Neurofilament light in serum and cerebrospinal fluid in hip fracture patients with delirium

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Abstract
BACKGROUND:
Delirium is associated with new-onset dementia, suggesting that delirium pathophysiology involves neuronal injury. Neurofilament light (NFL) is a sensitive biomarker for neuroaxonal injury.

METHODS:
NFL was measured in cerebrospinal fluid (CSF) (n = 130), preoperative serum (n = 192), and postoperative serum (n = 280) in hip fracture patients, and in CSF (n = 123) and preoperative serum (n = 134) in cognitively normal older adults undergoing elective surgery. Delirium was diagnosed with the Confusion Assessment Method.

RESULTS:
Median serum NFL (pg/mL) was elevated in delirium in hip fracture patients (94 vs. 54 pre- and 135 vs. 92 postoperatively, both $p < 0.001$). Median CSF NFL tended to be higher in hip fracture patients with delirium (1,804 vs. 1,636, $p = 0.074$). Serum and CSF NFL were positively correlated ($\rho = 0.56$, $p < 0.001$).

CONCLUSION:
Our findings support an association between neuroaxonal injury and delirium. The correlation between serum and CSF NFL supports the use of NFL as a blood biomarker in future delirium studies.

Key words: delirium; cerebrospinal fluid; Neurofilament light; hip fracture; neuronal cell death.

Introduction:
Delirium is a severe syndrome of acute brain dysfunction characterized by fluctuating awareness, inattention and cognitive symptoms and is associated with serious adverse outcomes and increased healthcare costs (1-3). Elderly hospitalized patients are at high risk of developing delirium, and up to 50% of hip fracture patients are affected (3, 4). Delirium seems to increase the risk of new-onset dementia and worsen the long-term trajectory of existing dementia (2, 5-7). The pathophysiological background for this association is unclear as delirium pathophysiology is still poorly understood.

Emerging literature suggests that delirium is associated with neuronal death, directly or as a mediator of deleterious insults (2, 8). Studies on biomarkers of brain cell injury have conveyed conflicting results in terms of their association with delirium. Several studies on S100B, a protein expressed primarily in astrocytes in the central nervous system (CNS) (9), have reported increased levels of S100B in delirium patients (10-14). Others studies, however, did not find similar associations (15-17). Increased neuron-specific enolase (NSE), an enzyme expressed in neurons in the CNS (18), has been reported in delirium patients in three studies (19-21), whereas one study in stroke patients could not replicate these findings (22). Dissimilar study populations as well as lack of tissue specificity for both S100B and NSE may partly explain the divergent reports. Only a minority of the published studies (13, 14, 23) has been performed in CSF and, since extracerebral sources of S100B and NSE may influence blood levels, serum changes may not solely reflect brain damage (24). One preliminary study on serum phosphorylated neurofilament heavy (pNFH), a structural protein in neurons, found an association between pNFH and delirium severity (25).

Neurofilament light (NFL) is the smallest of three structural filament proteins of the cytoskeleton in the axoplasm of neurons and is highly expressed in myelinated large-caliber axons (26-28). It is a sensitive marker for neuraxonal injury, increasing shortly after and in correspondence to the intensity of brain insult or disease activity (29, 30). In Alzheimer disease (AD), NFL has been shown to increase in relation to amyloid deposition prior to symptom debut in mouse models, and to correlate with lower cognitive scores as well as accelerated rates of cognitive decline in AD patients (31-33). NFL in blood and CSF are strongly correlated in patients with multiple sclerosis, raising the possibility of disease monitoring through blood rather than CSF sampling (34, 35).

To our knowledge, NFL has never been assessed in delirium. Our aim was to examine whether delirium was associated with higher levels of NFL in CSF and serum, and assess the correlation between NFL concentrations in these two sample types.

Methods

Cohorts

Hip fracture cohort

332 patients with proximal femur fractures were recruited to the Oslo Orthogeriatric Trial, a randomized controlled trial evaluating the effect of orthogeriatric care on cognitive function (4, 36). The participants were enrolled at Oslo University Hospital from September 2009 to January 2012. Patients were not included if they were terminally ill or if the fracture resulted from a high-energy trauma. The intervention had no effect on delirium incidence and all participants were pooled in the ensuing study.

Participants were evaluated daily for delirium preoperatively and until the fifth postoperative day (all) or discharge (patients with delirium), using the Confusion Assessment Method (CAM). The study physician or nurse scored CAM based on a 10- to 30-minute interview with participants combined with information from relatives, nurses and hospital records. Depending on time of onset and criteria fulfillment, delirium was categorized as preoperative, incident (i.e. no delirium preoperatively, but developed delirium during the hospital stay), subsyndromal (defined as at least two positive CAM features (pre- or postoperatively) but never fulfilled criteria for full delirium) and no delirium. Two specialists (one geriatrician and one geriatric psychiatrist) evaluated each participant's fulfillment of the ICD-10 criteria for dementia prior to the fracture (i.e. consensus diagnosis for dementia), based on all existing data (except delirium status during admission), including the Informant Questionnaire on Cognitive decline in the Elderly (IQCODE), hospital records and clinical follow-up.

Cognitively healthy control group

172 patients undergoing elective gynecological, orthopedic or urological surgery in spinal anesthesia, and turning 65 years or older the year of inclusion, were recruited to the COGNORM-study from 2012-2013 at Oslo University Hospital and Diakonhjemmet sykehus, Oslo, as previously described (37). Participants were evaluated with a multidomain battery of cognitive tests prior to surgery. Dementia, previous stroke with sequelae, Parkinson's disease and other acknowledged or suspected brain disease likely to influence cognition were exclusion criteria. Furthermore, we excluded patients without CSF or serum (n=6), suspected undiagnosed dementia with referral to a memory clinic by a geriatrician (based on test scores and clinical data) at any time point during five years of follow-up (n=15) or MMSE <28 at baseline (n=16).

Sampling and biochemical analyses
CSF was collected in propylene tubes in conjunction with anesthesia prior to administration of the anesthetic agent in both cohorts. Blood was collected by venous puncture preoperatively in both cohorts as well as postoperatively in hip fracture patients. For logistical reasons, samples were taken at irregular time intervals pre- and postoperatively and all types of sampling were not obtainable for all patients. Serum and CSF were centrifuged, aliquoted and stored at – 80° C. Samples were thawed, aliquoted and sent on dry ice (hip fracture patients) or sent directly on dry ice (controls) for analyses at the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital (Mölndal, Sweden). CSF NFL concentrations were measured using a commercial ELISA (UmanDiagnostics, Umeå, Sweden) (38). Serum NFL levels (sNFL) were analysed using a homebrew Single molecule array method (39).

Statistical methods
Changes in sNFL were calculated as the difference in serum NFL pre-to postoperatively. Data from CSF and serum were not normally distributed. Transformation improved fit to the normal distribution only for preoperative serum and did not change results; therefore non-transformed data are reported. Continuous variables were analyzed using Mann-Whitney *U* test (unpaired group comparisons), Kruskal-Wallis (unpaired subgroup analyses) and Wilcoxon signed-rank test (paired comparisons). Categorical variables were analyzed using Chi Square statistics. Correlations were calculated with Spearman's rho (ρ).
Since dementia is a major risk factor for delirium, we also carried out the analyses stratified on dementia. In order to adjust for confounding factors, linear regression analyses were performed using delirium and age as independent variables. To achieve normal distribution of residuals, serum NFL (sNFL)/CSF NFL and sNFL change were transformed with the natural logarithm in the regression analyses. The regression coefficient β is reported in logarithmic units of NFL.

We further wanted to explore whether time of symptom debut had any impact on NFL levels at time of sampling. Thus analyses on delirium status at the time of CSF sampling (preoperative versus incident versus subsyndromal versus no delirium) were performed posthoc. A recent study indicates that subsyndromal delirium may be symptomatologically closer to full-blown delirium than to no delirium (40), and all analyses were therefore repeated excluding patients with subsyndromal delirium. Furthermore potentially delirium-associated release of NFL likely begins prior to substrate sampling in patients with preoperative delirium. Measuring change of NFL pre-to postoperatively in this subgroup may therefore underestimate the release of NFL significantly. The analyses on change were therefore repeated and compared between incident delirium and no delirium, excluding patients with preoperative delirium (n=41).

All statistical analyses were performed using SPSS Statistics version 25 (IBM, Armonk, NY).

Statement of Ethics
The study was conducted in accordance with the Declaration of Helsinki. The data and serum/CSF samples were collected after informed consent from the patient and/or proxy (if the patient was unable to consent due to cognitive impairment), as approved by the Regional Committee for Ethics in Medical and Health Research (South-East Norway) (REK 2009/450 and REK 2011/2052).

Results

CSF NFL was analyzed in 128 hip fracture patients and 123 cognitively healthy controls. In serum, preoperative levels of NFL were analyzed in 192 hip fracture patients and 134 controls, and postoperative levels were analyzed in 280 hip fracture patients. Changes in serum NFL pre- to postoperatively were calculated in 162 hip fracture patients (figure 1).

Hip fracture patients

Patients with delirium were slightly older, had a higher median IQCODE score and higher percentage of dementia than delirium-free hip fracture patients (table 1).

Neurofilament in serum (sNFL)

Preoperative and postoperative serum

Patients with delirium had significantly higher pre- and postoperative sNFL compared to patients without delirium (median 94 vs. 54 pg/mL preoperatively, $p<0.001$ and 135 vs. 92 pg/mL postoperatively, $p<0.001$) (figure 2).The associations were upheld in regression analysis adjusting for age (preoperative, $\beta = 0.37$, $p=0.001$ and postoperative, $\beta = 0.27$, $p=0.001$) (supplementary table 1).

In analyses stratified according to delirium status at the time of surgery, patients without delirium had significantly lower NFL than all subgroups preoperatively ($p \leq 0.033$) and lower NFL than both delirium subgroups postoperatively ($p<0.001$). Moreover, sNFL seemed related to the time of onset of symptoms, with highest NFL values in preoperative serum measured in patients with preoperative delirium and in postoperative serum measured in patient with incident delirium (figure 3A-B).

Patients with dementia had significantly higher levels of NFL pre- and postoperatively than patients without dementia (median 98 vs 53 pg/mL preoperatively and 130 vs 93 pg/mL postoperatively, both $p<0.001$) (supplementary table 2). When stratifying for dementia, NFL remained significantly higher in delirium patients in the dementia-free stratum preoperatively (median 80 vs 46 pg/mL, $p=0.001$) and postoperatively (median 88 vs 136 pg/mL, $p<0.001$). This association was upheld in a regression analysis with age, $\beta = 0.43$, $p=0.014$ (preoperatively) and $\beta=0.33$, $p=0.009$ (postoperatively) (supplementary table 1). There was no difference between patients with and without delirium in the stratum with dementia (supplementary table 2).

Change in serum NFL

sNFL was increased significantly pre- to postoperatively in all groups independently of delirium and dementia status (45 % on average in the whole hip fracture cohort). No significant disparity in change was found in patients with delirium compared to those without in the overall population ($p=0.080$, Mann Whitney-*U*). However, in pairwise comparisons of delirium subgroups using Mann Whitney *U*-test, the increase of NFL was significantly larger in incident delirium (65 vs 27 pg/mL, $p=0.026$) compared to no delirium (figure 3C).

Within the dementia strata, patients with delirium had significantly larger increases of NFL (median 42 vs 23, $p=0.040$) (not shown). When excluding patients with preoperative delirium, the increase was significantly higher in patients with incident delirium than in patients without delirium both in the overall hip fracture group (median 65 vs 27, $p=0.006$) and in patients with dementia (median 94 vs 24, $p<0.001$) (figure 4). In a linear regression adjusting for age, the associations remained significant ($\beta=0.57$, $p=0.020$ in overall population; $\beta=0.93$. $p=0.009$ in the dementia stratum) (supplementary table 1).

Neurofilament in CSF
Patients with delirium (n=71) tended to have higher levels of NFL than patients without delirium (n=59), median 1804 vs 1636 pg/mL, p=0.074 (table 1). In pairwise subgroup comparisons using Mann Whitney *U*-test, NFL was higher in patients with preoperative (n=44) than no delirium (n=37), median 1845 vs 1267 pg/mL, p=0.011(figure 3c). Surprisingly, NFL was numerically highest in patients with subsyndromal delirium (n=20, median 2044 pg/mL IQR 1209-2537) (figure 3D). Analyses were repeated, excluding patients with subsyndromal delirium. NFL was found to be significantly higher in delirium compared to no delirium (n=37), median 1804 vs 1267 pg/mL, p=0.018. However the association with NFL did not hold up (β=0.21, p=0.16) when adjusted for age (supplementary table 1).

Patients with dementia (n=65) had significantly higher levels of NFL than patients without dementia (n=65), median 1961 vs 1526 pg/mL) pg/mL, p= 0.025.
No significant difference in CSF NFL was found between patients with and without delirium in either dementia strata (supplementary table 2).

Cognitively healthy controls
Patients in the control group were younger than the hip fracture patients (71 vs 85 years, p<0.001) with a more equal gender distribution (table 1).
NFL was significantly higher in the FCF patients than in the healthy controls, both in preoperative serum (153 % higher) and CSF (67 % higher) (table 1). A robust correlation between age and NFL in both serum and CSF (ρ =0.49 and ρ =0.47, p<0.001) was shown.

Correlations in blood and cerebrospinal fluid
NFL concentrations in serum and CSF correlated in both hip fracture patients (preoperative serum, ρ=0.56 and p<0.001) and cognitively healthy controls (ρ =0.69, p<0.001) (supplementary figure 1).

Discussion
In hip fracture patients, pre- and postoperative sNFL were significantly higher in delirium, also after adjusting for age. After stratification for dementia, similar findings were found in the dementia-free strata. Furthermore, CSF NFL was higher in delirium, but this difference did, however, not reach statistical significance. The association between elevated NFL and delirium supports the hypothesis that delirium may be connected with neuronal death (2, 8). Increased NFL in delirium patients may reflect brain vulnerability due to reduced neuronal capacity with increased risk of delirium, or that specific pathophysiological mechanisms leading to delirium result in cell death at the time of syndrome onset. Recent studies have suggested that delirium in dementia patients may multiplicatively contribute to the pathological processes in dementia by alternative unknown pathways causing neuronal death (6, 41).

Repeated measurements can yield increased insight into the relationship between delirium and cell death. A marked increase in serum NFL in patients with delirium between two time points would strengthen the direct association between delirium and cell death. Patients who were delirium-free initially, but developed delirium after surgery (i.e. incident delirium) had the highest increase of NFL, followed by patients with preoperative delirium, SSD and finally no delirium (figure 3D). Furthermore, highest preoperative levels of serum NFL (and CSF NFL) were measured in patients with preoperative delirium and highest postoperative levels in patients developing delirium postoperatively (figure 3 A-C). Hypothetically, this pattern of

NFL changes in serum subgroups could illustrate a process whereby cell death occurs prior to development of delirium symptoms, followed by a NFL peak during the episode and shortly thereafter, followed by a gradual decrease or stabilization of NFL release. A recent study on traumatic brain injury suggests that outflow of NFL may be bimodal (42). The bimodality may be due to initial outflow from acutely damaged neurons, followed by a decrease and a secondary peak due to secondary axonal degeneration (43). In posthoc analyses we therefore assumed that potential delirium-associated NFL release might already be in progression in patients with delirium preoperatively and that including these patients could presumably underestimate NFL changes. We thus compared NFL-change in incident delirium to the corresponding change in delirium-free patients. The NFL increase was significantly larger in patients who developed delirium subsequent to the first serum sampling, compared to patients without delirium. In the dementia stratum this difference was highly significant, also after age adjustment. This indicates that NFL release and neuraxonal death may be directly involved in delirium pathophysiology in patients with dementia, accounting for the increased risk of dementia progression. No difference was found in dementia-free patients, suggesting that different pathophysiological trajectories, comprising white matter change to a lesser degree, may be involved in development of new-onset dementia after delirium, or that the extent of neuronal death in delirium in the neurodegenerative brain may be more extensive and hence detectable (44, 45).

Robust correlations were found between CSF and serum NFL in the overall group, in delirium and in dementia patients, in line with findings in multiple sclerosis (34, 35). Spinal tapping is an invasive procedure that is clinically required only in a minor subset of delirium patients (46). Robust validity of NFL measurements in blood would greatly facilitate use of NFL as a biomarker in research on delirium pathophysiology.

Strengths of our study comprise daily bedside assessment of delirium based on validated instruments. Furthermore the large number of included patients allowed for subgroups analyses. We included both patients with and without dementia as well as a cohort of healthy controls in order to present a wide neurodegenerative specter. Moreover serum was sampled repeatedly and paired with CSF sampling preoperatively.

We were faced with several challenges when we explored NFL in this population.
Firstly, NFL levels in elderly hip fracture patients were significantly higher than in elective controls. This highlights the importance of using matched controls in delirium research. An exaggerated reaction to a generalized stress response following a hip fracture may possibly lead to cell death and NFL release in a vulnerable brain (44). Furthermore, comorbidities, such as dementia (47, 48) and greater age (37, 49) in an overall frailer hip fracture population are associated with increased levels of NFL in blood and CSF, as we confirmed. A stronger correlation found in the elective patients compared to the hip fracture patients further suggests that extracerebral sources of NFL may be of greater importance in the later population.

Secondly, these patients were subjected to spinal anesthesia and major surgery. In all patients and subgroups, serum NFL increased significantly from the preoperative to the postoperative phase. In elective surgery patients undergoing general anesthesia, NFL in serum has been found to increase to a maximum 48 hours postoperatively (50). Damage to peripheral nerves during surgery, noxious effects of anesthesia (51) and systemic physiological reactions could be possible underlying mechanisms.

Thirdly, due to logistical reasons, time between blood samplings varied and CSF and serum samples were not available from all patients at all time points. Bimodality of NFL release (42) could implicate that timing of serum samplings may have been inadequate to demonstrate maximal increases and change in serum NFL, reducing the sensitivity of our measurements.

This is the first study to show that delirium is associated with increased NFL in CSF and serum, suggesting an association between delirium and neuraxonal death. The increased outflow of NFL demonstrated in incident delirium suggests that mechanisms directly related to delirium pathophysiology may be responsible for the increase in NFL. Robust correlations found between CSF and serum NFL indicate that future analyses of NFL in delirium patients may be performed in serum, obsoleting spinal tapping.

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Conflict of Interest/Disclosure Statement
Dr. B.Halaas reports no disclosures
Dr. H. Zetterberg has served at scientific advisory boards for Eli Lilly, Roche Diagnostics and Wave, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg.

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