Title: Neurofilament light protein levels in cerebrospinal fluid predict long-term disability of Guillain-Barré syndrome: A pilot study

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Abstract

OBJECTIVES:

Although the recovery from Guillain-Barré syndrome (GBS) is good in most patients, some develop permanent severe disability or even die. Early predictors would increase the likelihood to identify patients at risk for poor outcome at the acute stage, allowing them intensified therapeutic intervention.

MATERIALS AND METHOD:

Eighteen patients with a history of GBS 9-17 years ago were reassessed with scoring of neurological disability and quality of life assessment (QoL). Their previous diagnostic work-up included clinical examination with scoring of disability, neurophysiological investigation, a battery of serology tests for infections, and cerebrospinal fluid (CSF) examination. Aliquots of CSF were frozen, stored for 20-28 years, and analyzed by ELISA for determination of neurofilament light protein (NFL) and glial fibrillary acidic protein (GFAP).

RESULTS:

Patients with poor outcome (n = 3) had significantly higher NFL and GFAP levels at GBS nadir than those with good outcome (n = 15, P < .01 and P < .05, respectively). High NFL correlated with more prominent disability and worse QoL at long-term follow-up (r = .694, P < .001, and SF 36 dimension physical component summary (PCS) (r = -.65, P < .05), respectively, whereas GFAP did not correlate with clinical outcome or QoL.
CONCLUSION:

*High NFL in CSF at the acute stage of GBS seems to predict long-term outcome and might, together with neurophysiological and clinical measures, be useful in treatment decisions and clinical care of GBS.*
Introduction

The Guillain-Barré syndrome (GBS) of rapidly evolving polyradiculoneuropathy is a rare condition but yet it accounts for the main part of acute flaccid paralysis in the world. The age-adjusted incidence of GBS in Sweden and Europe has previously been estimated to 1.45-2.30 /100 000 \textsuperscript{1,2}. Although demyelination of nerves and nerve roots is considered the dominating feature in acute GBS, axonal damage has been recognized as an important part of the pathophysiological process \textsuperscript{3,4}. The most common variant of GBS in North America and Europe is acute inflammatory demyelinating polyneuropathy (AIDP), and the axonal variants are rarely seen \textsuperscript{5}. Patients with evidence of axonal involvement tend to be more severely afflicted by disease and with poorer recovery than patients with demyelinating forms \textsuperscript{6}. In surveys of GBS approximately 20% have persistent and significant disability at long-term follow-up \textsuperscript{3,7,8}. Early therapeutic intervention can probably influence the prognosis in these patients. Thus, predictors that identify patients at risk for poor outcome would be desirable in the acute phase of GBS.

Previous investigations conclude that high age, preceding diarrhoea and high GBS disability score are predictors for poor recovery \textsuperscript{9}. High levels of neurofilament heavy protein (NFH), a biomarker for axonal damage in cerebrospinal fluid (CSF), obtained in close relation to GBS onset was related to severe disability at follow-up \textsuperscript{10-12}. The aim of this study was to investigate whether clinical features, neurophysiological examination, and/or neurospecific biomarkers in CSF, obtained at the acute stage of GBS, could predict patients at risk for poor outcome at long-term follow-up 9-17 years after GBS onset.
Material and methods

Study population

Fifty-one patients with a suspected diagnosis of GBS were consecutively admitted to the Department of Neurology at Sahlgrenska University Hospital, Gothenburg, Sweden from November 1989 to January 1997. The diagnostic work-up included; registration of a preceding infection or vaccination, acute and convalescent serology for infections (Mycoplasma, Cytomegalovirus, Adenovirus, RS-virus and Influenza type A, Campylobacter jejuni), electroneurography (ENG) and electromyography (EMG), and routine CSF analysis (cell count, protein level, albumin ratio\(^\text{13}\), oligoclonal IgG bands\(^\text{14}\)).

Medical records for these patients were reassessed 9-17 years after hospitalization in the year 2006. Only patients with a monophasic disease that fulfilled the diagnostic criteria of GBS\(^\text{6}\) were included in the study. At follow-up, 11 patients had died (by other causes than GBS) and 11 patients were excluded, 6 of these had other diagnosis (1 HIV-associated neuropathy, 1 toxic neuropathy, 1 arachnoiditis, 3 undefined neuropathy) and 5 had variants of GBS not fulfilling the classical criteria (1 Miller Fischer syndrome, 4 relapsing chronic inflammatory demyelinating polyneuropathy). Three patients were lost to follow-up and 8 declined participation in the study (Figure 1). Thus, 18 patients, 8 males and 10 females, participated in the present study. CSF from 28 healthy subjects served as controls when analysing NFL and 36 healthy subjects served as controls for GFAP. Demographic, clinical and laboratory characteristics of patients and healthy controls are presented in Table 1. The regional ethical board of Gothenburg University approved the study. All patients and controls gave their informed consent to inclusion in the study.
Clinical and neurophysiological assessment

Clinical examination of patients was performed shortly after disease onset (median 19 days, range 4-28 days) by either of two experienced neurologists. Results from these examinations and data from the medical records were used to assign a functional score at GBS nadir for each patient, in accordance to the Hughes functional score \(^{15}\) ranging from F-score 0 (no symptoms) over increasing functional deficit/walking restriction to a maximum of 6 (dead). The second assessment was made by a third neurologist at follow-up 9-17 years after GBS (median 13 years) with registration of Overall Disability Sum Score (ODSS) \(^{16}\) and F-score. An F-score of ≥3 (not being able to walk independently) at follow-up was classified as “poor outcome” and an F-score <3 as “good outcome”.

Neurophysiological examination included electro neurography (ENG) and electro myography (EMG) using standard protocols. Patients were classified either by ENG alone or supplemented with EMG \(^5,17\). The neurophysiological findings were categorized as demyelinating, axonal or mixed.

Quality of life (QoL)

QoL was assessed with Short Form 36 (SF-36) \(^{18}\), a patient reported survey of 36 items, contributing to eight scaled scores or sections: Physical Functioning (PF), Rolefunctioning-Physical (RP), Bodily Pain (BP), General Health Perceptions (GH), Vitality (VT), Social Role Functioning (SF), Role Function-Emotional (RE), and Mental Health (MH). A scale 0-100 are weighted sums of the items in each section. The eight sections are divided into two dimensions: Physical Component Summary (PCS) and Mental Component summary (MCS). The mean of the PCS is extracted from PF, RP, BP, GH, and VT and the mean of the MCS is extracted from MH, RE, SF, VT, and GH.
**Sampling of peripheral blood and CSF**

Samples of peripheral blood and CSF were obtained in close relation to the neurological examination. The lumbar puncture was made according to similar procedures that are recommended in the consensus protocol of the BioMS-EU network for CSF biomarker research in MS\(^\text{19}\). The first 12 ml of CSF were carefully mixed and after centrifugation, fractions were frozen and stored in 0.5 ml aliquots at \(-80^\circ\) C. Serological tests for infectious diseases and CSF routine analysis were done as part of the diagnostic investigation and analysis of CSF biomarkers, were done 2003 as part of a larger clinical project on polyneuropathies at the Department of Neurology, Sahlgrenska University Hospital (unpublished data).

**Neurofilament light protein assay**

NFL was determined in CSF 11 years ago with enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies against NFL (Rosengren, L.E. et al., J. Neurochem. 1996; 67, 2013–2018.) (unpublished data) and in the present study once again, using the UmanDiagnostics NF-light ELISA as previously described \(^\text{20}\). The latter test has improved analytical sensitivity that allows for quantification of NFL concentration in CSF from healthy controls, i.e. considerably lower concentrations. In brief, the assay is based on two highly specific monoclonal antibodies and a biotinstreptavidin horseradish peroxidase (HRP) system. Analysis was performed at room temperature. CSF samples were diluted 1:1 with sample dilution buffer to a total volume of 100 µL and incubated with agitation (800 rpm) for one hour in pre-coated anti-NFL ELISA plates. Thereafter, a 100 µL solution of tracer antibody (biotin anti-NFL) was added to each well and incubated for 45 minutes. Washing cycles were performed after all incubations. Detection was performed using 100 µL streptavidin-HRP incubated for 30 minutes, followed by another incubation with 100 µL 3,30,5,50-
tetramethylbenzidine for 15 minutes. A volume of 50 µL stop solution (8% v/v sulphuric acid) was added to each well, and absorbance was read at λ 490 nm. The analytical sensitivity of the NFL assay was 31 ng/L.

**Glial fibrillary acidic protein assay**

Glial fibrillary acidic protein (GFAP) was measured with a previously described ELISA procedure\(^2\). In brief: capturing antibody (hen anti-GFAP IgG) was absorbed to microplates. CSF samples or reference GFAP were added and incubated for 2 h at room temperature. Rabbit anti-GFAP IgG was then added and incubated for 1 h at room temperature. Captured secondary antibody was detected using peroxidase-conjugated donkey anti-rabbit IgG. The analytical sensitivity of the GFAP assay was 16 ng/l.

**Statistics**

The Mann-Whitney U-test was used to compare groups. Spearman’s rank correlation coefficient, was used to discern relationships between CSF neuro-specific proteins and clinical or laboratory variables. Analyses were made with SPSS 21 software.

**Results**

**Clinical measures for long-term outcome**

Clinical, neurophysiological and laboratory results, obtained during the acute phase of GBS (Table 1), were used to characterize patients with good and poor outcome at follow-up (Table 2). At the acute phase of GBS, 12 patients had severe disability (FS 4-6; bedridden, need of assisted ventilation), six had moderate (FS 2-3; able to walk 10 metres with or without
support) and none had mild or no disability (FS 0-1). In three patients, all in the severely
afflicted group, mechanical ventilation was needed. Eleven of the severely disabled patients
(92%), and three patients (50%) with moderate disability, were treated with 4-5 plasma
exchanges or 5 days of i.v. high dose of immunoglobulin infusions (IVIG). At follow-up 9-17
years after the GBS onset, one patient had severe residual disability (FS 4), four had moderate
(FS 2-3) disability, and 14 had mild (FS 0-1) disability; 7 of these were in complete remission
(FS-score 0, no pathological findings in neurological examination or ODSS-scoring, Table2).
The long-term outcome was considered good (FS <3, capable of independent walking) in
15/18, (83%) and poor (FS 3-6, in need of assisted walking), in 3/18 patients (17%). Higher
age, preceding diarrhoea and Campylobacter jejuni infection were associated with poor
outcome. The disability at nadir did not predict a worse outcome.

Neurophysiological assessments
The neurophysiological examination, performed 15 days (median, range 4-39) from symptom
onset included ENG and EMG (n=11) or only ENG (n=6). The ENG was compatible with
demyelinating neuropathy in 10 patients (5 pure motor, 1 pure sensory, and 4 sensory-motor),
and EMG indicated axonal loss in 8 of them, i.e. most patients had mixed axonal and
demyelinating neuropathy. Eight patients were either not examined or could not be
categorized. No association was found between the results of the neurophysiological
investigation and the severity or outcome of GBS.

NFL and GFAP concentrations for predicting long-term outcome
Patients had significantly higher NFL and GFAP levels in CSF compared with healthy
controls (p<0.0001, Table 1). Patients with poor outcome (F-score 3 or worse; incapable of
independent walking) had significantly higher NFL levels than those with good outcome
Patients with NFL levels above 10,000 ng/L (n=6) (five times the highest level of healthy controls) had persistent disability at follow-up with a median ODSS of 5.5 (range 2-10) as compared to 0 (range 0-3) in patients with NFL concentrations below this limit (p<0.001), Table 2. NFL and GFAP concentrations did not differ between patients in need of assisted ventilation at nadir and those with less respiratory impairment.

The influence on QoL from GBS

The severity of GBS influenced QoL at follow-up (Figure 2). PCS correlated inversely with F-score (r=-0.69, p=0.01) and ODSS (r=-0.66, p<0.05). Among the related PCS scores; PF correlated inversely with F-score (p=-0.82, p<0.001) and ODSS (r=-0.79, p=0.0001), GH only with F-score (r=-0.66, p<0.05), while other section scores did not correlate with clinical outcomes. Although MCS did not correlate with clinical outcomes, the related scores; MH correlated inversely with F-score (p=-0.60, p<0.05), SF with F-score (r=-0.61, p<0.05), and GH with F-score, as noted for the PCS score. All calculations were corrected for multiple comparisons (Bonferroni).

Correlations between NFL, GFAP, clinical outcomes, and QoL

The NFL level correlated with disability (F-score) in the acute phase of GBS (r=0.59, p=0.01) and were strongly predictive of persistent disability, as measured by the F-score (r=0.69, p<0.001) and the ODSS score (r=0.52, p<0.05) at follow-up. NFL levels were dependent on age in the control group (r=0.70, p<0.0001), independent of gender, and correlated with the albumin ratio (function of the blood-brain barrier) in patients (r=0.65, p<0.005). Although patients were of older age than controls the correlation between impairment and NFL at follow-up remained statistical significant also after correction for age; F-Score (r=0.81 p<0.0001) and ODSS (r=0.83 p<0.0001) and for both age and albumin ratio; F-score (r=0.71,
p<0.01) and ODSS (r=0.49, p=0.05). NFL predicted impairment of QoL (corrected for multiple comparisons according to Bonferroni) for PCS (r=−0.65, p<0.05) and the related score PF (r=−0.78, p=0.001). Although NFL did not correlate with MCS, there was a correlation with the section score SF (−0.60, p<0.05). GFAP had no predictive value for clinical outcomes or for QoL. No relationships were found between NFL or GFAP levels and gender or findings from neurophysiology examinations. Despite an interval of 11 years between the first analysis of NFL, using the polyclonal ELISA, and the second analysis, using the monoclonal ELISA, the correlation between the results from the immunoassays was almost perfect (r= 0.99, p<0.001).

4. Discussion
We showed that high NFL levels in CSF, obtained during the acute phase of GBS, were predictive of poor clinical outcome and worse QoL. Our results confirm the results of a previous investigation, demonstrating the predictive value of the heavy neurofilament subunit (NFH) in GBS [16]. It showed that patients with high NFH levels had worse functional and motor outcome at a median follow-up time of 175 days after GBS onset. Although muscle strength recovery in GBS occurs primarily during the first 6 months, recovery may continue even beyond 12 months [17] and significant improvement has been recorded between 6 months (57%) and 12 or 24 months of follow-up (70% and 82%, respectively) [18]. We based our results on both clinical and QoL assessments, done 9-17 years after GBS onset when no further improvement was expected.
In a previous investigation of GBS, patients with axonal involvement were more disabled and had poorer recovery than patients with demyelinating forms of the disease. Both NFL and NFH are structural elements of axons and increased levels of neurofilaments in CSF have also been observed in patients with traumatic, ischemic, degenerative and inflammatory injuries of the central nervous system (CNS) (ref till review, t.ex. Zetterberg H, Neuron, 2016). In contrast, GBS is considered an immune-mediated demyelinating disorder in the peripheral nervous system (PNS). The release of NFL to the CSF compartment is probably due to axonal damage of nerve roots, influenced by the disruption of the blood-nerve barrier (BNB) and the blood-CSF barrier (BCB). However, extensive deterioration of BNB/BCB may also allow NFL released to peripheral blood from damaged peripheral nerves, to enter the CSF. The importance of such peripheral contribution may be further analysed with the recently developed ultrasensitive immunoassay of NFL, using the Single Molecule Array platform (REF).

Although the number of included patients in this study was limited, there was a clear difference between NFL concentrations in patients who had severe disability at follow-up compared to those who recovered or had mild to moderate impairment. The correlations between NFL and clinical and QoL outcomes support NFL as a relevant predictive biomarker in GBS. Some patients were lost to follow-up, but due to the retrospective design with long-term of follow-up, we could exclude patients with initial symptoms and laboratory findings indicative of GBS who with time revealed other diseases, e.g., chronic inflammatory demyelinating neuropathies.

In conclusion, high CSF NFL concentrations at GBS onset were predictive of long-term disability and worse QoL. The results support that axonal damage is the major explanation of sustained disability in GBS. CSF NFL could better identify patients with poor long-term
outcome than clinical scoring. Neurophysiological evidence of axonal involvement was not related to poor prognosis [16]. Our findings suggest that NFL should be included as an early indicator of patients in need of extensive medical and rehabilitating efforts on a long-time basis. Moreover, patients with severe disability at GBS onset but with low NFL concentrations in CSF seem to have a much better chance of recovery than those with increased CSF NFL concentrations.

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**Conflicts of interest**

KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. HZ has served at advisory boards for Roche Diagnostics, Eli Lilly and Pharmasum Therapeutics.

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