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## Preoperative protein profiles in cerebrospinal fluid in elderly hip fracture patients at risk for delirium: A proteomics and validation study



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### ABSTRACT

**Background:** A neuroinflammatory response is suggested to play an important role in delirium, a common complication in older hospitalized patients. We examined whether hip fracture patients who develop postoperative delirium have a different proteome in cerebrospinal fluid (CSF) prior to surgery.

**Methods:** Patients ( $\geq 75$  years) were admitted for hip fracture surgery. CSF was collected during spinal anaesthesia; proteins were separated using gel electrophoresis and identified with mass spectrometry. We compared the proteome of patients with and without postoperative delirium. Findings were validated in an independent, comparable cohort using immuno-assays.

**Results:** In the derivation cohort 53 patients were included, 35.8% developed postoperative delirium. We identified differences in levels of eight CSF proteins between patients with and without subsequent delirium: complement factor C3, contactin-1, fibulin-1 and I-beta-1,3-N-acetylglucosaminyltransferase were significantly lower in patients with postoperative delirium, while neural cell adhesion molecule-2, fibrinogen, zinc- $\alpha$ -2-glycoprotein and haptoglobin levels were significantly higher. In the validation cohort 21.2% of 52 patients developed postoperative delirium. Immuno-assays confirmed contactin-1 results although not statistically significant. Complement factor C3 was significantly higher in patients with postoperative delirium.

**Conclusion:** Our results show the complexity of pathophysiological mechanisms involved in delirium and emphasizes the need of independent validation of findings.

**General significance:** This study highlights the challenges and inconsistent findings in studies of delirium, a serious complication in older patients. We analysed proteins in CSF, the most proximal fluid to the brain. All patients were free from delirium at the time of sampling.

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**Abbreviations:** 2D-DIGE, Two-dimensional difference gel electrophoresis; b3GNT3, N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase; C3, Third component of the complement system; CSF, Cerebrospinal fluid; ELISA, Enzyme linked immunosorbent assay; IQR, Interquartile range; LC-MS/MS, Liquid chromatography–mass spectrometry; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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### 1. Introduction

Delirium is the most common complication in hospitalized older patients and is known to be associated with both short- as well as long-term detrimental outcomes [1]. The exact pathophysiological mechanisms in delirium and its associated poor outcomes are still unknown, but a neuroinflammatory response has been suggested to play a role [2]. Neuroinflammation may arise in response to peripheral insults, such as infection, surgery and fracture [3]. The communication

pathways between periphery and the brain may lead to activation of microglia, the innate immune cells of the central nervous system. In response to activation, microglia release a range of inflammatory mediators, influencing neuronal function and possibly inducing delirium in susceptible individuals [4]. Aging and neurodegenerative disease are well known predisposing risk factors for delirium [3] and are both accompanied by a pro-inflammatory state [4]. Older people are especially vulnerable, since impaired cholinergic inhibition can elicit increased microglia activation and inflammation, which may result in neuronal damage [4].

Previous research revealed that hip fracture patients who develop postoperative delirium show lower preoperative cerebrospinal fluid (CSF) concentrations of anti-inflammatory cytokines compared to patients who do not develop postoperative delirium [5]. This finding is consistent with a role for a dysfunctional neuroinflammatory response in delirium. However, inflammation is a highly complex and dynamic process in which many different effectors interact. The state of inflammation is not likely to be dependent on the net effect of individual cytokines only, but on the balance between numerous pro- and anti-inflammatory mediators in the central nervous system [6].

A recent study assessed the CSF proteome in patients with delirium, and found 16 dysregulated proteins, including acute phase proteins like complement C3, fibrinogen and haptoglobin, providing supplementary evidence that inflammation is involved in delirium [7].

We hypothesized that elderly hip fracture patients who develop postoperative delirium, have specific CSF protein profiles prior to operation, as compared to patients who do not develop postoperative delirium. Differences in the CSF proteome can be assessed using two-dimensional difference gel electrophoresis (2D-DIGE), a technique frequently used in research on potential CSF biomarkers in neurological diseases such as Parkinson's and Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis [8–11]. We used this technique to compare the pre-operative CSF proteome of patients with postoperative delirium with that of patients without delirium after surgery. Proteomic findings were validated in a separate, comparable cohort with immunoassays.

## 2. Methods

The study was approved by the medical ethical committees of both hospitals where patients were included, and was conducted in accordance with the guidelines of Good Clinical Practice. All patients gave written informed consent.

### 2.1. Patients

CSF for proteomic analysis (derivation cohort) was collected in a teaching hospital in Alkmaar, The Netherlands between March 2008 and March 2009. Patients participated in a double blind randomized study comparing effectiveness of taurine versus placebo in reducing morbidity and 1-year mortality in elderly hip fracture patients [5]. Patient recruitment and study procedures for the proteomics study have been described in detail elsewhere [5]. In brief: all patients of 75 years or older who were admitted for surgical repair of a hip fracture were assessed for eligibility. Patients were excluded if they had no acute trauma or a pathological fracture, received total hip prosthesis, had contraindications regarding the administration of taurine (that is, renal failure defined as a creatinine clearance <30 ml/min), or did not provide consent. The Informant Questionnaire on Cognitive Decline Short Form (IQCODE-sf) was completed by the primary caregiver. Cognitive impairment was defined as a score of 3.4 or higher on this questionnaire or a record of dementia in the medical history.

The main outcome was postoperative delirium, defined according to the Confusion Assessment Method [12]. The presence of delirium was assessed daily from admission until the fifth postoperative day. To

assess risk factors for delirium instead of markers of delirium, all patients with preoperative delirium were subsequently excluded from analysis. Because all participants were at high risk of delirium, they all received routine care with prophylactic treatment of 0.5 mg haloperidol, three times daily from admission until postoperative day three, unless contraindications were present [13].

To check whether preoperative protein differences between groups represent reproducible, clinically relevant changes in patients with postoperative delirium, we performed a validation study in an independent cohort. CSF for validation, using immunoassays, was collected in a teaching hospital in Amsterdam, The Netherlands between March 2012 and April 2014. Patients were participants in a prospective descriptive cohort study assessing incidence, prevalence and pathophysiology of delirium. All patients of 65 years or older who were admitted for surgical repair of a hip fracture were checked for eligibility. Patients were excluded if they were discharged or died within 48 h after inclusion, did not receive spinal anesthesia or if no communication was possible (e.g. aphasia, language barrier, deafness). In the validation cohort, the IQCODE-sf was also completed by the primary caregiver. To make sure patients included in the validation cohort would be comparable to those in the derivation cohort, we subsequently excluded patients who were younger than 75 years, had no acute trauma or a pathological fracture, received total hip prosthesis, or had preoperative delirium. The main outcome was postoperative delirium, assessed daily by trained nursing staff using the delirium observation screening scale [14]. In case of a score of 3 or higher, a psychiatrist was consulted to assess delirium as defined in the fourth edition of the Diagnostic and Statistical manual of Mental disorders [15]. If necessary, patients received treatment with haloperidol according to local clinical practice.

### 2.2. CSF samples

CSF samples for the proteomics and validation studies were collected according to the same procedures: during cannulation for the introduction of spinal anaesthesia, always prior to administration of any anaesthetic. Of each patient CSF was collected in polypropylene tubes which were transported to the laboratory within 15 min after withdrawal. Upon arrival at the laboratory the CSF samples were centrifuged, aliquoted into polypropylene tubes, and stored at  $-80^{\circ}\text{C}$ .

### 2.3. 2D-DIGE

To analyse the CSF proteome, we adapted a protocol previously described in more detail [16]. In brief: of each patient, 2 ml of CSF sample was spun down and the supernatant was precipitated in 80% acetone. The protein pellet was resuspended and the protein concentration was estimated by the Bradford method using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA). Protein extracts were labelled using the fluorescent cyanine dyes developed for 2D-DIGE technology (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein extracts (40  $\mu\text{g}$ ) were labelled with 333 pmol of fluorescent dye (Cy2, Cy3 or Cy5). Samples were randomly labelled with Cy3 or Cy5 to avoid dye related bias. An internal control sample, to avoid inter-gel variations, was created by pooling 20  $\mu\text{g}$  of each protein sample and was labelled with Cy2. Three protein samples (Cy3, Cy5 and Cy2) were pooled and passively loaded onto 24 cm pH 3–10 NL strips (GE Healthcare), followed by isoelectric focussing using a manifold-equipped IPGphor IEF unit (GE Healthcare). Second dimensional SDS-PAGE was performed on hand-cast 12% SDS-PAGE gels using low fluorescence glass plates. Electrophoresis was carried out at 1 W per gel until completion using an Ettan DALT-12 unit (GE Healthcare). Gels were scanned with a Typhoon 9410 imager (GE Healthcare). Spot detection was performed with DeCyder 7.0 software (GE Healthcare): the Cy2, Cy3 and Cy5 images for each gel were merged, spot boundaries are automatically detected and spot volumes are calculated. This analysis was used to calculate

abundancy differences in given proteins between patients. Protein spots in the resulting 'spot-map' were matched after extensive landmarking and automatic matching. Dividing each Cy3 or Cy5 spot volume by the corresponding Cy2 (internal control) spot volume within each gel corrected for inter-gel variations.

For spot-picking, 600 µg of unlabelled protein extract was separated and stained with Flamingo fluorescent stain (Bio-Rad) following the manufacturer's protocol. We compared the Flamingo stained preparative gel with the DIGE master image, designated the differentially expressed spots manually in the preparative gel, and picked the protein spots with an automated spot picker (Bio-Rad).

#### 2.4. Liquid chromatography–mass spectrometry (LC–MS/MS) analysis

After picking, the gel spots were reduced and alkylated, and the proteins were digested. After incubation the peptides were extracted and dried in vacuo. Of each sample, five percent was injected on the LC–MS system, [17] a LTQ Finnigan (Thermo Fisher Scientific, Bremen, Germany) connected to an Agilent 1200 HPLC system (Agilent Technologies, Amstelveen, The Netherlands) adapted for nanoflow LC separation [18]. All columns were packed in-house [18,19]. Nanospray was achieved using a distally coated fused silica emitter made in-house biased to 1.7 kV. The mass spectrometer was operated in the positive ion mode and parent ions were selected for fragmentation in data dependent mode to automatically switch between MS and MS/MS. The most intense ions above the threshold of 500 were fragmented in the ion trap using collision-induced dissociation with collision energy of 35 at a target value of 300,000.

Raw data were analyzed by Proteome Discoverer version 1.3.0.339 (Thermo Fischer Scientific). The data were searched against the Swissprot database (version July 2013), using Mascot (Matrix Science, London, UK). The search parameters included the use of trypsin as proteolytic enzyme allowing up to a maximum of 2 missed cleavages. The precursor mass tolerance was 0.8 Da and product ion tolerance of 0.6 Da. The following peptide filters were applied: a low peptide confidence, a Mascot ion score of at least 20 and a search engine rank of 1.

#### 2.5. Immuno-assays

Proteomic results were validated with enzyme linked immunosorbent assays (ELISA) following the manufacturer's protocol (MyBioSource, San Diego, CA, USA, or USCNK life science Inc. Hubei, China). The researcher was blinded for clinical diagnosis of delirium. Optical density of samples was measured with an iMark microplate absorbance reader (Biorad), and compared to a standard curve run on the same plate. Protein concentrations were calculated with SoftMax Pro Software (version 5, Molecular Devices, Sunnyvale, CA, USA).

#### 2.6. Statistical analysis

Proteomics data were analysed with DeCyder software. Analysis was performed using paired Student t-tests with false discovery rate control, a statistical method used in multiple hypotheses testing to correct for multiple comparisons. It takes into account both the concern about multiple testing errors and the probability of a false rejection [20]. It is especially appropriate for exploratory analyses in which several significant results are expected in large numbers of tests. Compared to family wise error rate correction procedures (such as the Bonferroni correction), false discovery rate procedures have greater power, but at the cost of increased rates of type I errors.

All other statistics were performed using SPSS (SPSS for Windows, version 20, IBM Corporation, Armonk, NY, USA). Numerical variables are presented as median with interquartile range (IQR) or as mean with standard deviation (SD). Categorical data were analysed using Chi-square or Fisher-Exact test. Continuous variables were tested with

Mann–Whitney U tests or Student t-tests depending on sample size and distribution of the data. A  $p$ -value  $\leq 0.05$  was considered statistically significant. For visual presentation of ELISA data, GraphPad Prism 5 software was used (GraphPad Software Inc., La Jolla, CA, USA).

### 3. Results

#### 3.1. Patients

In the derivation cohort 53 patients were included, of whom 19 patients (35.8%) developed postoperative delirium (Table 1). In the validation cohort 52 patients were included, of whom 11 patients (21.2%) developed postoperative delirium. Patients' characteristics between the derivation and validation cohort were similar, although patients in the derivation study were younger (median age, 83.1 year vs 87.5 year, ( $p = 0.011$ ) on average with a large overlap of the age range. The median time between admission and surgery was longer in the derivation group ( $p < 0.001$ ). Within each cohort, patient's characteristics and time between admission and surgery were similar between patients with and without postoperative delirium (data not shown).

#### 3.2. Proteomics

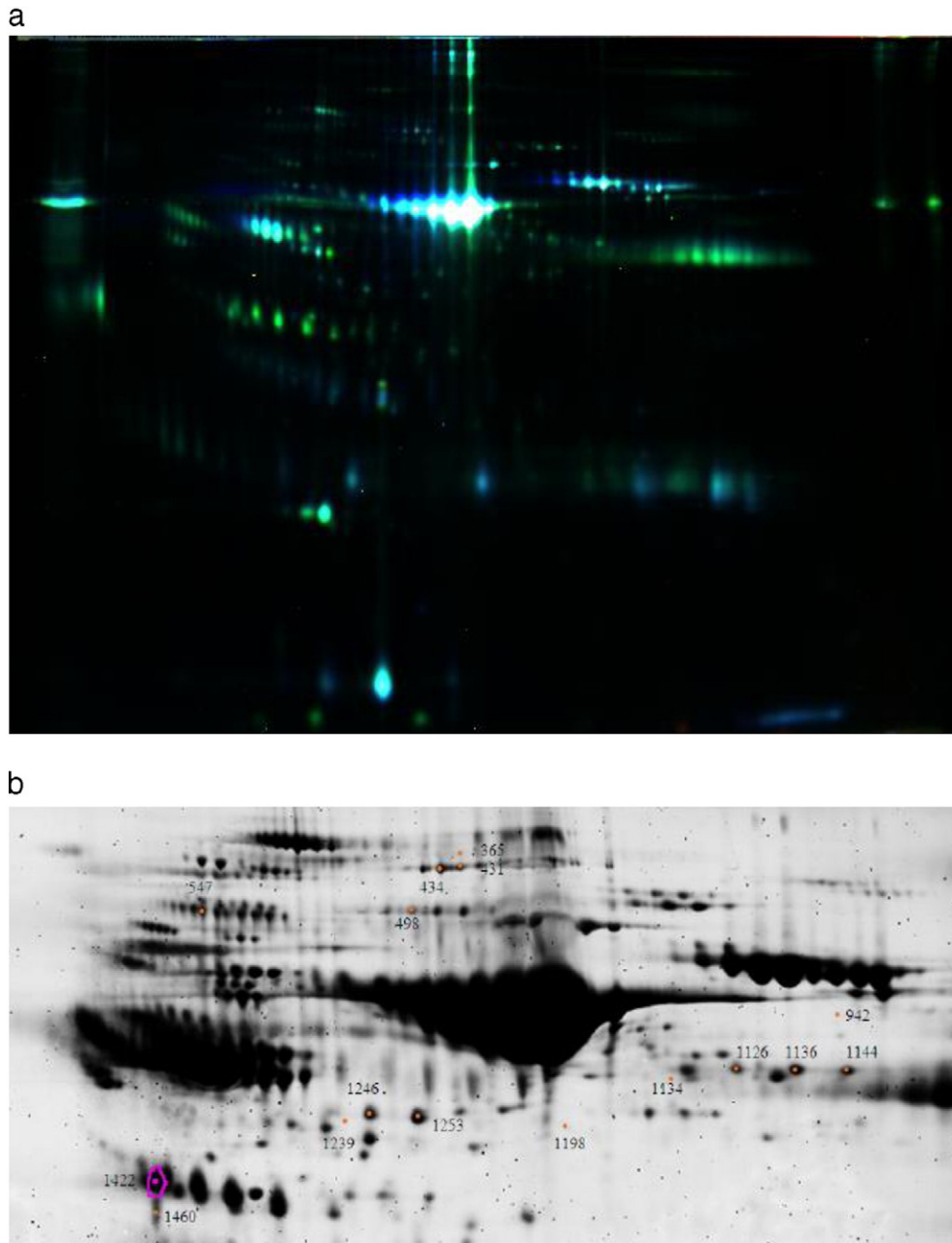
In the derivation group, CSF of 75 patients was collected. Eleven additional patients were excluded because of preoperative delirium ( $n = 3$ ), or unclear labeling of samples ( $n = 7$ ), or missing postoperative delirium assessment ( $n = 1$ ), leaving 64 patients for analysis in this exploratory study. Of these 64 samples, 11 samples were excluded because they had too low protein concentration, resulting in 53 CSF samples for analysis. Mean CSF protein concentration after precipitation did not differ between groups (delirium: 2.98 µg/µl (SD 1.27); control: 2.79 µg/µl (SD 1.35),  $p = 0.89$ ). Half of the protein samples in each group were labelled with Cy3, and half with Cy5. Seventeen proteins were significantly different between groups. Based on image analysis (spot map is shown in Fig. 1), 16 proteins were selected for mass spectrometry. LC–MS/MS revealed 8 different proteins in 16 spots (Table 2). Complement C3 was 1.4 fold lower in patients who developed postoperative delirium, as were contactin-1 (–1.3 fold), fibulin-1 (–1.2 fold) and l-beta-1,3-N-acetylglucosaminyltransferase (–1.2 fold). Patients with postoperative delirium had higher levels of neural cell adhesion molecule-2 (1.3 fold), haptoglobin (1.3 fold), zinc-α-2-glycoprotein (1.3 fold) and fibrinogen α-chain (1.2 fold), β-chain (1.4 fold) and γ-chain (1.4 fold).

#### 3.3. Validation

CSF of 67 patients was available for a validation study. Fifteen patients had to be excluded due to incomplete delirium scores ( $n = 1$ ), preoperative delirium ( $n = 1$ ), pathological fracture ( $n = 1$ ), or age

**Table 1**  
Patient characteristics.

	Derivation N = 53	Validation N = 52	<i>p</i> -Value
Age (years), median, (IQR)	83.1 (79.6–87.0)	87.5 (81.0–90.5)	0.011
Female, n (%)	36 (67.9)	37 (71.2)	0.833
Living independently, n (%)	34 (64.2)	35 (67.3)	0.839
Number of co-morbid diseases. Median (IQR)	2 (1–2)	2 (1–3)	0.420
Cognitive impairment, n (%)	14 (26.4)	13 (25.0)	0.868
Time between admission and surgery (days), Median (IQR)	1 (1–2)	1 (0–1)	<0.001
Postoperative delirium, n (%)	19 (35.8)	11 (21.2)	0.130



**Fig. 1.** a. Fluorescent spot map: CSF proteins stained with fluorescent dye b. Spot picking map with spot numbers: 600  $\mu$ g of unlabelled protein extract, separated and stained with Flamingo fluorescent stain. The Flamingo stained gel was compared to DIGE master image, and differentially expressed spots manually designated.

younger than 75 years ( $n = 12$ ), leaving 52 patients for analysis. Results of validation can be found in Table 3 and Fig. 2. Patients with postoperative delirium had significantly higher C3 pre-operative concentrations than control patients. Contactin-1 levels were lower in patients with postoperative delirium, although not statistically significant. C3a, C5a, C5b-9, fibrinogen, haptoglobin, neural cell adhesion molecule 2, fibulin 1, zinc- $\alpha$ -2-glycoprotein and b3GNT3 levels did not differ between groups. Analyses were repeated without cognitively impaired patients, this did not reveal different outcomes. No statistical relevant correlation was found between protein levels and age or time-to-surgery.

#### 4. Discussion

We performed for the first time 2D-DIGE on preoperative CSF from hip fracture patients with and without postoperative delirium

and identified differences with respect to the levels of eight proteins.

Only one prior study used proteomics on CSF of patients with delirium [7]. Compared to Alzheimer's patients and hip fracture patients, the presence of delirium was associated with dysregulation of acute phase proteins C3, fibrinogen and haptoglobin, which is in agreement with the results of our proteomics study.

We found that concentrations of C3 were lower in patients with postoperative delirium, which could be explained by complement activation, consistent with a role for neuroinflammation in delirium. The complement system plays an important role in immunity [21]. Uncontrolled complement activity, however, has been associated with a wide variety of inflammatory conditions and immune-complex diseases such as autoimmune diseases, sepsis, Alzheimer's disease and rejection of transplants [22]. No studies have been published on complement



**Table 2**  
2D-DIGE results: altered protein quantity in CSF of patients with postoperative delirium.

Protein	Spot nr	MW (kDa)	PSMs	Mean score	Av. ratio*
Contactin-1	365	113.2	19	6	−1.34
Complement C3	431	187	53	3.5	−1.35
	434		63		
NCAM-2	498	93	22	4	1.25
Fibulin-1	547	77.2	22	4	−1.2
Fibrinogen α-chain	942	94.9	12	8	1.19
	1126		23		
	1134		5		
	1136		36		
	1144		38		
b3GNT3	1198	47.1	4	12	−1.17
	1239		10		
Fibrinogen γ-chain	1246	51.5	30	5	1.36
	1253		37		
	1253		1		
Zinc-α-2-glycoprotein	1422	34.2	21	2	1.34
Haptoglobin	1460	45.2	8	6	1.31

Av. ratio: average spot volume ratio between patients with postoperative delirium and no postoperative delirium; b3GNT3: N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase; MW: molecular weight (kilo Dalton); NCAM2: neural cell adhesion molecule; PSMs: peptide-spectrum matches.

activation in patients with delirium. However, activated complement proteins belong to the first neuropathological changes in dementia [23]. Old age and cognitive impairment are important risk factors for delirium. To increase the external validity of our study, we did not exclude patients with cognitive impairment. The underlying pathologic mechanisms that increase the risk for delirium in older and cognitively impaired individuals are unknown. Our hypothesis was that differences in CSF protein profiles could explain the difference in vulnerability in delirium.

However, validation of these results in an independent, comparable cohort, showed higher C3 concentrations in patients with postoperative delirium. Our results and the variable findings in previous research illustrate the complexity of the complement system. Especially C3 is hard to investigate, as it is subject to continuous low grade spontaneous activation, the so called 'C3 tickover' [24]. Studies on CSF complement in patients with dementia show equally contradictory results [25–28]. A possible explanation is that complement activation is dependent on age and on stage of dementia [25,26]. Our two cohorts did not differ in the number of patients with cognitive impairment, indicating that inconsistent results do not seem to be attributable to differences in the prevalence of dementia. Moreover, our results did not change once we repeated analyses without cognitively impaired patients in the validation cohort.

**Table 3**  
Validation of 2D-DIGE results with ELISA: CSF preoperative protein concentrations.

	No postoperative delirium median (IQR)	Postoperative delirium median (IQR)	p-Value	LLD
C3	1411.8 (1037.1–1748.6)	2103.07 (1320.7–2763.0)	0.008	3.2
C3a	2.57 (1.58–3.89)	4.56 (1.37–5.11)	0.329	0.064
C5a (pg/ml)	231.4 (144.5–308.7)	158.40 (39.1–249.0)	0.257	29
C5b-9	574.3 (468.8–724.9)	584.82 (561.8–710.6)	0.779	12.86
Fibrinogen	3213.2 (2076.7–4265.5)	3645.37 (2632.0–4162.6)	0.533	4.1
Haptoglobin	2530.0 (1664.1–3109.9)	2652.43 (1789.7–4782.5)	0.319	4.7
NCAM2	9.58 (5.77–11.63)	9.20 (6.0–12.3)	0.884	0.057
Fibulin-1	1206.5 (935.2–1686.6)	1582.45 (955.9–1695.7)	0.720	0.18
Contactin-1	53.6 (45.4–63.5)	45.76 (43.3–51.8)	0.086	0.108
ZAG (µg/ml)	17.3 (15.9–19.9)	18.30 (14.4–18.7)	0.712	0.48
b3GNT3	0.08 (0.06–0.11)	0.09 (0.04–0.13)	0.866	0.066

Results are presented in nanograms per millilitre, unless stated otherwise. b3GNT3: N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase; C3: complement factor 3; IQR: Interquartile range; LLD: Lower limit of detection; and ZAG: zinc-alpha-2-glycoprotein.

Another explanation for our inconsistent C3 results is the difference in time between admission and surgery for both groups. It is known that C3 levels rise due to the acute phase reaction after fracture [29]. Possibly this initial inflammatory reaction is exaggerated in patients who are prone to develop postoperative delirium. After some time, the complement system is activated, which in these patients is characterized by excessive splicing of C3, thereby inducing lower levels later on. This sequence of events might explain why the proteomics findings, in patients having longer waiting times, suggested lower CSF C3 levels, where the immunoassay, in patients with shorter waiting times, suggested increased CSF C3 levels in patients prone to develop delirium. However, we did not find a statistical relevant correlation between waiting time and complement levels in our validation cohort.

To investigate whether complement activation might be held responsible for low C3 levels, we assayed C3a, C5a and C5b-9. We did not find statistical relevant differences between groups.

Contactin-1 was also associated with the development of postoperative delirium. Contactin-1 is a cell adhesion/recognition molecule of the immunoglobulin superfamily [30]. Contactin-1 is expressed exclusively on neurons [30]. It is critical for establishment of paranodal axoglial junctions and for communication between axons and oligodendrocytes, the glial cells that form the myelin sheath [31]. We found lower levels of contactin-1 in preoperative CSF of patients with postoperative delirium in the proteomics cohort. The importance of this finding for delirium is not understood. We were able to replicate the lower levels of contactin-1 in patients with postoperative delirium in the validation cohort, though the trend did not reach statistical significance ( $p = 0.086$ ), which might be due to the limited power of our analysis.

Validation with immunoassays in an independent cohort with a similar design as the derivation cohort showed no significant differences in any of the remainder of the identified proteins with different proteomic profiles between patients with and without delirium.

The number of patients (i.e., 11 patients with delirium in the validation cohort) precludes multivariable analysis. Although we corrected for multiple testing, it is possible that our proteomic analysis generated false positive results. Another option is contributable to methodological aspects of 2D-DIGE. Some markers can be considered proteins that are relatively easy to detect with this technique, which would account for their frequent observation in proteomic studies on different diseases, while they may not be especially specific indicators for the underlying pathophysiological processes [32].

We deliberately validated results in a separate yet comparable cohort to assess the external validity of our findings. Both cohorts included elderly patients with surgery after a hip fracture, all of whom were free of delirium at the time of CSF sampling, resulting in a study of associated factors rather than markers of delirium.

There were some minor differences between the two cohorts. Patients in the validation group were somewhat older than patients in the proteomics group. However, it is difficult to understand how alleged

age effects, expected to be subtle over a period of 4 years only, could obscure effects of processes driving the risk for delirium, which can be hypothesized to be quite strong operators. Moreover, we did not find a statistical relevant correlation between age and C3 levels in our validation cohort. Although patients in the derivation cohort were younger, they more frequently developed postoperative delirium, yet this did not reach statistical significance. Prospective cohorts show comparable wide ranges, estimating an incidence stretching from 10 to 65% [33].

Furthermore, participants in the derivation cohort, but not in the validation cohort, received routine care with low dose prophylactic haloperidol. No studies have been published on the effects of haloperidol on acute phase proteins, yet it has been shown that long-term high-dose haloperidol use does not affect serum and CSF levels of cytokines in both healthy volunteers and schizophrenic patients [34–37]. It is therefore not likely that low doses as administered in our study influenced protein levels. Since patients in the derivation cohort participated in a clinical trial comparing taurine with placebo, we examined if taurine administration might have influenced our results. We found no significant differences in CSF protein levels or in delirium incidence between the intervention and placebo group.

More and more studies focus on CSF proteins in patients who are prone to develop delirium or are acutely suffering from delirium [5,7,38–40]. Although all of these studies of markers of trait or state, respectively, report results that tend to support neuroinflammatory or neurodegenerative hypotheses, the results are not consistent. One previous study assessed the CSF proteome in patients with delirium, and found evidence for a neuroinflammatory hypothesis [7]. However, this study compared a very heterogeneous group and had only small numbers of patients. Our findings are based on a larger sample of patients and validation has been performed in a very comparable cohort.

The present study illustrates the complexity of pathophysiological mechanisms involved in delirium, emphasizing the importance of validation in independent and largely comparable cohorts.

## 5. Conclusion

Based on a proteomic analysis, we found alterations in 8 preoperative CSF protein levels in patients with postoperative delirium, but we were not able to replicate these results by immuno-assay in an independent validation cohort. Our results show the complexity of pathophysiological mechanisms involved in delirium. Furthermore, this study emphasizes the need of independent validation of findings in proteomic analyses.

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## Author's contributions

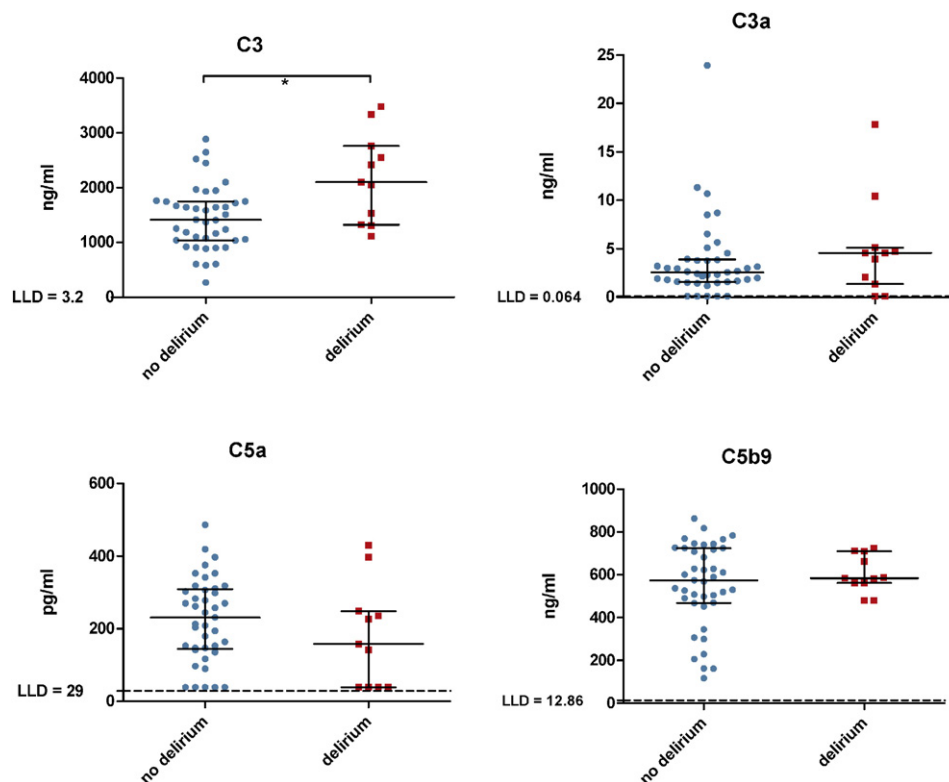
JFdJ and APH designed the TAUP study. DW, JW, LK, WAvG and DvdB designed the study of the derivation cohort. BvM and SEdR designed the study of the validation cohort. DW, JW and RMS acquired and interpreted clinical data. DW and CvA carried out 2D analysis. DW performed ELISA validation and statistical analyses and drafted the manuscript. DjvW and PE critically revised the manuscript. All authors read and approved the final version of the manuscript.

## Conflicts of interests

The authors declare that they do not have any competing interests.

## Transparency document

The [Transparency document](#) associated with this article can be found, in online version.



**Fig. 2.** Validation of proteomics with ELISA: CSF protein concentrations shown as median with IQR b3GNT3: N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase; C3: Complement component 3; LLD: lower limit of detection; NCAM2: neural cell adhesion molecule 2; and ZAG: zinc- $\alpha$ -2-glycoprotein.

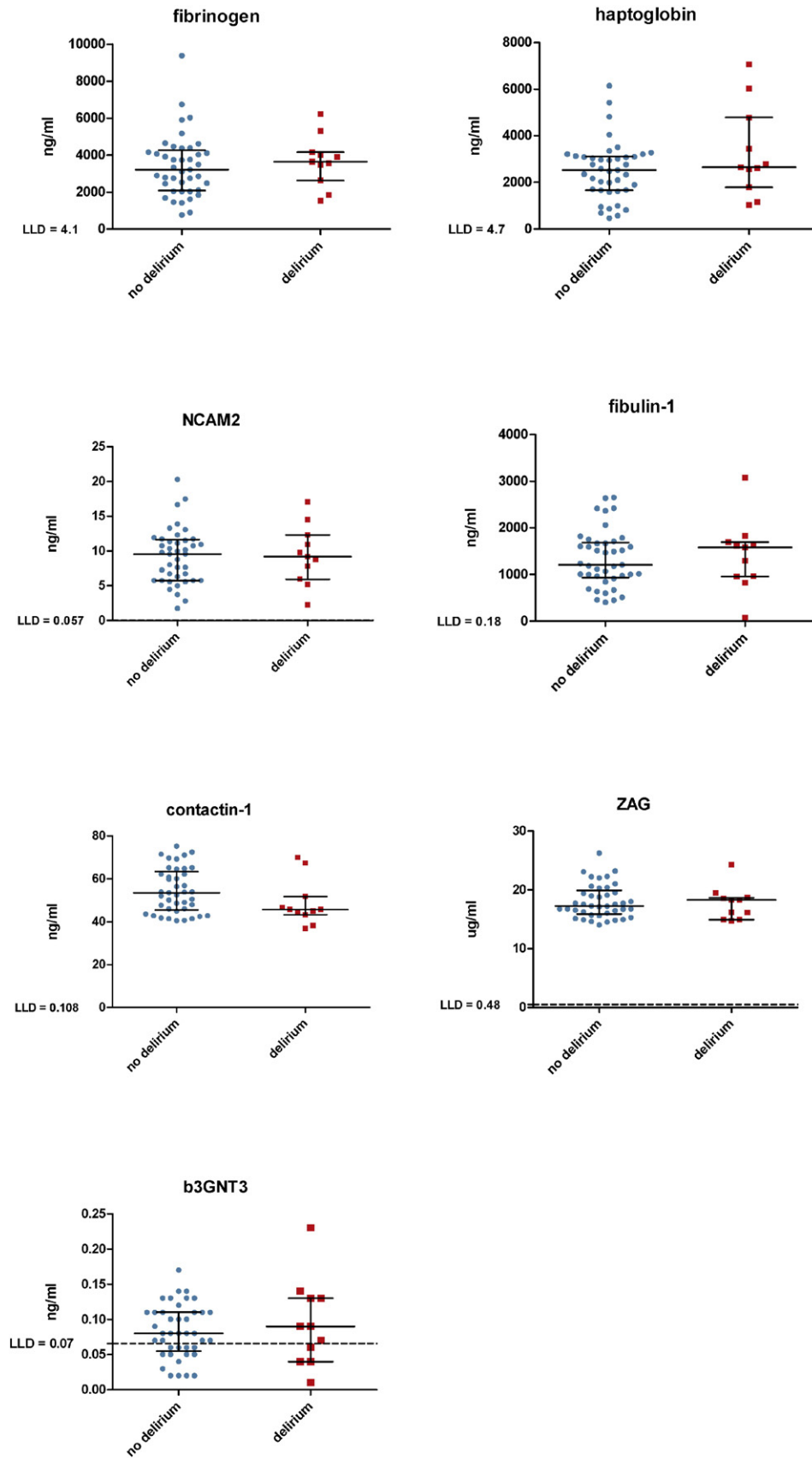


Fig. 2 (continued).

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Sponsor's role

None.

Trial registration

The Effect of Taurine on Morbidity and Mortality in the Elderly Hip Fracture Patient.

Registration number: NCT00497978. Local ethical protocol number: NL16222.094.07.

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