer (10 mmol/L Tris pH 7 containing 0.1% triton X-100 ± 1× protease inhibitor cocktail) (Roche Catalogue No. 04693159001); lysates were stored at ≤−65°C until analysis. ASA CRIM in cell lysates was determined by ELISA with a paired

Table 2. Biomarkers in 13 patients with late infantile MLD.

| Patient ID | %mRNA ASA | CRIM (ng/mg) | SN sulfatide (ng/mg dw)1 | SN lysosulfatide (ng/mg dw)2 | CSF sulfatide (nmol/L) | NAA in CS |
|------------|------------|--------------|--------------------------|----------------------------|------------------------|-----------|
| 1          | 16.2       | 0.04         | 7670                     | 5.32                       | ND                     | 2.65      |
| 2          | 48.1       | 0.28         | 3430                     | 1.48                       | 200                    | 0.63      |
| 3          | 35.7       | 0.31         | 5630                     | 1.99                       | 750                    | 0.28      |
| 4          | 18.5       | 0.03         | 7620                     | 1.18                       | 550                    | 0.59      |
| 5          | 28.3       | 0.58         | No SN                    | No SN                      | 1450                   | 0.74      |
| 6          | ND         | ND           | 4700                     | 2.47                       | 2300                   | 0.35      |
| 7          | 45.9       | 0.09         | 3800                     | 0.65                       | 700                    | 1.20      |
| 8          | 15.9       | 0.11         | 2160                     | 0.53                       | 225                    | 1.42      |
| 9          | 20.1       | 0.92         | 9530                     | 8.50                       | 1200                   | 0.87      |
| 10         | 34.6       | 0.02         | 7850                     | 4.94                       | 2200                   | 0.42      |
| 11         | 6.0        | 0.10         | 11,300                   | 9.85                       | 1930                   | 1.04      |
| 12         | 4.9        | 0.02         | 7190                     | ND                         | 1150                   | 2.34      |
| 13         | 3.0        | 0.04         | 9400                     | 2.73                       | 675                    | 0.55      |
| NV         | 100        | 73.57        | 935 ± 157                | 0.14 ± 0.12                | <50                    | 3.49 ± 0.39|

MLD, metachromatic leukodystrophy; ASA, arylsulfatase A; CRIM, cross-reactive immunological material; SN, sural nerve; dw, dry weight; CSF, cerebrospinal fluid; NAA, N-acetylaspartate; CS, centrum semiovale; No SN, no sural nerves; ND, not done; NV, normal values.

1Of the 19 molecular species monitored during LC-MS/MS: liquid chromatography–tandem mass spectrometry analysis of sural nerve sulfatide, the four most abundant species on a mass basis are C24:1 (23%), C24:0 (20%), C24:0-OH (9%), and C22:0 (7%); the distribution in MLD and control nerves is similar. C24:1 sulfatide has a molecular weight of 889.

2Lysosulfatide has a molecular weight of 541.
polyclonal goat anti-ASA antibody (R&D system, AF2485, BAF2485, Minneapolis, MN, USA) followed by detection of bound ASA CRIM with streptavidin-horseradish peroxidase and TMB peroxidase substrate. Cell lysates from MLD patients were tested at 1:5 and 1:10 dilution, whereas lysates from controls were tested at 1:50 dilution. ASA concentration in cell lysates (ng/mL) was calculated based on the calibration curve of recombinant human ASA in the same assay. Protein concentrations of cell lysates were determined by Pierce™ Life Technologies (Rockford, IL, USA) BCA protein assay kit according to the manufacturer’s instructions; results are reported as ng ASA CRIM/mg total protein (Table 2).

**LC-MS/MS analysis of sural nerve sulfatide and lysosulfatide**

Tissue samples (~2–10 mg of wet weight) were weighed, dried, and homogenized in 1 mL of water using a 24-bead homogenizer. An aliquot of the homogenate equivalent to ~0.2 mg of dry tissue was used for analysis. Briefly, after adding C12:0 sulfatide (Avanti Polar Lipids, Alabaster, AL) as the internal standard (ISTD), samples were extracted with acetonitrile, subjected to alkaline hydrolysis in 4 N sodium hydroxide, quenched with acetic acid, and then further purified by solid phase extraction (SPE) (Oasis WAX 1 mL cartridge; Waters, Milford, MA). The eluate from SPE was dried under nitrogen and the residue was reconstituted in 200 μL of methanol prior to LC-MS/MS analysis (Table 2).

Calibration curve standards and quality control samples were prepared by spiking cynomolgous monkey sural nerve homogenate with C16:0 sulfatide (analyte; Matreya, Pleasant Gap, PA) in the range of 20–2000 ng/mg of dry tissue and 400 ng/mg of C12:0 sulfatide (ISTD, Avanti Polar Lipids).

Sample preparation for lysosulfatide detection followed the same extraction procedure, except that acetonitrile/methanol (1:1 v/v) was used as the extraction solvent, and neither alkaline hydrolysis nor SPE were carried out. To generate the standard curve, cynomolgous monkey sural nerve homogenate was spiked with lysosulfatide as the analyte (Matreya) in the range of 0.3–17 ng/mg of dry tissue and 5 ng/mg of C2:0 sulfatide as ISTD (Matreya). Control values for sural nerve lysosulfatide and sulfatide concentration were determined by analysis of glutaraldehyde-fixed nerves from eight adult patients (age 37–61; 7F/1M) who underwent biopsy at Johns Hopkins Medical Institutions for suspected neuromuscular disorders. Pilot experiments demonstrated that sulfatide and lysosulfatide levels in cynomolgous monkey sural nerves were comparable in freshly harvested and glutaraldehyde-fixed tissue.

For quantitation of sulfatide species, a 10-μL aliquot of reconstituted sample was injected onto an XBridge BEH 300 2.1 × 50 mm reversed-phase C4 column (3.5 μm particle size, Waters) connected to an Accela HPLC system (Thermo Electron, San Jose, CA). Elution was performed using a linear gradient from 40% 2 mmol/L aqueous ammonium formate: 60% acetonitrile to 100% acetonitrile (pH 8.5) over 5 min at a flow rate of 0.5 mL/min. The eluent from the column was introduced into a triple quadrupole mass spectrometer operated in negative-ion electrospray mode (TSQ Quantum Ultra; Thermo Electron). Sulfatide species were identified by precursor ion scan for m/z 97 (the most abundant fragment ion). The monitored SRM transitions were m/z 722 (for the ISTD), 778, 804, 806, 822, 832, 834, 850, 860, 862, 874, 876, 878, 886, 888, 890, 902, 904, 906, and 916. Calibration curves were constructed by linear regression analysis of the peak area ratios of C16:0/C12:0 versus the amount of C16:0 sulfatide. Sulfatide concentration was calculated from the summed values of each of the 19 molecular species.

For quantitation of lysosulfatide, the same LC-MS/MS system was used. The solvent gradient consisted of water/acetonitrile each containing 5 mmol/L of ammonium formate ramped from 20 to 100% acetonitrile over 5 min at a flow rate of 0.4 mL/min. The SRM transitions monitored for lysosulfatide and the ISTD C2:0 sulfatide were m/z 540 to m/z 97 and m/z 582 to m/z 97, respectively.

**CSF sulfatide**

CSF sulfatide was determined by immunostaining of thinline chromatograms with the Sulph I antisulfatide antibody as previously described (Table 2).10,11

**Proton MRSI studies**

Magnetic resonance spectroscopy imaging (MRSI) was performed under general anesthesia utilizing standard institutional scanning protocols. Echo-planar spectroscopic imaging provided multislice metabolic maps covering deep white matter from which centrum semiovale NAA levels were measured as previously described (Table 2).12

**Nerve conduction studies**

The children were sedated with chloral hydrate rectally (30–50 mg/kg body weight) and temperature of the limbs was kept at 35–38°C using a thermocoupled infrared heating lamp. Motor fibers of the right median nerve were stimulated supramaximally at the wrist and elbow through surface electrodes. The compound muscle action potential (CMAP) was recorded through a surface electrode placed...
over the abductor pollicis brevis (APB) muscle. The CMAPs were amplified (frequency range 20 Hz–10 kHz) and latencies and amplitudes were measured off-line. The distal motor latency (DML) from wrist to APB was measured and the motor nerve conduction velocity (MNCV) from elbow to wrist calculated. F-waves were recorded by stimulating the median nerve at the wrist and the shortest latency was measured. Sensory fibers in digit 2 were stimulated supramaximally through ring surface electrodes. The orthodromic compound SNAP was recorded through surface electrodes at the wrist and amplified (frequency range 200 Hz–4 kHz); amplitudes were measured peak-to-peak. The sensory nerve conduction velocity (SNCV) from digit 2 to wrist was calculated (Table 3).

Motor fibers of the right peroneal nerve were stimulated supramaximally at the ankle and fibular head. The CMAP was recorded from the extensor digitorum brevis muscle (EDB). The DML from ankle to EDB was measured, and the MNCV from the fibular head to ankle was calculated. F-waves were recorded by stimulating the peroneal nerve at the ankle.

Both sural nerves were stimulated supramaximally at the lateral malleolus through insulated needles with a 3-mm bared tip. The orthodromic SNAP was recorded unipolarly through a needle electrode placed close to the nerve at midcalf and referenced to a needle placed medially at a transverse distance of 2 cm, amplified (frequency range of 200 Hz–4 kHz) and averaged in two runs to ensure reproducibility. Latency to the first positive phase was measured and the SNCV calculated. The amplitude was measured peak-to-peak (Fig. 1).

Motor and sensory conduction study values were compared to our own laboratory controls13 and other published values14 in normal children (Table 3). In normal children, there is a gradual increase in MNCV and decrease in DML with age from birth to 3–4 years at

### Table 3. Electrophysiological studies in 13 patients with late infantile MLD.

| Patient ID | Median nerve conduction studies | Peroneal nerve conduction studies | Sural nerve conduction studies | Median SSEP | Tibial SSEP |
|------------|---------------------------------|-----------------------------------|--------------------------------|-------------|------------|
|            | CMAP (mV)¹  | DML (msec)² | MNCV (m/sec)³ | SNAP (µV)⁴ | SNCV (m/sec)⁵ | CMAP (mV)⁶ | DML (msec)⁷ | MNCV (m/sec)⁸ | SNAP (µV)⁹ | SNCV (m/sec)¹⁰ | N20 (msec)¹¹ | P28 (msec)¹² |
| 1          | 7.0        | 5.7        | 23          | 5.4         | 46          | 0.9        | 8.2        | 19          | 12.4        | 38          | 21.4        | 50.7        |
| 2          | 5.2        | 3.5        | 27          | 2.8         | 33          | 5.1        | 5.1        | 34          | 5.7         | 24          | 17.0        | 36.0        |
| 3          | 3.5        | 5.8        | 16          | 2.2         | 18          | 1.3        | 7.2        | 17          | 0.5         | 27          | 25.4        | 54.9        |
| 4          | 7.1        | 5.3        | 13          | 1.4         | 34          | 2.8        | 7.9        | 14          | 1.2         | 33          | 23.6        | 45.6        |
| 5          | 8.2        | 8.0        | 11          | 1.1         | 24          | 0.7        | 9.5        | 10          | ND          | ND          | 38.5        | 88.6        |
| 6          | 5.5        | 6.6        | 12          | 1.6         | 20          | 3.0        | 8.2        | 11          | 2.3         | 16          | 33.7        | 113.5       |
| 7          | 7.5        | 3.4        | 47          | 21.1        | 54          | 4.3        | 4.5        | 46          | 100.9       | 52          | 17.2        | 34.7        |
| 8          | 11.2       | 2.7        | 48          | 26.6        | 53          | 9.2        | 3.2        | 49          | 97.5        | 51          | 15.7        | 56.2        |
| 9          | 4.2        | 6.4        | 19          | 1.9         | 19          | 1.8        | 7.6        | 23          | 4.2         | 34          | 23.1        | 46.0        |
| 10         | 5.1        | 5.8        | 13          | 1.7         | 12          | 0.2        | 10.3       | 9           | NR          | NR          | NR          | NR          |
| 11         | 2.9        | 7.7        | 10          | 0.8         | 16          | 1.5        | 11.6       | 10          | 0.1         | 18          | 28.2        | 39.3        |
| 12         | 5.2        | 4.7        | 23          | 2.6         | 40          | 1.6        | 6.3        | 16          | 1.9         | 43          | 19.8        | 46.7        |
| 13         | 8.9        | 5.3        | 17          | 0.7         | 33          | 4.0        | 7.7        | 16          | 0.3         | 33          | 26.5        | 37.0        |
| NV         | 2–10       | 2–3        | 40–60       | 5–15        | 50–60       | 1–7        | 2–3        | 42–52       | >19         | 45–55       | 13–17       | 24–33       |

MLD, metachromatic leukodystrophy; SSEP, somatosensory evoked potential; CMAP, compound muscle action potential; DML, distal motor latency; MNCV, motor nerve conduction velocity; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; ND, study not done; NR, no response; NV, normal values (ranges in children aged 2–5 years)¹³,¹⁴.

¹Compound muscle action potential from abductor pollicis brevis muscle (APB).
²Distal motor latency from wrist to APB.
³Motor nerve conduction velocity from elbow to wrist.
⁴Compound sensory action potential (SNAP) from digit 2 to wrist.
⁵Sensory nerve conduction velocity from digit 2 to wrist.
⁶Compound muscle action potential from extensor digitorum brevis muscle (EDB).
⁷Distal motor latency from ankle to EDB.
⁸Motor nerve conduction velocity from fibular head to ankle.
⁹Sural nerve SNAP from lateral malleolus at midcalf.
¹⁰Sensory nerve conduction velocity from lateral malleolus to midcalf.
¹¹Latency of median nerve somatosensory evoked potential from wrist to cortex.
¹²Latency of tibial nerve somatosensory evoked potential from medial malleolus to cortex.
which time the MNCV is similar to young adults. The increase in conduction velocity in normal children from 2 to 5 years of age is in the order of 5–10%. The amplitude of the CMAP increases slightly from birth to adulthood. SNCV of the median and sural nerves increases with age from birth to 3 years of age at which time the SNCV is similar to young adults. The amplitudes of the SNAPs recorded from the median and sural nerves increase gradually to adult values by 3 years of age.

**Somatosensory evoked potentials**

Bilateral median and tibial nerve SSEPs were examined. Stimulation of the median nerve through surface electrodes at the wrist was adjusted to elicit a movement of the thumb whenever possible. Surface electrodes were placed over the median nerve at the elbow and supraclavicular fossa (Erb’s point) to record the nerve action potential. Surface electrodes were placed over the C7 spinous process to record the spinal response and over the contralateral C3/C4 cranial site to record the cortical response. The tibial nerve was stimulated through surface electrodes at the medial malleolus. Surface electrodes were placed at the popliteal fossa to record the nerve action potential, over the T12 spinous process to record the spinal response, and over the vertex at Cz to record the cortical response. The latencies were compared to published values from normal children (Table 3).

**Pathology of sural nerve**

Right sural nerve biopsies were examined in 12 children. The nerves were fixed by immersion in glutaraldehyde (2.0% in 0.1 mol/L cacodylate buffer) for 24 h. Fixed
nerves were postfixed in 1% osmium tetroxide in 0.1 mol/L Sørensen’s buffer, dehydrated in graded alcohol (30–100%), cleared in propylene oxide, and embedded in increasing concentrations of epoxy resin until polymerized in pure epon in a heated cabinet. Cross sections of the sural nerve were cut with dry glass knives at 2–3 μm thickness, overstained with p-phenylenediamine, mounted and investigated with a light microscope (Olympus BX51; Olympus Danmark A/S, Ballerup, Denmark). Transverse sections were stained with toluidine blue to assess the presence of metachromatic inclusions in Schwann cells and macrophages. Studies in the MLD patients were compared with three control children aged 2, 3, and 11 years examined at Johns Hopkins Medical Institutions to exclude giant fiber neuropathy (Table 4).

### Light microscopic examination

Overview micrographs at 40× and detailed micrographs at 100× were photographed with a digital camera (Olympus DP71), and images were processed using the MNERVE custom made nerve morphometry software developed at MATLAB (Version 2010a; MathWorks, Inc., Natick, MA). The total nerve area was traced from the overview micrographs and myelin rings were traced from at least 10% of the total nerve area; fiber diameters were calculated as the diameter of the circle having the same area as the outer contours of the “myelin rings.” Fiber density within the measured area was then used to calculate the total number of myelinated fibers (Fig. 1).

### Electron microscopic evaluation

For ultrastructural analyses, 70-nm thick cross sections of the nerves were obtained and stained with citrate/uranyl acetate. Five to seven electron micrographs at 2000× were acquired from each nerve using a Hitachi 7600 electron microscope (Hitachi 7600 TEM, Tokyo, Japan). A micrograph was taken from each fascicle within the nerve. Measurements of the outer and inner diameters of the myelin sheath were obtained from 150 to 400 fibers to obtain the axon and fiber diameters as described above. The g-ratio and the myelin thickness were calculated from the diameters (Fig. 1).

### Data analysis

The goal of this exploratory analysis was to assess important associations between a broad range of disease biomarkers in a cross-sectional sample of 13 children with late infantile MLD. In addition, sural nerve samples were obtained from three control children for morphometric analyses. Due to the small sample size, model building and statistical comparisons between the MLD and control patients were not appropriate. Therefore, the exploratory analyses were only descriptive in nature. A measure of the linear association between two variables was obtained using Pearson correlation. The resulting estimates were tested for statistical significance at the 0.05 level; P-values were not adjusted for multiplicity. Correlation estimates for the SNAP amplitudes were obtained after logarithmic

### Table 4. Morphometric values from sural nerves of 12 patients with late infantile MLD.

| Patient ID | Total number of fibers | Number fibers >7 μm | Percent fibers >7 μm | Number fibers >9 μm | Percent fibers >9 μm | Mean diameter of 10 largest fibers (μm) | Mean axon diameter (μm) | Mean myelin thickness (μm) | Mean g-ratio | Myelin-fiber slope |
|------------|------------------------|---------------------|---------------------|---------------------|---------------------|----------------------------------------|------------------------|------------------------|----------------|------------------|
| 1          | 2092                   | 536                 | 26                  | 172                 | 8                   | 11.5                                   | 3.3                    | 0.39                   | 0.82           | 0.10             |
| 2          | 4735                   | 839                 | 18                  | 102                 | 2                   | 10.3                                   | 3.1                    | 0.33                   | 0.83           | 0.10             |
| 3          | 3591                   | 399                 | 11                  | 9                   | 0                   | 8.8                                    | 2.0                    | 0.23                   | 0.82           | 0.13             |
| 4          | 4050                   | 871                 | 21                  | 120                 | 3                   | 10.0                                   | 2.3                    | 0.27                   | 0.80           | 0.08             |
| 5          | ND                     | ND                  | ND                  | ND                  | ND                  | ND                                     | ND                     | ND                     | ND             | ND               |
| 6          | 1587                   | 188                 | 12                  | 3                   | 0                   | 8.5                                    | 3.3                    | 0.29                   | 0.85           | 0.08             |
| 7          | 5051                   | 1820                | 36                  | 637                 | 13                  | 12.0                                   | 3.2                    | 0.43                   | 0.79           | 0.12             |
| 8          | 6400                   | 1947                | 30                  | 867                 | 14                  | 11.5                                   | 2.6                    | 0.37                   | 0.78           | 0.13             |
| 9          | 5010                   | 1166                | 23                  | 159                 | 3                   | 10.0                                   | 2.8                    | 0.29                   | 0.83           | 0.09             |
| 10         | 1829                   | 165                 | 9                   | 5                   | 0                   | 8.5                                    | 3.0                    | 0.32                   | 0.82           | 0.07             |
| 11         | 5020                   | 255                 | 5                   | 0                   | 0                   | 8.0                                    | 3.0                    | 0.29                   | 0.83           | 0.09             |
| 12         | 3230                   | 614                 | 19                  | 55                  | 2                   | 9.5                                    | 3.1                    | 0.31                   | 0.84           | 0.07             |
| 13         | 6160                   | 501                 | 8                   | 0                   | 0                   | 9.0                                    | 3.0                    | 0.30                   | 0.84           | 0.13             |
| NV         | 7422–9397              | >1800               | >25                 | >400                | >5                  | 10.0–12.5                               | 2–3                    | 0.68–1.20              | 0.65           | 0.13–0.23        |

MLD, metachromatic leukodystrophy; ND, study not done; NV, normal values.

1Slope of myelin thickness (μm) versus fiber diameter (μm).

2Sural nerve was previously biopsied in connection with diagnosis at another institution; not included in the present study.
transformation based on previous studies. Empirical cumulative distributions plots were produced to contrast the g-ratio data between the MLD patients and the three controls.

Results

Demographic, clinical and genotypic characteristics

All 13 children (Table 1) had pathologically low leukocyte ASA enzymatic activities (2.2–9.4 nmol/h per mg cell protein; normal >20 nmol/h per mg cell protein) and elevated urinary sulfatide excretion. They all presented before age 4 with progressive symptoms of late infantile MLD including spasticity, impaired gait, late motor milestones, or evidence of cognitive impairment. On motor function evaluation, three children were able to walk (GMFM-88 scores: 131–180), two were able to sit without support (GMFM-88 scores: 36–38), and eight were unable to sit (GMFM-88 scores: 13–47); the maximum normal score is 264. The abnormalities observed on NCS did not correlate with CNS disease burden as measured by motor function assessment (GMFM-88) and NAA levels in the centrum semiovale.

The diagnosis of MLD was confirmed by molecular analysis (Table 1). Fifteen different mutations were identified; the common c.459+1G>A splice site mutation, which is frequent in the late infantile phenotype, accounted for 38% of the alleles (10/26). Nine of the 13 patients carried at least one allele predicted to be targeted by the nonsense mediated mRNA decay pathway (NMD) due to a disrupted reading-frame. Three novel mutations were identified (c.1171A>G, c.1240T>G, and c.1304C>G) and will be described in detail elsewhere. All novel mutations were identified in a compound heterozygous state with a known pathogenic mutation. Patients 4, 5, and 13 were in addition heterozygous for the pseudo-allele (c.*96A>G), whereas patient 12 was homozygous. Overall, mutation analysis confirmed the association of splice site and frame shift mutations with the late infantile phenotype.

Quantitation of ASA mRNA

The relative amount of ASA mRNA varied from 3.0% (Pt 13) to 48.1% (Pt 2) compared to control values, which may be explained in part by the genotype of the individual patients (Tables 1, 2). Except for Pt 12, patients carrying one or two alleles predicted to undergo NMD exhibited the lowest expression levels. Examination of arylsulfatase CRIM levels in fibroblasts obtained from skin biopsies showed that all patients had extremely low values (<1 ng/mg) compared to the control reference value (74 ng/mg); there was no apparent relationship between CRIM levels and other measures of disease severity.

Analysis of (lyso)sulfatides in CSF and sural nerve

CSF sulfatide varied from 200 to 2300 nmol/L (normal <50 nmol/L). The two oldest patients (two and eight) had the lowest values (200 and 225 nmol/L); no relationship between disease course, age, motor function or central white matter NAA levels, and the amount of sulfatide in spinal fluid was apparent (Table 2). The patients who were still able to walk had values of 700 and 1150 nmol/L.

Sural nerve sulfatide concentration was increased 2- to 12-fold (2160–11,300 ng/mg dry weight) compared with normal values (Table 2); lysosulfatide levels were increased 4- to 70-fold (0.53–9.85 ng/mg dry weight), and the two concentrations were strongly correlated (P < 0.005). The correlation between CSF sulfatide and sural nerve sulfatide concentration did not reach statistical significance (P = 0.1), whereas the correlation between CSF sulfatide and sural nerve lysosulfatide was significant (P < 0.02).

Proton MRSI findings

NAA provides a marker of neuronal and axonal integrity. Inspection of cerebral NAA maps revealed marked signal attenuation in the deep white matter (centrum semiovale) compared to findings in normal controls (Table 2). We have previously reported a significant relationship between progressive attenuation of the NAA signal and loss of motor function in this cohort. Central white matter NAA levels did not correlate with CSF or sural nerve (lyso)sulfatide concentrations, molecular measurements (ASA mRNA expression levels and CRIM), neurophysiological parameters, or sural nerve pathology.

Electrophysiological findings

Nerve conduction studies

NCS of the sural, median, and peroneal nerves revealed pronounced abnormalities of sensory and motor fibers in 11 patients, whereas two patients had normal values (Table 3). In normal children the stimulus strength required to obtain maximal responses is <20 mA, but in patients with MLD the threshold was often markedly increased requiring stimulus strengths of 50–100 mA and prolonged duration above the usual 0.2 msec to ensure supramaximal stimulation.

The median nerve DMLs in 11 children with neuropathy were 40–220% prolonged and near normal in two patients (Fig. 2) (normal range 2–3 msec). The median MNCVs
were 46–78% reduced in 11 patients and normal in two (normal values in children aged 2–4 years, 45–55 m/sec\textsuperscript{13,14}). The F-wave latencies were prolonged at 28–75 msec in nine and normal in two patients; F-waves were absent in two patients (normal range 15–20 msec\textsuperscript{14}). The amplitudes of the CMAPs evoked at the wrist were within normal range in all patients (Fig. 2, Table 3) and when evoked at the elbow were 31 ± 5% smaller than at the wrist. The orthodromic median nerve SNCVs from digit 2 to wrist (Table 3) were 9–76% reduced in 11 and normal in two patients; SNAP amplitudes were 76–93% reduced in 10 and normal in three (Fig. 2).

The peroneal nerve DMLs were 104–364% prolonged in 11 children and near normal in two. The MNCVs were 15–78% reduced in 11 patients and normal in two (Fig. 2, Table 3). F-waves could only be recorded in two patients with abnormal NCS and were markedly prolonged at 57 and 73 msec; they were normal in two patients with normal NCS (normal range 25–35 msec\textsuperscript{20}). The amplitudes of the CMAPs evoked at the ankle were reduced in three patients and within normal range in 10 patients (Table 3); when evoked at the fibular head they were 21 ± 8% smaller than at the ankle.

The sural nerve SNAPs were absent in one patient, reduced by 75–99% in nine, and normal in two patients. The SNCVs were reduced by 17–47% in nine and normal in two patients (Fig. 3).

In order to ascertain the variability in conduction abnormalities in the tested nerves, correlations between amplitudes, latencies and conduction velocities were examined. The amplitudes of CMAPs and logSNAPs were linearly correlated ($r = 0.522$, $n = 24$, $P < 0.01$) as were the MNCVs and the SNCVs ($r = 0.797$, $n = 24$, $P < 0.0005$). The DMLs and MNCVs of the peroneal and median

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**Figure 2.** Motor and sensory nerve conduction studies from the right median nerve (A and B, left column) and motor conduction studies from the right peroneal nerve (C and D, right column). The patient on top (A and C) was a 5-year-old boy (patient 8) without electrophysiological evidence of neuropathy, and the patient below (B and D) was a 3-year-old boy with severe demyelinating neuropathy (patient 4). The compound sensory nerve action potential (SNAP) of the median nerve was evoked by stimulation at Digit2 (Dig2) and recording at the wrist (Wr), the compound muscle action potential (CMAP) of the median nerve was evoked by stimulation at wrist (Wr) and elbow (Elb) and recorded at the abductor pollicis brevis muscle (APB). The CMAP of the peroneal nerve was evoked by stimulation at the ankle and distal to the fibular head (DCF) and recorded from the extensor digitorum brevis muscle (EDB). Latencies, conduction velocities, and amplitudes of SNAPs and CMAPs are indicated below traces.
nerves were linearly correlated \( (r = 0.898–0.966, n = 13, P < 0.0005) \) as were the CMAP amplitudes \( (r = 0.634; n = 13, P = 0.02) \). Similarly, the SNCVs of the sural and the median nerves were linearly correlated \( (r = 0.870, n = 11, P < 0.0005) \) as were the logSNAP amplitudes \( (r = 0.904, n = 11, P < 0.005) \).

**Somatosensory evoked potentials**

Cortical responses to median and tibial nerve stimulation could be recorded in 12 of the 13 patients. The median nerve SSEPs had prolonged latencies (range 19.8–38.5 msec) to N20 in nine patients, they were borderline in two, and normal in one patient (Table 3). The tibial nerve SSEPs had prolonged latencies (range 35–114 msec) to P28 in all 12 patients. The central conduction times with median nerve stimulation could be ascertained in 10 patients in whom an N13 response could be recorded at C7, and they were found to be abnormal in nine. With tibial nerve stimulation, the central conduction times could only be ascertained in four patients, and they were abnormal in all.

**Morphometric analysis of sural nerve biopsies**

Light and electron microscopic studies of sural nerve biopsies from 12 patients were compared with findings in three control sural nerves from children aged 2, 3, and 11 years and with values referenced in the literature.21,22 The total number of myelinated fibers ranged from 1587 to 7422.}

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**Figure 3.** Comparison of morphological (A and B) and electrophysiological (C) findings of the right sural nerves in three patients with late infantile metachromatic leukodystrophy (MLD). The three patients had either no evidence of neuropathy (top, 3-year-old girl, patient 7), moderate neuropathy (above middle, 4-year-old girl, patient 2), or severe neuropathy (below middle, 4-year-old boy, patient 11). The lowermost section was from a 3-year-old control without evidence of neuropathy. The diameter distribution and total number of fibers (B, center column) were calculated from light microscopy of p-phenyldiamine semithin sections. Sural nerve conduction studies are shown in the right column (C). The amplitudes and conduction velocities are indicated above the traces.
to 6400 and was lower than normal in all patients compared with the values from three control children (7422–9397); the number of large fibers >7–9 μm was reduced in 10 patients. The fiber distribution was bimodal in five and unimodal in seven patients (Fig. 3); the diameter of the 10 largest fibers in the patients’ nerves ranged between 8 and 12 μm and were smaller than control in six and within normal range in six patients. Average axonal diameters were similar in the patients and controls, whereas myelin thickness, g-ratio, and the slope of myelin thickness versus fiber diameter plots in control nerves differed substantially from the patient values. Cumulative g-ratio plots showed complete separation between control and MLD nerves consistent with evidence of thinned myelin in all patients (Fig. 4, Table 4).

Relationship between electrophysiological and morphometric results

The sural and median nerve SNAP amplitudes were closely related to the number of myelinated fibers ≥7–9 μm in diameter ($r > 0.8$, $P < 0.001$, Fig. 5A) and to the diameters of the 10 largest fibers in the sural nerve ($r = 0.88$, $P < 0.001$, Fig. 5B), whereas there was no correlation with the total number of myelinated fibers. Moreover, the maximal median and sural SNCVs were closely related to the number of large myelinated nerve fibers ($r = 0.82$, $P < 0.005$). Factors in addition to the number of large myelinated fibers contributed to reduction in SNCV. The relationship between maximum SNCV and the diameter of the 10 largest fibers in normal sural nerves (conversion factor of 4.3 m/sec per μm) could not be applied to the sural nerves of the patients (Fig. 5C). SNCV as a function of fiber diameter was less than expected for the patients, which indicates that demyelination contributed to the slowing of conduction.

Relationship between (lyso)sulfatide levels and neuropathy

The number of myelinated sural nerve fibers ≥9 μm in diameter correlated with sulfatide concentration ($P < 0.05$) as did the log of the median and sural SNAP amplitudes ($P < 0.05$) and the median and sural SNCVs ($P < 0.05$).
The MNCV and CMAP of the median and peroneal nerves also correlated with sural nerve sulfatide concentration ($P < 0.05$, Fig. 6).

The median and tibial SSEP cortical latencies and central conduction times did not correlate with sural nerve sulfatide content, whereas the median peripheral latencies

Figure 6. Relationships between sural nerve sulfatide concentration and myelinated fibers and nerve conduction studies in late infantile metachromatic leukodystrophy. (A) Number of myelinated fibers in the right sural nerve (fibers $>9 \mu m$, $P < 0.05$; fibers $>7 \mu m$, $P = 0.07$; all fibers, $P = 0.9$). (B) Pooled amplitudes of sensory nerve action potentials (SNAP) in the right median, left sural, and right sural nerves. (C) Pooled sensory nerve conduction velocities (SNCV) in the right median, left sural, and right sural nerves. (D) Pooled amplitudes of compound muscle action potentials (CMAP) in the right median and peroneal nerves. (E) Pooled motor nerve conduction velocities (MNCV) in the right median and peroneal nerves.
Disorder is associated with accumulation of 3-
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during the first years after onset of symptoms.24 The
and cognitive decline leading to loss of speech and ambula-
the PNS and the CNS are involved with devastating motor
by progressive neurodegeneration and early mortality. Both
The disease process in late infantile MLD is characterized
Discussion
Cortical latency
Peripheral latency
Central conduction time

Figure 7. Relationship between the cerebrospinal fluid sulfatide
concentration and median nerve somatosensory evoked potentials
(SSEP). The latencies of the cortical responses correlated with the
sulfatide concentration (P < 0.01) and the peripheral latencies trended
toward significance (P = 0.06). The central conduction time was not
correlated with the sulfatide concentration.
correlated with the sural nerve content. The median SSEP
cortical (P < 0.01) and peripheral nerve (P = 0.056)
latencies correlated with cerebrospinal sulfatide concentra-
tion; central conduction time, however, did not (Fig. 7). The tibial SSEP cortical latency trended toward signifi-
cance (P < 0.1).

The abnormalities observed in CSF and sural neve
(lyso)sulfatide levels and NCS did not correlate with CNS
disease burden as measured by motor function assessment
(GMFM-88) and NAA levels in the centrum semiovale.
Similar discordance between NCS studies and clinical
examination has previously been reported in a large
cohort of MLD patients36 and in other demyelinating dis-
orders of the PNS.34 In our case series, this point is illus-
trated by two patients (1 and 12) who were still able to
walk despite motor conduction velocities of 23 m/sec.
Furthermore, two patients had normal peripheral nerve
conduction; one was still able to walk and the other was
only able to sit. LM of the sural nerves in the latter
patients showed minimal demyelination, whereas electron
microscopy (EM) showed inclusion bodies in Schwann
cells and abnormal g-ratios. SSEPs were delayed suggest-
ing central involvement. To our knowledge normal NCS
and LM findings have been described in only a few cases
of adult-onset MLD and EM examination of the sural
nerve in one revealed demyelination.39,40 The two chil-
dren with normal NCS in our series had mildly increased
sural nerve sulfatide levels raising the possibility of a
distinctive genotype/phenotype correlation. However, in
approved therapy for late infantile MLD, but gene and
enzyme replacement therapies are now being tested in clini-
cal trials.30–33 In this context, it is important to have sen-
tive biomarkers that correlate with disease severity and are
dose-responsive to therapeutic interventions.
Slowing of conduction was uniform in different nerves
in the arms and legs as has been described in other stud-
ies of patients with MLD35 and other types of hereditary
demyelinating neuropathy.34 Moreover, the amplitudes
and conduction velocities of motor and sensory responses
were linearly correlated supporting the generalized distri-
bution of demyelination. In contrast, we did not find evi-
dence of multifocal involvement as has been claimed in
other studies.35 However, the excitability of peripheral
nerves was reduced as indicated by the extremely large
increase in threshold, which in some cases erroneously
may be interpreted as conduction block if maximal stim-
ulation cannot be attained.

The process of demyelination and fiber loss in the PNS
of patients with MLD has been well documented in the lit-
erature.36–38 Electron micrographs show characteristic
inclusion bodies and light microscopy (LM) reveals meta-
chromatically stained sulfatide granules within the cyto-
plasm of Schwann cells in all nerves. In the present study,
we found impaired PNS function with very low nerve con-
duction velocities and SNAPs associated with loss of large
fibers, thin myelin sheaths (Fig. 5), and increased g-ratios
as indicated by the correlation between the g-ratios and the
right sural nerve SNAP log-amplitudes and SNCVs
(P < 0.05).

The disease process in late infantile MLD is characterized
by progressive neurodegeneration and early mortality. Both
the PNS and the CNS are involved with devastating motor
and cognitive decline leading to loss of speech and ambula-
tion during the first years after onset of symptoms.24 The
disorder is associated with accumulation of 3-O-sulfogalac-
tosylceramide (sulfatide) due to reduced activity of ASA,
which is caused by mutations in the gene located on chro-
mosome 22q13.31–qter. The mutations reported here are
heterogeneous and not predictive of phenotype as previ-
sously described in the literature.1 Studies in murine models
of MLD indicate that disease pathology is due in part to the
accumulation of sulfatide. Sulfatide is normally present in
oligodendrocytes and Schwann cells and is necessary for
the development and maintenance of myelin25–27; in its
absence fibers show severe dysmyelination and ion-channel
dysregulation.28 Recently it has become clear that a deriva-
tive of sulfatide, lyso(sulfatide, is involved in cellular cal-
cion homeostasis. When lyso(sulfatide is added to cells
in vitro, Ca2+ levels increase causing activation of intracel-
lar proteases and cellular injury.29

We examined the relationship between motor and
sensory function and CSF and sural nerve (lyso)sulfatide
levels in 13 children with late infantile MLD. There is no

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the present series of patients with several different mutations, there was no discernible relationship between the (lyso)sulfatide levels, the electrophysiological findings, or the clinical presentation and the particular mutations. The EM pictures of all other patients showed pronounced demyelination and numerous inclusion bodies and masses of cell debris.37,38

In a recent investigation of regeneration in injured nerves, it was concluded that sulfatide plays a major role in the inhibition of axonal outgrowth.41 This observation together with the understanding that sulfatide also has a role in oligodendrocyte differentiation and the communication between Schwann cells and axons could explain how (lyso)sulfatide content influences nerve function.26 The correlations between standardized measures of PNS function and levels of sulfatide are intriguing since they underscore the pathophysiologic relationship between a biomarker and functional status. Our findings suggest that the main pathological abnormalities are demyelination with preserved axonal caliber; axonal loss occurs with advanced disease. Our data indicate that the extent of sulfatide and lysosulfatide accumulation within sural nerve and CSF is directly proportional to the severity of damage to peripheral nervous tissue, whereas these markers do not reflect the extent of CNS damage. Similar findings have been reported in an ASA-deficient mouse model where accumulation of sulfatide increased with age and correlated with histological damage to peripheral nerves.42 Sulfatide and lysosulfatide levels provide a measure of the extent of local tissue injury. For the patients reported here the average sural nerve sulfatide concentration is estimated to be ~35,000-fold greater on a molar basis than the corresponding CSF values. We suggest that the CSF sulfatide level provides a marker reflective of disease burden in the intraspinal nerve roots and PNS; it does not mirror the extent of central white matter injury as stated previously.

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Conflict of Interest

Dr. i Dali has been salaried by Shire, and Dr. Krarup has received consulting honorarium from Shire. Drs. Barton, Cao, Pan and Sellos-Moura are employees of Shire. Dr. Nair was an employee of Shire at the time of performing the work in the paper.

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