Detection of biomarkers for lysosomal storage disorders using novel technologies
van Breemen, M.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Addendum One

Proteomic profiling of plasma and serum of elderly patients with postoperative delirium


a Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
b Department of Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
c Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
d Department of Anesthesiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Abstract

Delirium is a frequently observed postoperative complication in elderly patients, the pathophysiology of it is still poorly understood. The aim of this study was to compare plasma and serum protein profiles in patients with and without postoperative delirium and to identify discriminating protein(s). Patients aged 65 years or more admitted for surgery following a hip fracture were included. Serum and plasma samples of eight patients with and eight patients without delirium were selected as testing group. A second group of eight patients with and eight without delirium was selected for validation. An additional sample was collected after the delirious episode. Protein profiles were generated by SELDI-TOF MS using CM10 and Q10 ProteinChip® Arrays. Demographical and clinical characteristics of patients with delirium were not significantly different from patients without delirium, except for the number of medications before admission. After correction for multiple testing, significant protein profile differences were found in the testing group. The largest difference was found in EDTA plasma using CM10 ProteinChip® Arrays, which was confirmed in the validation group. Taking both groups together, three discriminating peaks were found in delirious patients. These peaks presumably correspond to hemoglobin-β (15.9 kDa), the doubly charged ion of the unmodified protein (7.97 kDa) and its glycosylated form (16.0 kDa). Diagnostic accuracies of the three peaks expressed as area under the curve were 0.84, 0.88, and 0.83, respectively (P-values 0.001).
Introduction

Delirium is a neuropsychiatric syndrome characterized by the rapid onset of fluctuating changes in consciousness and attention, caused by physiologic consequences of a medical condition [1]. A hip fracture and orthopaedic surgery are both important etiologic factors for developing delirium. The incidence of postoperative delirium following orthopaedic surgery for hip fracture varies between 16% and 62%, with a mean duration of 3 days [2-4]. Though patients usually recover after treating the provocative factor, having delirium is associated with a three-times increased mortality risk, higher morbidity risk, and increased health care costs [5,6].

The pathophysiology of delirium is still poorly understood although several mechanisms have been proposed [7-9]. Some studies looked at individual proteins in relation to delirium to unravel the pathophysiology, such as S-100 β protein [10], Tau-protein [11], and β-endorphin [12]. These candidates were studied based on a priori models of pathophysiology, thus running the risk of missing possible alternative mechanisms. Proteomics, the large-scale study of proteins, provides the opportunity to identify proteins potentially involved in the pathophysiological mechanism for example by comparing protein expression profiles [13].

Differences in protein profiles in comparable psychiatric syndromes have been found in a rat model of cocaine withdrawal [14] and in the post-mortem prefrontal cortex in humans with schizophrenia and bipolar disorder [15]. In subjects with Alzheimer disease, proteomic techniques have revealed specific oxidized proteins in blood plasma [16].

We hypothesize that differences in protein profiles also occur in patients who develop delirium. Since orthopaedic surgery after a hip fracture is a time-defined trigger for postoperative delirium with in-hospital recovery, this setting provides a good opportunity to study protein expression before, during and after delirium in elderly patients. The aim of the current study was to compare the protein profiles found in plasma and serum in patients during a postoperative delirium with the profiles of patients without postoperative delirium and to identify discriminating protein(s).

Materials & methods

Patients

All consecutive patients aged 65 years or more suffering from a hip fracture and scheduled for operation at the Department of Orthopedic Surgery or Traumatology of the Academic Medical Center, Amsterdam, were invited to participate in this cohort study from May 2005 till September 2006. Informed consent was obtained from the patient or from the substitute decision-maker in case of cognitive impairment. Patients were excluded if they were unable to speak or understand Dutch or English. The Institutional Medical Ethics Committee approved the study.

For the current pilot-study, we selected a random group of eight patients with delirium
and eight patients without delirium as a testing group. We selected the patients without delirium to resemble the delirious patients as much as possible with respect to type of anesthesia (spinal or general), sex, day of blood collection since operation, and blood transfusion status. In case this testing group would result in significant differences in protein profile between patients with and without a delirium, a second random group consisting of eight patients with and eight without delirium (validation group) would be selected. In case the validation group confirmed the findings from the testing group, both groups would be taken together in the subsequent analysis.

**Procedures**
Two geriatric physicians, a fellow in geriatric medicine, and a team of research nurses trained in geriatric medicine collected demographic and clinical data from all study participants. The presence or absence of delirium was scored with the Confusion Assessment Method (CAM) [17]. We based our information for the diagnosis on our psychiatric examination of the patient, medical and nursing records, including the Delirium Observation Screening Scale (DOS) [18], and information given by relatives.

Possible confounding factors were registered for all patients; e.g. fracture characteristics, type of anesthesia, type of surgery, time between hip fracture and surgery, blood transfusions, demography, number of medications taken before admission, cognitive impairment and functionality. Cognitive functioning was scored by medical history and IQCODE (Informant Questionnaire on COgnitive DEcline). The IQCODE assesses the possible presence of global cognitive decline before admission based on the response of an informant who had known the patient for at least 10 years [19]. The informant was asked to recollect the situation 2 weeks before the hip fracture and to compare it with the situation 10 years before. Patients with a mean score of 3.9 or more were considered to have cognitive impairment [20]. To measure functionality we asked the relative to complete the 15-item KATZ ADL scale based on the situation 2 weeks before the hip fracture [21].

For all patients several blood samples, serum and ethylenediamine tetraacetic acid (EDTA) plasma, were collected under similar strict conditions around 11.00 am. For the patients with delirium we used a sample taken during delirium and for the patients without delirium the sample collected on average 2 days after surgery was taken. Of all delirious patients an additional blood sample was collected after the delirious episode. Blood was collected in tubes containing anticoagulants, and in tubes without anticoagulants and kept on ice. Serum was obtained after allowing blood samples to clot at room temperature for 30 minutes. After centrifugation for 15 minutes at 4000 RPM (1780g) at 4°C the aliquots were stored at -80°C.

**Proteomic analysis**
Protein profiles of serum and EDTA plasma samples were generated making use of the anionic surface of CM10 ProteinChip® Arrays, and the cationic surface of Q10 ProteinChip® Arrays (Ciphergen Biosystems Inc., Fremont, CA, USA). Experiments were
Protein profiling of plasma and serum of patients with delirium

blinded for sample type and samples were applied in random order. First 10 μL of EDTA plasma/serum was mixed with 90 μL of denaturation solution (9 M urea [Sigma Chemical Company, St Louis, MO, USA], 2% CHAPS [Fluka Biochemika, Buchs, Switzerland], and 1% DTT [Sigma Chemical Company, St Louis, MO, USA]). After incubation for 1 hr at room temperature (RT), 10 μL of this solution was mixed with 90 μL binding buffer (binding buffer for CM10 ProteinChip® Arrays: 50 mM sodium phosphate [Merck, Darmstadt, Germany] pH 6.5 + 0.1% Triton X-100 [BHD Laboratory Supplies, Poole, Dorset, UK], binding buffer for Q10 ProteinChip® Arrays: 50 mM Tris [Sigma Chemical Company, St Louis, MO, USA] pH 8.0 + 0.1% Triton X-100 [BHD Laboratory Supplies, Poole, Dorset, UK]). Before application of a sample to ProteinChip Arrays, all spots were equilibrated. All washing and binding was performed using a platform shaker. To equilibrate the ProteinChip Arrays, spots were washed with 200 μL of binding buffer on a Ciphergen Biosystems 96-well bioprocessor (2 x 5’). After equilibration, buffer was removed and samples were added. The samples were allowed to bind to the surface for 40 min at room temperature. Subsequently the ProteinChip Arrays were washed with 200 μL binding buffer (2 x 5’). Next the ProteinChip Arrays were washed with 200 μL binding buffer without Triton X-100 (2 x 5’). After a brief wash with deionised water (to remove salts) ProteinChip Arrays were dried on air. Prior to SELDI-TOF MS analysis, matrix was added to each spot (2 times 0.5 μL of sinapinic acid [Fluka Biochemika, Buchs, Switzerland] in 50% aqueous acetonitrile [Merck, Darmstadt, Germany] containing 1% TFA [Fluka Biochemika, Buchs, Switzerland]). After co-crystallization of the (bound) proteins with matrix, a pulsed nitrogen laser was used for sample ionization. ProteinChip Arrays were analyzed using a PBSIIc ProteinChip Reader (Ciphergen Biosystems Inc., Fremont, CA, USA), a linear laser desorption/ionization time-of-flight mass spectrometer equipped with time-lag focussing. This resulted in mass spectra composed of mass to charge ratios (m/z values) and intensities of the desorbed (poly)peptide ions. All spectra were acquired in positive-ion mode.

Pre-processing of SELDI-TOF MS data for further analysis

Data was pre-processed using Ciphergen ProteinChip® Software 3.1.1. Spectra were externally calibrated against a mixture of known peptides (All-in-1 Peptide Standard, Ciphergen Biosystems Inc., Fremont, CA, USA). Spectra from the validation group samples were calibrated with testing group calibration coefficients.

Spot-to-spot calibration is a feature of the ProteinChip® Software that accounts for the spot to spot variation that can occur on an individual array. To determine the correction factors for the different positions on an array we used a set of peaks that is always present in our spectra. The correction factors for the different positions on an array were applied to the corresponding mass spectra and used in the recalculation of the masses. Baseline subtraction was applied to all spectra in order to remove offsets in the spectra that were the result of how the signal was collected electronically and of chemical noise contributed from the energy absorbing molecules in the matrix. The algorithm implemented in the ProteinChip® Software is a modified piecewise convex-hull method that attempts to find
the bottom of the spectra and correct the peak height and area. Spectra were normalized to
the average total ion current in the mass range from 1.7 to 50 kDa.

Peaks in the mass range from 1.7 to 50 kDa were detected with Biomarker Wizard™. Biomarker Wizard™ is a feature of the ProteinChip® Software used for preparing data generated by ProteinChip® Software for further analysis. Biomarker Wizard™ groups peaks of similar molecular weight from across sample groups of spectra. For peak extraction on the first group, the testing group, we used the following parameter settings: first pass: 10 S/N, min peak threshold: 20%, cluster mass window: 0.4%, second pass: 2 S/N for CM10 EDTA plasma and first pass: 12 S/N, min peak threshold: 20%, cluster mass window: 0.4%, second pass: 5 S/N for the other experiments. For peak extraction in the second group, the validation group, we projected the peaks extracted from the first group onto the second group and estimated the corresponding peak intensities. Finally, for peak extraction on first and second group together we used the following parameter settings: first pass: 10 S/N, minimal peak threshold: 20%, cluster mass window: 0.6%, second pass 2 S/N. Resulting peak intensities were log2-transformed in order to stabilize their variance. Any peak intensity which was zero or negative after baseline subtraction is set equal to half the minimum of the positive corrected intensities for that peak.

Data analysis

We tested for differences in demographic and clinical characteristics in patients with and without delirium using T-tests, Mann Whitney Tests and Chi-squared tests. A two-tailed P-value < 0.05 was considered statistically significant.

We compared profiles to identify peaks differentially expressed between both groups and corrected them for chip effect, type of anaesthesia, and whether patients received a blood transfusion. Between-group comparisons were done using a moderated t-test [22]. This test is similar to a standard t-test for each peak except that the standard errors are moderated across peaks to ensure more stable inference for each peak. The resulting P-values were corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate adjustment (FDR) [23]. Tests were considered to be significant if the adjusted P-values were <0.05.

Results

During the inclusion period 149 patients aged 65 years or more were admitted for acute hip surgery, of which 74 patients gave informed consent for participation in the study. Of these 74 patients, 35 (47%) developed delirium after orthopaedic surgery. For the current study, a random group of 16 patients with and 16 patients without postoperative delirium were selected. For the patients with delirium the mean age was 85 yrs (SD:7.6) and 19% were male, while for the patients without delirium mean age was 83 yrs (7.8) and 38% were male. The number of medications taken before hospital admission was significantly higher in patients with delirium, P-value 0.01 (Table 1).
Protein profiling of plasma and serum of patients with delirium

Table 1. Baseline characteristics of elderly patients with and without delirium after acute admission.

<table>
<thead>
<tr>
<th></th>
<th>Delirium (n=16)</th>
<th>No delirium (n=16)</th>
<th>P-value (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – yrs</td>
<td>84.6 (7.6)</td>
<td>83.2 (7.8)</td>
<td>0.59</td>
</tr>
<tr>
<td>Male – n (%)</td>
<td>3 (19%)</td>
<td>6 (38%)</td>
<td>0.24</td>
</tr>
<tr>
<td>Living at home – n (%)</td>
<td>12 (75%)</td>
<td>15 (94%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Katz ADL – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (no)</td>
<td>1 (7%)</td>
<td>3 (20%)</td>
<td>0.18</td>
</tr>
<tr>
<td>1-3 (mild)</td>
<td>1 (7%)</td>
<td>5 (33%)</td>
<td></td>
</tr>
<tr>
<td>4-6 (medium)</td>
<td>5 (36%)</td>
<td>3 (20%)</td>
<td></td>
</tr>
<tr>
<td>7 (severe)</td>
<td>7 (50%)</td>
<td>4 (27%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Number of medications before admission</td>
<td>6 (1-12)</td>
<td>2 (0-9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Cognitive impairment – n (%)</td>
<td>5 (36%)</td>
<td>3 (19%)</td>
<td>0.24</td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days between fracture and operation</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
<td>0.51</td>
</tr>
<tr>
<td>Spinal anesthesia – n (%)</td>
<td>5 (31%)</td>
<td>9 (56%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Fracture characteristics – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral neck</td>
<td>9 (56%)</td>
<td>5 (31%)</td>
<td>0.15</td>
</tr>
<tr>
<td>Intertrochanteric</td>
<td>6 (38%)</td>
<td>6 (38%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (6%)</td>
<td>5 (31%)</td>
<td></td>
</tr>
<tr>
<td>Type of surgery – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal fixation</td>
<td>7 (44%)</td>
<td>11 (69%)</td>
<td>0.15</td>
</tr>
<tr>
<td>Hip replacement</td>
<td>9 (56%)</td>
<td>5 (31%)</td>
<td></td>
</tr>
<tr>
<td>Preoperative hemoglobin – mg/L</td>
<td>8.1 (1.1)</td>
<td>7.8 (0.6)</td>
<td>0.45</td>
</tr>
<tr>
<td>Bloodtransfusion – n (%)</td>
<td>6 (38%)</td>
<td>4 (25%)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Mean values (SD) are given for continuous variables with a normal distribution. Median values (range) are given for continuous variables that are not normally distributed.

Blood samples were taken on average 2 days (range 1-3) after surgery for both patients with and without delirium. After correction for multiple testing a discriminating peak was found (15.9 kDa (P=0.002, FDR)) in EDTA plasma applied to a CM10 chip (Table 2). Next, the protein profiles of the samples taken after the delirious episode, were compared with the samples of the patients without delirium. Discriminating peaks were found with EDTA plasma (15.9 kDa, FDR=0.042) and serum (5.8 kDa, FDR=0.004) applied to CM10 ProteinChip® Arrays. Another discriminating peak (4.5 kDa, FDR=0.017) was found with EDTA plasma applied to Q10 ProteinChip® Array. Finally, the protein profiles of eight patients during and after delirium were compared in a paired analysis. We found a discriminating peak (5.8 kDa) with serum applied to CM10 ProteinChip® Array. No significant differences in protein profile were found with serum applied to Q10 ProteinChip® Arrays. For all four combinations of chip type and kind of blood sample, no significant interaction effect of ProteinChip® Array and delirium-group (FDR>0.50) or type of anaesthesia and delirium-group (FDR>0.80) was found. Moreover, after stratifying for having had blood transfusions or not, similar results were found (data not shown).
Table 2. Main results from the testing group; eight patients with delirium (during and after delirium) and eight patients without delirium.

<table>
<thead>
<tr>
<th>Significant peaks (Da)</th>
<th>Overall test P-value (FDR)</th>
<th>During delirium vs. without delirium P-value (FDR)</th>
<th>After delirium vs. without delirium P-value (FDR)</th>
<th>During delirium vs. after delirium* P-value (FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM10 EDTA plasma</td>
<td>M15852</td>
<td>0.007</td>
<td>0.002</td>
<td>0.042</td>
</tr>
<tr>
<td>CM10 serum</td>
<td>M5809</td>
<td>0.011</td>
<td>0.946</td>
<td>0.004</td>
</tr>
<tr>
<td>Q10 EDTA plasma</td>
<td>M4485</td>
<td>0.054</td>
<td>0.357</td>
<td>0.017</td>
</tr>
<tr>
<td>Q10 serum</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Paired analysis

No interaction with chip or anesthesia observed.

Since the largest effect between patients with and without delirium was found in the profiles obtained with EDTA plasma applied to CM10 ProteinChip® Arrays, we decided to concentrate on this finding, i.e. to validate it. The intensity of the discriminating peaks observed with EDTA plasma of the testing group using CM10 ProteinChip® Arrays was also determined in the validation group. The intensity of the 15.9 kDa peak was again significantly higher in patients during delirium vs. patients without delirium (P=0.03). The intensity of this peak was also significantly higher after delirium vs. patients without delirium (P=0.041). No significant difference was found for this peak comparing the same patients during and after delirium (P=0.88). Since the results of the testing group were validated in the second group, we considered the two groups as one large group consisting of 16 delirious patients and 16 patients without delirium for the subsequent analyses.

A total of 35 peaks were detected when EDTA plasma was applied to CM10 ProteinChip® Arrays. Three peaks were significant in the overall testing. We found no significant interaction with chip effect, and adjustment for type of anaesthesia or stratification for blood transfusion, did not alter our findings. The three peaks with masses of 15.9, 16.0 and 7.97 kDa correlated significantly with each other, with correlations of 0.91, 0.93 and 0.95 respectively; P <0.001. Fig. 1 shows representative SELDI-TOF mass spectra of EDTA plasma samples of a patient during and after delirium and a patient without delirium. In the best spectra, a difference of about 162 Da between the 15.9 and the 16.0 kDa peaks could be measured, which is strongly indicative of glycosylation. The 7.97 kDa peak results from a doubly charged ion. It is the same protein as detected at 15.9 kDa (singly charged). This could be deduced from the absolute mass values as well as from the correlation in relative intensities of the two peaks (with the 7.97 peak intensities being only a fraction of the 15.9 peak intensities). Based on the observed mass and the efficient glycosylation we deduced the peak to represent hemoglobin-β. The spectra obtained in this mass range closely resemble those obtained previously, including its efficient glycosylation (e.g. with hemoglobin from plasma) [24,25]. In order to confirm our assumption that the observed peaks represent hemoglobin-β, we attempted to deplete the plasma samples with the aid of anti-hemoglobin antibodies to show that this resulted in the specific disappearance of all three peaks. However, the antibodies did not precipitate efficiently enough for a conclusive experiment in this regard.
Mature $\alpha_2\beta_2$ hemoglobin levels were compared between patients with and without delirium. Hemoglobin levels of delirious patients 2 days after surgery (mean 6.5 mg/L (SD:0.9) were comparable to patients without delirium (mean 6.7 mg/L (SD:1.2) (P=0.62). Fig. 2 shows the log2-transformed sum of the peak intensities of the combination of 7.97, 15.9, and 16.0 kDa peaks of 16 patients during delirium and after delirium and 16 patients without delirium in EDTA plasma applied to CM10 ProteinChip® Arrays. Based on the total intensity of the 15.9 kDa and related peaks, we calculated the diagnostic potential of this candidate biomarker (Table 3). Diagnostic accuracy expressed as area under the curve (AUC) showed a good diagnostic value of the three different representatives and the combined value of the significant peaks for all 16 patients during delirium vs. 16 patients without delirium with AUC’s above 0.83.

Figure 1. SELDI-TOF mass spectra. EDTA plasma samples of a patient during and after delirium and a patient without delirium were applied to CM10 ProteinChip® Arrays. Representative spectra are shown. Mass ranges 15500 to 16500 m/z.

Figure 2. Peak intensity, log2-transformed, of the combination of 7.97, 15.9, and 16.0 kDa peaks of patients during delirium, after delirium and patients without delirium in EDTA plasma applied to CM10 ProteinChip® Arrays.
Table 3. Diagnostic value of the significant peaks observed in EDTA plasma applied to CM10 ProteinChip® Arrays of 16 patients during delirium compared to 16 patients without delirium.

<table>
<thead>
<tr>
<th>Peak M</th>
<th>AUC</th>
<th>P-value (FDR)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0 kDa</td>
<td>0.83</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.9 kDa</td>
<td>0.84</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.97 kDa</td>
<td>0.88</td>
<td>~0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Peaks</td>
<td>0.86</td>
<td>0.001</td>
<td>0.94*</td>
<td>0.62*</td>
</tr>
</tbody>
</table>

* cut-off -1.20

Discussion

In this study we found a significant difference in protein profiles during postoperative delirium following orthopaedic surgery for hip fracture in elderly patients as compared to patients who underwent the same operation but did not become delirious after the operation. We found a significant difference in three related peaks, which we identified as hemoglobin-β, its glycosylated form and the doubly charged ion of the unmodified protein. This identification however, is still not absolutely certain because it is only based on the spectrum as such. Collision induced fragmentation followed by ms/ms sequencing is not an option with proteins of this size, so we tried to obtain independent confirmation of our identification with the aid of an immunodepletion approach. However, the antibodies available did not allow us to get detectable levels of precipitation. Still we are quite convinced that the peaks indeed represent (forms of) hemoglobin-β, due to the mass value observed, its abundance in plasma, and its efficient glycosylation, characteristic of hemoglobin subunits.

Both groups did not significantly differ on risk factors for delirium except for the number of medications taken before admission. Although prior functional and cognitive impairment and fracture characteristics, anesthesia, and type of surgery are not completely similar between groups with and without delirium, these differences were not significant. In this respect we think it is unlikely that the observed difference in SELDI patterns is related to risk factors for developing delirium [26]. In our study population, the somatic trigger (a hip fracture combined with surgery) leading to delirium was equal for all patients. In the complete cohort the fraction of patients with delirium (34%) was in line with the expectation based on the literature (16-62%). Moreover, both groups received treatment irrespective whether they became delirious or not, thus a relation between the observed differences in protein profile and peri-operative medication or surgery is highly unlikely. There were more patients in the delirium group with blood transfusion, as anaemia is one of the most important complications of surgery precipitating delirium. Yet, after excluding all patients with a blood transfusion, the SELDI pattern still showed significant differences. All patients with delirium were treated with haloperidol, so hemoglobin-β differences could in theory be related to this treatment and not to the underlying disorder. Haloperidol attaches to the D2 dopamine receptors in the brain, so a direct pathophysiological link with hemoglobin levels is hard to envisage. The blood
samples used were not from the same postoperative day for all patients, since patients did not become delirious at the same postoperative time-point and venapunction was only possible during weekdays. However, since there was no significant difference in relative collection time between patients with and without delirium and no correlation between time of collection and peak intensity was observed, this is unlikely to have influenced the results.

Previously, some different proteins related to delirium have been described [10-12]. Based on their molecular weights (MW), we excluded that the observed differences in protein profiles could be due to one of these proteins; S-100 β protein has a MW of 10.7 kDa, and MW’s of Tau-protein and beta-endorphin are above the studied maximum weight of 20 kDa.

Hemoglobin consists of four firmly attached polypeptide chains, each with a heme-group. Hemoglobin A, the predominant hemoglobin in adults, has α₂β₂ subunit structure. Free hemoglobin-β is usually not present in plasma and it is only possible to detect small amounts in hemolysis.

One would expect comparable intensities for hemoglobin-α (with an estimated mass of 15.1 kDa) and hemoglobin-β, since these are patients without known β-thalassemia. Possible reasons for the absence of a peak representing hemoglobin-α could be the breakdown of hemoglobin-α during sample preparation and/or inefficient ionization. Although we do not understand the role of elevated hemoglobin-β in patients with a delirium, a relation between delirium and low hemoglobin has been described [27]. However, in our study comparable hemoglobin levels were found in patients with and without delirium. No correlation was found between the intensity of the hemoglobin-β peak and hemoglobin blood levels. Patients with indicators of oxidative dysfunction developed delirium more frequently, and this was not linked to illness severity [28,29]. The relation between oxidative dysfunction and low hemoglobin is established, but a specific role for hemoglobin-β in this relation is difficult to explain with our current knowledge.

Hemoglobin-β has recently been discovered as a biomarker for early diagnosis of ovarian cancer [30,31]. This was ascribed to increased susceptibility of erythrocyte membrane to hemolysis due to biochemical modifications in women with ovarian cancer. If this biomarker would reflect common stress factors, it explains the higher intensity in our hip fractured patients with delirium as compared to without delirium. Hemolytic parameters as such were not determined, but some hemolysis may have occurred during venapunction. This may have happened especially in delirious patients since the punction is more difficult in these patients due to restlessness. We doubt that this can explain the increased levels of hemoglobin-β as they are still elevated in the same group post delirium.

Based on the literature, we expected to find differences in expression of cytokines [9]. This SELDI analysis may not reveal all differences, since only a selection of ProteinChip® Arrays is used and mainly molecular masses below 20 kDa are detected. In addition, the identification of other differences between both groups could be hampered by the small sample size of the study population, the relative amount present of possible differences in protein expression as well as their ionization efficiency. Finally, delirium is a
predominantly cerebral process and it probably would have been better to sample cerebrospinal fluid or even brain tissue, but this was not feasible with elderly (delirious) patients. We could have missed cerebral proteins that could not pass the blood-brain barrier.

Poor reproducibility of proteomics due to the combination of detection sensitivity, large impact of impurities and lack of methodological rigour is one of the difficulties that must be overcome in order for proteomic technology to become a robust tool. Results obtained so far in the search for biomarkers for cancer have been often disappointing as this technique still has difficulties to detect low-abundant plasma and serum proteins. Despite the relatively high sensitivity of 0.94 and independent validation with a second group, the diagnostic potential of the candidate biomarker hemoglobin-β should be tested further. For this purpose a rapid assay measuring hemoglobin-β concentration should be developed. Moreover, bootstrapping or comparable techniques should be applied to improve accuracy of this candidate diagnostic tool.

Given the small sample size of the total pilot group, analysis of a larger group is warranted. The discriminative value of the 15.9 kDa protein in patients with and without delirium could thus be more firmly established.

Acknowledgements

The authors wish to thank the research nurses, A.W.J. Giesbers, J.L. Popma, C.M.M. van Rijn, A.C.L. Scheffer and M.J.A. van der Zwaan and the geriatric physician J.L. Parlevliet for collecting data.

References

Protein profiling of plasma and serum of patients with delirium


