

**SUPPLEMENTAL INFORMATION FOR****CSF sphingomyelin: a new biomarker of demyelination in the diagnosis and management of CIDP and GBS**

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## **METHODS**

### **Inclusion/exclusion criteria and patient cohorts' characteristics**

Adult male and female patients with suspected diagnosis of CIDP or GBS were enrolled in the study, independently from disease severity and treatment.

Moreover, adult male and female patients affected by non-demyelinating neurological diseases, independently from disease severity and treatment, were also enrolled as control group, namely OND.

Patients with clinical conditions contraindicating a spinal tap and patients positive for HCV, HIV, bacterial and viral encephalitis and meningitis were excluded from the study. Patients with documented demyelinating lesions of the CNS and patients with any medical condition that could influence study results were also excluded.

The diagnosis of definite CIDP was based on the clinical and electrodiagnostic EFNS/PNS criteria.<sup>1</sup> In particular, the clinical criteria allowed to discern between typical and atypical CIDP and excluding other forms of neuropathy. According to electrodiagnostic criteria, patients included in the study were first classified as definite, probable or possible CIDP; the diagnostic certainty for probable or possible CIDP to reach a final diagnosis of definite CIDP was done when patients fulfilled one or more supportive criteria.<sup>1</sup> Suspected CIDP, not fulfilling EFNS/PNS diagnostic criteria, were not included in the control group owing to their confounding phenotype and sometimes undefined diagnosis. Instead, this group of patients, also referred to as “no EFNS/PNS CIDP”, was used to emphasise the diagnostic value of SM testing especially by avoiding misdiagnosis for CIDP patients.

The diagnosis of definite GBS was based on published criteria and confirmed according to Brighton criteria.<sup>2,3</sup> Based on clinical and neurophysiological features, we distinguished patients with demyelinating (ie, AIDP) from those with axonal forms of GBS.

### **Data collection**

We collected data on demographical, laboratory, clinical and neurophysiological features.

Immunochemical examination of CSF and serum was performed according to the recommendations of the Italian Association for Neuroimmunology and included CSF to serum albumin concentration quotient (QA<sub>lb</sub>), CSF to serum IgG concentration quotient (QIgG), CSF protein (gr/L), CSF cell count (number of cells/ $\mu$ L), oligoclonal IgG bands (OCBs) in CSF and serum.<sup>4</sup> In particular, albuminocytologic dissociation and increased proteins are considered a pathological index in GBS and supportive diagnostic criteria for CIDP by EFNS/PNS guidelines.<sup>5</sup>

Clinical data encompassed disease duration and activity, response to treatment, and clinical scores. General scores and scales evaluated for grading the severity of peripheral polyneuropathy included Overall Neuropathy Limitations Scale (ONLS, range 0-12), Medical Research Council sum score (MRC sum score, range 0-60), Inflammatory Neuropathy Cause and Treatment (INCAT) disability scale (range 0-10), INCAT sensory Sum Score (ISS) (range 0-20), and GBS disability scale (range 0-6).<sup>6-10</sup>

Nerve conduction studies (NCS) were performed within the first week of patients' enrolment according to standardised shared protocols.<sup>11-14</sup> Both motor and sensory evaluations were performed bilaterally on different nerves of legs and arms. In particular, motor NCS examined median, ulnar, peroneal and tibial nerves. For each nerve, we collected data including motor conduction velocity (MCV) (m/s), distal motor latency (DML) (ms), proximal compound muscle action potential (elbow/FH/knee CMAP) amplitude (mV), distal compound motor action potential (wrist/ankle CMAP) amplitude (mV), presence of conduction block (>50% drop in proximal CMAP to distal amplitude), absence or increased minimal F-wave latency, and number of nerves that have lost excitability (ie, not recordable, NR). Sensory (antidromic) NCS examined median, ulnar and sural nerves. For each nerve, we collected data including sensory conduction velocity (SCV) (m/s), sensory nerve action potential (SNAP) ( $\mu$ V), and number of NR nerves.

### **SM assay**

We recently optimised a fluorescence-based assay to quantify SM in tissue homogenates and biological fluids. All chemicals used in the following procedures were purchased by Sigma-Aldrich.

This assay is based on lipid extraction from tissues, cells and fluids followed by enzymatic hydrolysis of SM to hydrogen peroxide which is able to react 1:1 with dihydroxyphenoxazine (Amplex Red, AR) to generate resorufin, a highly fluorescent product.<sup>15</sup> In the present manuscript, 10  $\mu$ L of lipid extract from human CSF by Bligh and Dyer method<sup>16</sup>, were added to individual wells of a 96-well microtiter plate that contained an enzymatic cocktail consisting of 12.5 mU of *Bacillus cereus* sphingomyelinase, 400 mU of alkaline phosphatase, 120 mU of choline oxidase, 200 mU of horseradish peroxidase, and 20 nmol of AR in 100  $\mu$ L of reaction buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, pH 7.4). For each sample, the relative negative control obtained removing sphingomyelinase by the reaction mixture was also analysed. After 20 min incubation at 37°C in the dark, the microtiter plate was read using a fluorescence microplate reader with excitation and emission wavelength at 560 and 587 nm, respectively (Infinite 200 PRO, Tecan Italia Srl). A standard curve was prepared by making serial dilutions (from 0.0125 to 1.6 nmol) from a 2.8 nmol/ $\mu$ L of SM standard stock solution. For each sample SM, levels were calculated from the difference in fluorescence between the sample and its relative negative control. Resulting values were interpolated with the related standard curve to obtain absolute SM concentration (nmol).

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## SUPPLEMENTAL TABLES

Table S1 Motor NCS

	Typical CIDP (n = 30)	Atypical CIDP (n = 17)	AIDP (n = 11)	Axonal GBS (n = 3)
<b>MEDIAN</b> (assessed nerve/patients with motor NCS available, n)	15/30	7/17	1/11	2/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	0/15	0/7	0/1	0/2
<b>MCV</b> (m/s)	35.57 ± 12.49 (28.65-42.49)	47.26 ± 3.48 (44.04-50.49)	34.2	42.85 ± 0.21 (40.94-44.76)
<b>DML</b> (ms)	5.36 ± 2.49 (3.98-6.70)	4.10 ± 0.51 (3.62-4.57)	4.95	3.87 ± 0.24 (1.65-6.00)
<b>CMAP elbow</b> (mV)	5.54 ± 4.91 (2.82-8.26)	8.05 ± 4.68 (3.71-12.39)	0.30	0.60 ± 0.56 (0.20-1.00)
<b>CMAP wrist</b> (mV)	6.57 ± 4.81 (3.90-9.23)	9.06 ± 4.59 (4.82-13.31)	2.00	0.70 ± 0.84 (0.10-1.30)
<b>Conduction block</b> (nerves with conduction block/assessed nerve, n)	3/15 (20%)	0/7 (0%)	1/1 (100%)	0/2 (0%)
<b>Absent or increased minimal F-wave latency</b> (%)	11%	0%	/	/
<b>ULNAR</b> (assessed nerve/patients with motor NCS available, n)	30/30	14/17	10/11	2/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	2/30	0/14	0/10	0/2
<b>MCV</b> (m/s)	38.34 ± 14.05 (32.89-43.79)	49.48 ± 8.12 (44.79-54.17)	45.84 ± 11.29 (37.76-53.92)	50.33 ± 5.90 (46.15-54.15)
<b>DML</b> (ms)	3.94 ± 2.05 (3.15-4.7)	2.98 ± 0.74 (2.55-3.42)	2.90 ± 0.72 (2.40-3.44)	3.16 ± 0.86 (2.55-3.77)
<b>CMAP elbow</b> (mV)	4.79 ± 4.38 (3.09-6.49)	8.40 ± 4.83 (5.60-11.2)	4.50 ± 4.50 (1.27-7.72)	0.12 ± 0.03 (0.10-0.15)
<b>CMAP wrist</b> (mV)	6.15 ± 4.26 (4.49-7.80)	9.43 ± 4.78 (6.67-12.2)	6.17 ± 3.93 (3.35-8.98)	0.25 ± 0.07 (0.20-0.30)
<b>Conduction block</b> (nerves with conduction block/assessed nerve, n)	6/28 (21%)	1/14 (7%)	3/10 (30%)	0/2 (0%)
<b>Absent or increased minimal F-wave latency</b> (%)	27%	0%	50%	50%
<b>PERONEAL</b> (assessed nerve/patients with motor NCS available, n)	30/30	17/17	11/11	3/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	7/30	1/17	1/11	1/3
<b>MCV</b> (m/s)	32.26 ± 6.57 (29.42-35.11)	38.20 ± 6.52 (34.73-41.67)	36.85 ± 7.90 (31.19-42.51)	35.63 ± 1.59 (34.5-36.75)
<b>DML</b> (ms)	5.99 ± 2.14 (5.06-6.92)	5.06 ± 1.51 (4.26-5.87)	4.69 ± 0.93 (4.02-5.36)	4.75 ± 0.98 (4.05-5.45)
<b>CMAP elbow</b> (mV)	2.52 ± 3.90 (0.83-4.21)	2.51 ± 2.18 (1.35-3.68)	2.32 ± 2.54 (0.50-4.14)	1.60 ± 1.98 (0.20-3.00)
<b>CMAP wrist</b> (mV)	2.93 ± 3.80 (1.28-4.58)	3.42 ± 3.11 (1.76-5.08)	2.86 ± 3.05 (0.67-5.04)	2.00 ± 2.54 (0.20-3.80)
<b>Conduction block</b> (nerves with conduction block/assessed nerve, n)	1/23 (4.3%)	0/16 (0%)	1/10 (10%)	0/2 (0%)
<b>Absent or increased minimal F-wave latency</b> (%)	42%	50%	66%	/
<b>TIBIAL</b> (assessed nerve/patients with motor NCS available, n)	29/30	10/17	8/11	2/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	7/29	0/10	1/8	0/2
<b>MCV</b> (m/s)	34.50 ± 7.00 (31.22-37.77)	38.79 ± 7.48 (33.44-44.14)	40.73 ± 6.20 (34.99-46.47)	34.98 ± 3.50 (32.50-37.45)
<b>DML</b> (ms)	5.67 ± 2.16 (4.66-6.68)	5.64 ± 1.39 (4.64-6.63)	5.96 ± 2.18 (3.94-7.99)	4.83 ± 0.76 (4.30-5.37)
<b>CMAP elbow</b> (mV)	2.33 ± 3.09 (0.88-3.70)	3.55 ± 3.65 (0.94-6.16)	5.30 ± 5.20 (0.40-10.16)	0.47 ± 0.45 (0.15-0.80)

<b>CMAP wrist (mV)</b>	3.55 ± 3.26 (2.02-5.07)	5.80 ± 6.31 (1.29-10.32)	6.53 ± 7.04 (0.64-12.43)	0.80 ± 0.98 (0.1-1.5)
<b>Conduction block</b> (nerves with conduction block/assessed nerve, n)	5/20 (25%)	2/10 (20%)	1/8 (12.5%)	1/2 (50%)
<b>Absent or increased minimal F-wave latency (%)</b>	60%	33%	25%	50%

Data were expressed as mean ± SD and CI (values between parentheses), unless otherwise specified.

AIDP, acute inflammatory demyelinating polyradiculoneuropathy; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; GBS, Guillain-Barré syndrome; CMAP, compound motor action potential; DML, distal motor latency; MCV, motor conduction velocity; NR, not recordable.



Table S2 Sensory NCS

	Typical CIDP (n = 30)	Atypical CIDP (n = 17)	AIDP (n = 11)	Axonal GBS (n = 3)
<b>MEDIAN</b> (assessed nerve/patients with sensory NCS available, n)	12/30	5/17	0/11	1/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	3/12	2/5	/	0/1
<b>SCV</b> (m/s)	39.97 ± 8.32 (30.53-43.37)	41.02 ± 11.53 (12.39-69.65)	/	55.20
<b>SNAP</b> (µV)	8.03 ± 4.53 (4.54-11.52)	4.03 ± 1.10 (1.20-6.70)	/	7.1
<b>ULNAR</b> (assessed nerve/patients with sensory NCS available, n)	22/30	10/17	8/11	2/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	7/22	3/10	2/8	0/2
<b>SCV</b> (m/s)	38.16 ± 10.25 (32.49-43.84)	44.54 ± 6.90 (38.09-51.00)	48.72 ± 15.86 (32.08-65.36)	54.75 ± 2.61 (52.90-56.60)
<b>SNAP</b> (µV)	7.41 ± 5.98 (4.09-10.72)	12.06 ± 11.76 (1.18-22.94)	12.10 ± 11.42 (1.11-24.08)	14.55 ± 5.58 (10.60-18.50)
<b>SURAL</b> (assessed nerve/patients with sensory NCS available, n)	26/30	17/17	10/11	2/3
<b>NR nerves</b> (NR nerves/assessed nerve, n)	12/26	2/17	2/10	0/2
<b>SCV</b> (m/s)	36.76 ± 4.33 (34.25-39.26)	38.91 ± 7.48 (34.76-43.06)	37.63 ± 16.18 (25.20-50.07)	46.15 ± 5.44 (42.30-50.00)
<b>SNAP</b> (µV)	5.14 ± 3.76 (3.05-7.23)	7.63 ± 7.02 (3.73-11.52)	14.57 ± 8.96 (6.28-22.86)	11.85 ± 8.13 (6.10-17.6)

Data were expressed as mean ± SD and CI (values between parentheses), unless otherwise specified.  
AIDP, acute inflammatory demyelinating polyradiculoneuropathy; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; GBS, Guillain-Barré syndrome; SCV, sensory conduction velocity; SNAP, sensory nerve action potential; NR, not recordable.

## SUPPLEMENTAL FIGURE LEGENDS

### Figure S1 CSF SM levels in typical and atypical CIDP at different stage and ROC curve analysis

(A) SM amount was profiled in patients with CIDP grouped according to the exhibited clinical form (ie, typical or atypical CIDP) and disease stage (ie, active or stable CIDP). A comparison of SM levels in the CSF of these cohorts of patients with CIDP and the control group (ie, OND) displayed a significant increase of SM just in patients with CIDP in the active stage of the disease, independently of the clinical form. Typical and atypical active CIDP cohorts showed increased SM levels compared to both the OND group and the two cohorts (ie, typical and atypical) of patients with CIDP in the stable stage of the disease ( $1.73\pm 0.21$  and  $1.34\pm 0.17$  vs  $0.41\pm 0.03$  and  $0.58\pm 0.07$  and  $0.59\pm 0.14$  pmol/ $\mu$ L). These latter groups did not show any difference in terms of CSF SM content compared to the OND group. (B) QAlb, a routine CSF index, was used to test its reliability to distinguish between patients with CIDP in the active or stable stage of the disease. QAlb was significantly increased in active CIDP compared to stable CIDP ( $17.31\pm 2.04$  vs  $8.94\pm 0.70$ ). Most of patients with CIDP in the stable stage of the disease displayed QAlb values over the threshold (ie,  $7.5 \times 10^{-3}$ ), which prevented their unambiguous identification. (C) SM test performance in terms of accuracy was evaluated using ROC curve analysis. SM test exhibited high sensitivity (80.85%) and specificity (98.82%) at an optimum cut-off of 0.9819 pmol/ $\mu$ L. An AUC of 0.94 defined SM testing as a very good discriminatory biomarker.

Data were presented as mean  $\pm$  SEM. Unpaired 2-tailed t-test was used for statistical comparison between two groups. Holm-Sidak multiple comparison test after one-way analysis of variance was used for statistical comparison of multiple groups. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ . All statistical analysis was performed using the Graph Pad V.7.0 (Prism) software.

AUC, area under the curve; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; OND, other neurological diseases; QAlb, CSF to serum albumin concentration quotient; ROC, receiver operating characteristic; SM, sphingomyelin.

### Figure S2 Correlation of CSF concentrations of protein and SM with CSF indexes

(Panel A) Patients affected by active CIDP and AIDP exhibited high and significant correlation of the most commonly used CSF indexes to track a blood CSF barrier dysfunction, including QAlb, QIgG, and protein. (Panel B) In the same patients, CSF SM displayed a low correlation with QAlb and QIgG, and no correlation with protein concentration.

Spearman's rank correlation test was used for statistical analysis. \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ . All statistical analysis was performed using the Graph Pad V.7.0 (Prism) software.

QAlb, CSF to serum albumin concentration quotient; QIgG, CSF to serum IgG concentration quotient; SM, sphingomyelin.

### **Figure S3 Correlation of CSF protein levels with clinical scales in CIDP and AIDP**

(Panel A) Protein levels were correlated with clinical scales, including INCAT and ONLS to grade disease severity, and MRC sum score to grade muscle strength in the CSF of patients affected by active CIDP. Patients did not display any correlation of protein amount with all clinical scales. (Panel B) Protein levels were correlated with clinical scales, including GBS disability scale and ONLS to grade disease severity, and MRC sum score to grade muscle strength in the CSF of patients affected by AIDP. Patients did not display any correlation of protein amount with all clinical scores.

Spearman's rank correlation test was used for statistical analysis. All statistical analysis was performed using the Graph Pad V.7.0 (Prism) software.

AIDP, acute inflammatory demyelinating polyradiculoneuropathy; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; INCAT, inflammatory neuropathy cause and treatment; MRC, Medical Research Council; ONLS, overall neuropathy limitations scale.

### **Figure S4 Correlation of CSF SM levels with motor NCS in active CIDP and AIDP**

SM amount in the CSF of patients affected by active CIDP and AIDP was correlated with DML (ms), proximal CMAP (elbow/FH/knee CMAP) amplitude (mV), distal CMAP (wrist/ankle CMAP) amplitude (mV) and MCV (m/s) of median (A-D), ulnar (E-H), peroneal (I-L), and tibial (M-P) motor nerves. A value of 0 mV was assigned to the proximal and distal CMAP amplitude of nerves that have lost the excitability. DML and MCV of the same nerves were excluded from the analysis. (N,

O) Proximal and distal CMAP amplitude of tibial nerve displayed a low but significant correlation with CSF SM levels. Correlation coefficient was estimated by the Spearman's rank correlation test.

CMAP, compound motor action potential; DML, distal motor latency; FH, fibular head; MCV, motor conduction velocity; NCS, nerve conduction studies; SM, sphingomyelin.

**Figure S5: Correlation of CSF SM levels with sensory NCS in active CIDP and AIDP**

SM amount in the CSF of patients affected by active CIDP and AIDP was correlated with SNAP amplitude ( $\mu\text{V}$ ), and SCV (m/s) of median (A, B), ulnar (C, D) and sural (E, F) sensory nerves. (A, C, E) A value of 0  $\mu\text{V}$  was assigned to SNAP amplitude of nerves that have lost the excitability. (B, D, F) SCV of the same nerves was excluded from the correlation analysis. (A, D) Both SNAP amplitude of median nerve, and SCV of ulnar nerve displayed a moderate but significant correlation with CSF SM levels. Correlation coefficient was estimated by the Spearman's rank correlation test. NCS, nerve conduction studies; SM, sphingomyelin; SCV, sensory conduction velocity; SNAP, sensory nerve action potential.