Cerebrospinal fluid markers of extracellular matrix remodelling, synaptic plasticity and neuroinflammation before and after cranial radiotherapy

E. Fernström1,†, K. Minta2,†, U. Andreasson2,4, Å. Sandelius2, P. Wasling3, A. Brinkmalm2,4, K. Höglund2,4, K. Blennow2,4, J. Nyman1, H. Zetterberg2,4,5,6 & M. Kalm7

From the 1Department of Oncology, Institute of Clinical Sciences, Sahlgrenska Academy at the University of Gothenburg, Gothenburg; 2Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology at the Sahlgrenska Academy at the University of Gothenburg, Mölndal; 3Department of Physiology, Institute of Neuroscience and Physiology at the Sahlgrenska Academy at the University of Gothenburg; 4Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden; 5Department of Molecular Neuroscience, UCL Institute of Neurology; 6UK Dementia Research Institute at UCL, London, UK; and 7Department of Pharmacology, Institute of Neuroscience and Physiology at the Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden

Abstract. Fernström E, Minta K, Andreasson U, Sandelius A, Wasling P, Brinkmalm A, Höglund K, Blennow K, Nyman J, Zetterberg H, Kalm M (Sahlgrenska Academy at the University of Gothenburg, Gothenburg; Institute of Neuroscience and Physiology at the Sahlgrenska Academy at the University of Gothenburg, Mölndal; Institute of Neuroscience and Physiology at the Sahlgrenska Academy at the University of Gothenburg; Sahlgrenska University Hospital, Gothenburg, Sweden; UCL Institute of Neurology; UK Dementia Research Institute at UCL, London, UK). Cerebrospinal fluid markers of extracellular matrix remodelling, synaptic plasticity and neuroinflammation before and after cranial radiotherapy. J Intern Med 2018; 284: 211–225.

Background. Advances in the treatment of brain tumours have increased the number of long-term survivors, but at the cost of side effects following cranial radiotherapy ranging from neurocognitive deficits to outright tissue necrosis. At present, there are no tools reflecting the molecular mechanisms underlying such side effects, and thus no means to evaluate interventional effects after cranial radiotherapy. Therefore, fluid biomarkers are of great clinical interest.

Objective. Cerebrospinal fluid (CSF) levels of proteins involved in inflammatory signalling, synaptic plasticity and extracellular matrix (ECM) integrity were investigated following radiotherapy to the brain.

Methods. Patients with small-cell lung cancer (SCLC) eligible for prophylactic cranial irradiation (PCI) were asked to participate in the study. PCI was prescribed either as 2 Gy/fraction to a total dose of 30 Gy (limited disease) or 4 Gy/fraction to 20 Gy (extensive disease). CSF was collected by lumbar puncture at baseline, 3 months and 1 year following PCI. Protein concentrations were measured using immunobased assays or mass spectrometry.

Results. The inflammatory markers IL-15, IL-16 and MCP-1/CCL2 were elevated in CSF 3 months following PCI compared to baseline. The plasticity marker GAP-43 was elevated 3 months following PCI, and the same trend was seen for SNAP-25, but not for SYT1. The investigated ECM proteins, brevican and neurocan, showed a decline following PCI. There was a strong correlation between the progressive decline of soluble APPα and brevican levels.

Conclusion. To our knowledge, this is the first time ECM-related proteins have been shown to be affected by cranial radiotherapy in patients with cancer. These findings may help us to get a better understanding of the mechanisms behind side effects following radiotherapy.

Keywords: cranial irradiation, CSF biomarkers, neuronal damage, small-cell lung cancer.

†Equal contribution
Introduction

Advances in the treatment of malignant brain tumours in children and adults have significantly increased the number of long-term survivors. Current treatment alternatives often include surgery, chemotherapy and radiotherapy, or most commonly a combination of all three. The price for survival is unfortunately high. Clinical side effects range from neurocognitive deficits to outright tissue necrosis. Various neurologic or neuropsychological adverse effects have been described after radiotherapy of many cancer types, including glioma [1], brain metastases [2], head and neck cancer [3], as well as paediatric tumours [4]. The severity of cognitive deficits ranges from mild or moderate cognitive deficits all the way to dementia. Despite improvements in radiotherapy delivery techniques, the healthy brain tissue is still exposed to various doses of incidental irradiation.

Small-cell lung cancer (SCLC) is a subtype of lung cancer with a high propensity for brain metastasis. There is evidence that prophylactic cranial irradiation (PCI) of the entire brain can prolong overall survival in patients with a favourable response to thoracic radiotherapy with concomitant chemotherapy who are free from brain metastasis at evaluation [5], and also in patients with extensive disease with response to chemotherapy [6]. Whole-brain radiotherapy at similar doses, as given to the SCLC patients, has shown to have adverse effects on cognitive performance, primarily learning and memory [7]. In a previous study from our group, neurochemical alterations in cerebrospinal fluid (CSF) were investigated in this group of patients with focus on neuronal degeneration and glial activation. For example, concentrations of the neuronal injury marker neurofilament light (NFL) were highly elevated in CSF 3 months after prophylactic radiotherapy to the brain of SCLC patients [8]. In frontotemporal dementia, the concentrations of NFL have been correlated with longitudinal changes in brain volumes and cognitive functioning [9]. Other markers that were highly affected following PCI were the peptides of amyloid precursor protein (APP), sAPPα and sAPPβ, but also Aβ38–42 [8]. They all showed a progressive decline following PCI, suggesting that radiotherapy affects the precursor protein in a general manner. APP, which is a key protein in Alzheimer’s disease, also plays a role in synaptogenesis and synaptic plasticity [10]. The function of the peptides is highly depending on what enzyme that cleaves APP and generates peptides as some are neurotoxic (amyloid β fragments). The peptides are divided into two different pathways, the amyloidogenic pathway (sAPPβ and Aβ38–42) and the nonamyloidogenic pathway (sAPPα). Interestingly, it has been shown that the secreted form of sAPPα has positive effects on neuronal proliferation, differentiation and synapse formation [11]. The CSF levels of sAPPα decreased by 44% 3 months after PCI. One year after cranial radiotherapy, the levels had decreased even further to 54% compared to baseline levels. The involvement of APP in the progressive decline in cognition that patient’s experience following cranial radiotherapy needs to be further investigated. The use of biomarkers to evaluate disease progression and interventional effects during and after cranial radiotherapy is of great clinical interest. SCLC patients who receive PCI do not have a tumour in the brain, which means that alterations seen in the CSF would reflect radiation effects rather than tumour-induced damage. With the help from this group of patients, we can increase the knowledge of the mechanisms behind radiation-induced neurotoxicity.

The mechanisms behind the long-term side effects are partly known and were recently reviewed by Makale et al. [12]. Loss of oligodendrocytes or neuronal precursor cells, inflammation and altered signalling are some examples of negative effects seen after cranial radiotherapy. Cortical thinning and a smaller hippocampus are anatomical effects seen in patients following cranial radiotherapy [13, 14]. The cause of learning difficulties after cranial radiotherapy is believed to partly depend on the injury seen in the neurogenic areas, particularly the subgranular zone of the dentate gyrus in the hippocampus [15]. However, little is known about how the extracellular matrix (ECM) is involved in the irradiation-induced injury. ECM is a scaffold in extracellular space of the central nervous system (CNS), which does not only play a structural role in the brain. It is also involved in regulating both cell migration and synaptic formation, which are two functions that are fundamental for brain plasticity [16]. The part of ECM that is tightly surrounding the soma and the dendrites of specific neurons are referred to as the perineuronal nets (PNN) [17]. The PNN has been suggested to be important for the control of plasticity by providing a suitable microenvironment for highly active neurons [18]. However, for synaptic plasticity to occur, the ECM and PNN need to be degraded, as they create a physical barrier for diffusion of synaptic molecules, hence blocking plasticity and axon regeneration [19].
PNNs are composed of hyaluronic acid, link proteins, tenasin and chondroitin sulphate proteoglycans (CSPGs), the most abundant proteoglycan family in the CNS [18]. Two CNS-specific CSPGs have been identified: brevican and neurocan. They play an important role in cell proliferation, differentiation, migration, neurite outgrowth and synaptogenesis [20]. Emerging evidence suggests the involvement of PNNs and related ECM proteins, as well as matrix metalloproteinases (MMPs), in the pathophysiology of several neurologic and psychiatric disorders, such as schizophrenia, Alzheimer’s disease, stroke, epilepsy, autism and drug addiction [21, 22].

As mentioned, the PNN is highly involved in plasticity and repair mechanisms and it is known that neuronal injury is measurable following PCI, which should be associated with or followed by activation of such protective mechanisms. Cognition is often negatively affected following radiotherapy towards the brain, and so is plasticity. However, it is not known whether alterations in markers reflecting plasticity are measurable in CSF. SNAP-25 (synaptosomal-associated protein of 25 kDa) and SYT1 (synaptotagmin-1) are two synaptic proteins, and both are part of or interacting with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex [23]. SNAP-25 is a well-known controller of exo/endocytic processes at the presynaptic terminal, but it also has a postsynaptic role that includes receptor trafficking, spine morphogenesis and plasticity [24]. The role of SNAP-25 in pathology is not clear, but it has been shown that acute postsynaptic downregulation of SNAP-25 causes long-term potentiation (LTP) impairment [25]. In CSF from patients with Alzheimer’s disease, the levels of SNAP-25 are increased already at an early stage of the disease, which is thought to reflect synaptic degeneration [26]. Further, SYT1 is located in the synapse and is a Ca²⁺-binding protein that regulates synaptic vesicle exocytosis. It has been shown that SYT1 regulates the formation of axonal filopodia and branching before becoming a functional synapse [27]. Another interesting marker for plasticity is GAP-43 (growth-associated protein 43 kDa). GAP-43 is found in axonal growth cones and immature synaptic terminals [28, 29]. Low levels of GAP-43 have been detected in severe brain atrophy, but also in neurodegenerative disorders involving movement disorders, such as Parkinson’s disease and neuropathy [30, 31]. In contrast, GAP-43 is increased in CSF in patients with Alzheimer’s disease [32]. Another marker that is related to plasticity and regeneration is neurogranin. Neurogranin is a postsynaptic protein and is localized in the dendritic spines, and is involved in the enhancement of synaptic strength [33, 34]. The levels of neurogranin in CSF from patients with Alzheimer’s disease are also increased compared with healthy controls [35]. SNAP-25, SYT1, GAP-43 and neurogranin were therefore chosen as candidates to measure alterations in plasticity following PCI.

Remodelling of PNN and ECM following cranial irradiation is a largely uninvestigated field, despite the abundance of these proteins in the brain. In this study, the aim was to investigate proteins involved in inflammatory signalling, synaptic plasticity and ECM integrity in CSF following PCI. The markers used in this study were measured with the methods giving the best analytical sensitivity and specificity to date. Therefore, different approaches had to be used within the study, for example, in-house ELISA for GAP-43 and neurogranin, and an in-house immunoprecipitation-mass spectrometry assay for SNAP-25 and SYT1. The inflammatory and ECM markers were possible to quantify using commercially available kits.

**Methods**

**Experimental outline**

The study was approved by the regional ethics committee (194-7, year 2007), Gothenburg, Sweden. Prior to PCI, each patient underwent magnetic resonance imaging (MRI) of the brain, lumbar puncture and Mini-Mental State Examination (MMSE) of cognitive function [36]. These examinations were repeated at approximately 3 and 12 months post-radiotherapy, respectively. MRI was performed as a general cerebral and cerebellar screening for possible neurological pathological findings, that is, brain metastases.

**Patients**

This cohort of patients, as well as inclusion and exclusion criteria, has been described earlier [8]. During the period from January 2010 to November 2011, patients from the Western region of Sweden with histologically confirmed SCLC, who were eligible for PCI after thoracic chemoradiotherapy or chemotherapy, were reviewed for potential inclusion in the study.

Twenty-two patients with complete or partial response to this treatment were referred to the Department of
Oncology at Sahlgrenska University Hospital for PCI. Eighteen of these patients were included in the study. Due to withdrawn consent (4 patients) and inability to undergo lumbar puncture (3 patients), 11 patients were ultimately left for baseline assessment. Patient characteristics are shown in Table 1. One patient underwent lumbar puncture before the MRI report showing initial brain metastases, and three patients were diagnosed with brain metastases at follow-up. These patients are reported separately.

Cranial radiation therapy

Following baseline registration, radiotherapy was prescribed either as 2 Gy/fraction to a total dose of 30 Gy to patients with limited disease or 4 Gy/fraction to 20 Gy if extensive disease was present. Treatment was planned with conformal 3D dose planning with the whole brain as planning target volume (PTV) and delivered by 6 MV photons on a linear accelerator from Varian Medical Systems™.

Controls

Nine controls were age-matched neurologically healthy individuals who underwent lumbar puncture in preparation for spinal anaesthesia in preparation for knee surgery at the Sahlgrenska University Hospital (Table 1).

CSF collection and biochemical measurements

CSF was collected by lumbar puncture (LP) in the L3/L4 interspace. Ten to twelve millilitres of CSF was collected in polypropylene tubes to avoid adsorption of proteins to the test tube wall, and aliquoted and stored at −80°C pending analysis. Serum was collected at the same time by venipuncture. Albumin levels in serum and CSF were measured by immunonephelometry on a Beckman Image Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA). The albumin ratio was calculated as CSF albumin (mg L⁻¹)/serum albumin (g L⁻¹) and was used as a measure of the blood–brain barrier function [8].

Table 1 Demographic characteristics of participants and controls

| Characteristics | Controls | Patients without metastases | Patients with metastases |
|-----------------|----------|------------------------------|--------------------------|
| n               | 9        | 7 (5 post-PCI 3 months)     | 4 (1 post-PCI 12 months) |
| Gender (% Male) | 89 %     | 29 % (0 % post-PCI 3 months) | 25 % (0 % post-PCI 3 months) |
| Mean age at baseline | 63.1 (± 8.7) | 63.7 (± 9.3) | 72.3 (± 5.0) |

**In-house GAP-43 Sandwich ELISA NM4-ABB**

Nunc-Immuno Polysorp microwell modules (Thermo Fisher Scientific, Waltham, MA, USA) were coated with mouse anti-GAP-43 antibody (0.77 µg mL⁻¹ NM4, Fujirebio, Tokyo, Japan) in carbonate buffer pH 9.6, overnight at 4°C. After washing, wells were blocked with PBS/0.05% Tween/1xCasein (10 × Casein blocking buffer, B6429, Sigma-Aldrich, Missouri, USA) for 1 h at room temperature. Thereafter, in-house recombinant full-length GAP-43 calibrators (78–5000 pg mL⁻¹), blanks, prediluted control samples and CSF samples in assay diluent (%BSA/PBS/0.05%Tween) were co-incubated with a rabbit detector antibody (0.14 µg mL⁻¹ ABB-135, Nordic Biosite, Täby, Sweden) overnight at 4°C. After additional washes, plates were incubated with anti-rabbit HRP (1:30 000, Promega, Wisconsin, USA) for 1.5 h. After subsequent washes, wells were incubated for 20 min with 3,3',5,5'-tetramethylbenzidine (TMB-ONE, KemEnTech Diagnostics, Taastrup, Denmark) in the dark. The colour reaction was stopped by addition of 0.2 M H₂SO₄, and the absorbance was read in a Sunrise™ microplate absorbance reader (Tecan group, Männedorf, Switzerland) at 450 nm (650 nm as reference value). CSF sample concentration was calculated via interpolation from the calibrator curve (4PL weighted 1/Y²).

**Brevican and neurocan**

Brevican and neurocan ELISA kits (RayBiotech, Norcross, GA, USA) were used according to the instructions from the manufacturer. In brief, 96-well strip microwarets were precoated with monoclonal mouse anti-brevican (immunogen: aa23-911) or polyclonal sheep anti-neurocan (immunogen: aa23-1321). As a calibrator, recombinant protein was used (0.041–10 ng mL⁻¹ for brevican and 0.41–40 ng mL⁻¹ for neurocan). CSF samples were diluted 1:400 or 1:10, for detection of brevican or neurocan, respectively. Calibrators, blanks and prediluted CSF were incubated in the
CSF markers after irradiation / E. Fernström et al.

assay plate for 2.5 h. Biotinylated antibody was added and incubated for 1 h. It was followed by the incubation of HRP-streptavidin concentrate (diluted 1:200 for brevican, 1:500 for neurocan) for 45 min. Repeated washes were performed between these steps. Next, wells were incubated with TMB for 30 min in the dark. After the reaction was stopped, the absorbance was read in a Sunrise™ microplate absorbance reader (Tecan group, Männedorf, Switzerland) at 450 nm.

Inflammatory markers

The inflammatory markers were measured using V-plex kits from Meso Scale Discovery (Chemokine Panel 1 for MCP-1, cat#: K15047D-1; Cytokine Panel 1 for interleukin [IL]-15 and IL-16, cat#: K15050D-1; Proinflammatory Panel 1 for IL-6, cat#: K15049D-1). All reagents were included in the kit, and the same procedure was used for the three panels as instructed by the manufacturer. In brief, the plates were washed three times before the addition of calibrators and samples (50 μL diluted 1:1 in Diluent 43) followed by an incubation for 2 h on a plate shaker (700 rpm) at room temperature. After a wash, 25 μL of SULFO-TAG labelled detection antibodies was added followed by incubation another 2 h. Finally, the plates were washed and 150 μL of 2 × Read Buffer T added before the plates were immediately read on a QuickPlex SQ 120 (Meso Scale Discovery, Rockville, MD, USA). Data were analysed using the software Discovery Workbench 4.0 (Meso Scale Discovery, Rockville, MD, USA).

Neurogranin

An in-house ELISA was used for the neurogranin measurements, and a detailed description of the procedure has previously been published [BMC Neurol. 2017; 17:170].

In-house SNAP-25/synaptotagmin-1 IP-MS assay

The SNAP-25/synaptotagmin-1 assay consists of enrichment with immunoprecipitation (IP) followed by quantitation with liquid chromatography/tandem mass spectrometry (LC-MS/MS), see Data S1 for details. Briefly, the IP was performed on a KingFisher™ Flex System (Thermo Fisher Scientific), which uses magnetic rods to move particles through the various binding, mixing, washing and elution phases in a 96-well plate format. CSF samples (200 μL diluted in 0.0625% Tween in PBS) were incubated 90 min at 22°C with mouse monoclonal antibodies clone 41.1 (Synaptic Systems) [37] and SMI81 ( Biosite) [38] added (0.5 g L⁻¹) to IgG-coated magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG (Thermo Fisher scientific)). After washing in 0.025% Tween in PBS, PBS and 50 mM NH₄HCO₃, captured proteins were eluted with 0.5% 0.1% aqueous formic acid, and dried in a vacuum centrifuge overnight. The plate was stored at −20°C until use. Digestion was performed by reconstituting the sample in a mixture of Trypsin/Lys-C (Promega) in 50 mM NH₄HCO₃ and incubating at 37°C overnight. Each sample was subsequently transferred to LC-vials (SUN-SRi) and loaded into the LC autosampler. Stable heavy isotope-labelled standards (IS) were added to the sample before IP and at reconstitution. High-resolution parallel reaction monitoring (HR-PRM) analyses were performed on a Q Exactive quadrupole–orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 standard liquid chromatography system (Thermo Fisher Scientific). The samples (50 μL) were loaded directly onto a Hypersil GOLD HPLC C18 column (Thermo Fisher Scientific) with 0.1% aqueous formic acid at 100 μL min⁻¹. The instrument was set to acquire scheduled pairs or triplets of fragmentation scans (PRM scans) in profile mode, allowing simultaneous detection of the CSF peptide and the corresponding IS. LC-MS/MS raw files acquired with Xcalibur software version 2.2 SP1.48 (Thermo Fisher Scientific) were imported into Pinpoint software version 1.3.0 (Thermo Fisher Scientific), and peak areas of the CSF and IS peptides were generated. CSF levels of SNAP-25tot, SNAP-25aa40 and synaptotagmin-1 were calculated by multiplying the ratio of the LC-MS peak areas with the concentration of the corresponding IS.

Statistical analysis

To compare baseline levels between patient and control groups, Student’s t-test was used. To investigate changes over time for patients treated with radiotherapy, repeated-measures ANOVA with a Greenhouse–Geisser correction was used. Pairwise comparisons between time-points were performed with Bonferroni post hoc test. The 1-year time-point was not investigated for the patients with metastases due to n = 1. Therefore, paired-samples t-test was used for the patients with metastases. This statistical analysis was performed using SPSS 25.0 (SPSS, Chicago, IL, USA), with P < 0.05 considered statistically significant. Repeated-measures
correlation analysis as described by Bland and Altman [39] was used to assess the common intra-individual correlation of paired biomarker levels sampled at multiple occasions. Analysis was performed using the rmcorr package [40] in R (R Core Team 2017). Only biomarkers that displayed a significant longitudinal change were included in the correlation analysis.

Results

Baseline values of patients and controls are presented in Table 2. None of the baseline biomarker values was significantly different between the groups. Longitudinal changes for patients receiving PCI are described in Table 3. There were no changes in CSF cell counts or blood–brain barrier function, as reflected by the CSF/serum albumin ratio, during the study.

Markers for ECM

Brevican and neurocan were studied to analyse effects on ECM/PNN induced by PCI. Brevican showed a chronic decrease following PCI for patients without brain metastases ($P = 0.002$, Fig. 1a, Table 3). Three months after PCI, the levels of brevican had decreased by 32% ($P = 0.045$). One year after PCI, the levels had dropped even further by 47% compared to baseline ($P = 0.020$). Neurocan showed a similar pattern as brevican with a significant decrease over time following PCI for patients without brain metastases ($P = 0.003$, Fig. 1b). Neurocan had decreased by 27% 3 months after PCI compared to baseline ($P = 0.048$). One year following PCI, the levels were still 29% below the baseline values ($P = 0.026$). For patients with metastases, the same tendency was seen for both brevican (14% decrease) and neurocan (16% decrease) 3 months following PCI compared to baseline. However, these changes were not significant.

Plasticity and inflammatory markers

A panel of markers reflecting neuronal plasticity and dysfunction were investigated. GAP-43 was significantly altered over time for patients without metastases ($P = 0.028$, Fig. 1c). For these patients, GAP-43 increased by 49% 3 months following PCI compared to baseline ($P = 0.025$).

Table 2 Mean values for measured parameters in CSF and serum

| Biomarker                  | Control Baseline (n = 9) | SCLC Baseline (all patients, n = 11) |
|----------------------------|-------------------------|--------------------------------------|
| Mean ± SD                  |                         |                                      |
| Brevican (ng mL$^{-1}$)    | 351.9 ± 107.2           | 372.7 ± 113.9                        |
| Neurocan (ng mL$^{-1}$)    | 32.2 ± 11.3             | 28.4 ± 10.0                          |
| Neurogranin (pg mL$^{-1}$) | 235.9 ± 63.7            | 239.2 ± 81.8                         |
| GAP43 (pg mL$^{-1}$)       | 2802.6 ± 1135.7         | 2312.0 ± 1036.4                      |
| SYT1 (pmol L$^{-1}$)       | 264.4 ± 83.1            | 245.9 ± 86.2                         |
| SNAP25(b) (pmol L$^{-1}$)  | 67.5 ± 17.5             | 65.0 ± 22.3                          |
| SNAP25aa40 (pmol L$^{-1}$) | 8.4 ± 2.1               | 8.1 ± 3.4                            |
| MCP1/CCL2 (pg mL$^{-1}$)   | 492.9 ± 129.1           | 523.5 ± 241.7                        |
| IL-6 (pg mL$^{-1}$)        | 3.3 ± 5.7               | 1.4 ± 0.7                            |
| IL-15 (pg mL$^{-1}$)       | 4.6 ± 1.8               | 3.7 ± 1.6                            |
| IL-16 (pg mL$^{-1}$)       | 9.5 ± 3.3               | 9.9 ± 4.7                            |
| Erythrocytes (cells $L^{-1}$) | 28.1 ± 46.8          | 59.4 ± 161                           |
| Lymphocytes (cells $L^{-1}$) | 3.0 ± 0.0              | 5.2 ± 6.6                            |
| Monocytes (cells $L^{-1}$) | 3.0 ± 0.0               | 3.1 ± 1.1                            |
| Neutro/poly (cells $L^{-1}$) | 3.0 ± 0.0              | 2.8 ± 0.6                            |
| s-albumin (g L$^{-1}$) serum | 33.0 ± 6.6             | 36.7 ± 5.8                           |
| Albumin ratio              | 9.6 ± 6.3               | 8.6 ± 4                               |
Table 3  Protein levels in CSF during longitudinal follow-up

| Development of brain metastasis | No development of brain metastasis |
|--------------------------------|-----------------------------------|
|                                | Pre-PCI (n=5)                      |
|                                | Post-PCI (n=4)                     |
|                                | 3 months (n=5)                     |
|                                | 12 months (n=4)                    |

| CSF markers after irradiation / E. Fernström et al. | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
|----------------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Brevican (ng mL⁻¹)                                 | 354.3 ± 76.2 | 240.7 ± 41.0 | 189.1 ± 22.6 | 275.8 ± 66.2 | 369.6 ± 160.9 | 323.9 ± n.a. | 314.0 ± 100.9 | 31.4 ± n.a. |
| Neurocan (ng mL⁻¹)                                 | 186.8 ± 50.0 | 198.0 ± 54.4 | 164.8 ± 72.8 | 165.8 ± 121.8 | 275.0 ± 52.3 | 292.0 ± n.a. | 213.0 ± 55.4 | 31.4 ± n.a. |
| SNAP-25aa40 (pmol L⁻¹)                             | 5.8 ± 1.9 | 7.2 ± 2.9 | 5.9 ± 2.9 | 5.9 ± 2.9 | 10.1 ± 3.7 | 12.0 ± 2.3 | 14.1 ± n.a. | 14.1 ± n.a. |
| MCP-1/CCL2 (pg mL⁻¹)                               | 0.4 ± 0.1 | 2.7 ± 0.8 | 1.7 ± 0.5 | 1.7 ± 0.5 | 1.7 ± 0.5 | 1.7 ± 0.5 | 2.2 ± n.a. | 2.2 ± n.a. |
| IL-15 (pg mL⁻¹)                                    | 159.1 ± 20.7 | 227.6 ± 61.8 | 165.6 ± 64.5 | 165.6 ± 64.5 | 258.0 ± 121.6 | 363.4 ± 160.9 | 318.9 ± 160.9 | 318.9 ± 160.9 |
| GAP-43 (pg mL⁻¹)                                   | 62.2 ± 15.8 | 61.8 ± 15.8 | 61.8 ± 15.8 | 61.8 ± 15.8 | 64.5 ± 28.0 | 52.3 ± 29.2 | 52.3 ± 29.2 | 52.3 ± 29.2 |
| SYT1 (pmol L⁻¹)                                    | 2.7 ± 0.8 | 4.0 ± 1.2 | 3.0 ± 0.4 | 3.0 ± 0.4 | 4.1 ± 0.8 | 4.9 ± 0.9 | 5.6 ± n.a. | 5.6 ± n.a. |
| SNAP-25tot (pmol L⁻¹)                              | 3.5 ± 0.8 | 2.6 ± 0.8 | 1.9 ± 0.8 | 1.9 ± 0.8 | 2.7 ± 0.4 | 2.7 ± 0.4 | 2.7 ± 0.4 | 2.7 ± 0.4 |
| SNAP-25aa40 (pmol L⁻¹)                             | 634.8 ± 276.5 | 852.4 ± 297.4 | 580.6 ± 166.9 | 580.6 ± 166.9 | 455.4 ± 229.0 | 713.5 ± 276.6 | 514.5 ± 276.6 | 514.5 ± 276.6 |
| MCP-1/CCL2 (pg mL⁻¹)                               | 0.4 ± 0.1 | 2.7 ± 0.8 | 1.7 ± 0.5 | 1.7 ± 0.5 | 1.7 ± 0.5 | 1.7 ± 0.5 | 2.2 ± n.a. | 2.2 ± n.a. |
| IL-16 (pg mL⁻¹)                                    | 8.8 ± 2.8 | 26.6 ± 10.3 | 18.2 ± 7.1 | 18.2 ± 7.1 | 15.7 ± 6.3 | 21.7 ± n.a. | 15.7 ± 6.3 | 21.7 ± n.a. |

For patients without metastases, SNAP-25aa40 showed a tendency to increase (24%) 3 months following PCI compared to baseline (Fig. Id). The same trend was seen for SNAP-25aa40 in patients with metastases, where the increase was 18% 3 months following PCI compared to baseline. This pattern was not seen with SNAP-25tot (Fig. 1e). Neither neurogranin nor SYT1 showed any significant differences over time.

Inflammatory markers were also investigated to get a better understanding of the inflammatory signalling following PCI. Markers that were not altered following PCI are presented in Table S1. IL-15 showed a transient increase following PCI for patients without metastases (P = 0.041). The levels had increased by 47% compared to baseline at 3 months. One year following PCI, the levels were elevated with 9% (Fig. 2a). For patients with metastases, both IL-16 and monocyte chemoattractant protein-1/ C-C Motif Chemokine Ligand 2 (MCP-1/CCL2) were significantly increased 3 months following PCI compared to baseline (Fig. 2b,c). IL-16 showed an increase of 208% from baseline to 3 months following PCI (P = 0.018). The same was seen for MCP-1/CCL2 which showed an increase of 57% (P = 0.013). For patients without metastases, there was a similar pattern of a transient increase in IL-16, MCP-1/ CCL2 and IL-6 following PCI (Fig. 2b-d).

One patient had a minor stroke before the second lumbar puncture. This patient’s values were the highest for GAP-43, SNAP-25aa40, SNAP-25tot, neurogranin, neurocan and SYT1 during the study (value points are marked as ● in Figs 1 and 2). This patient was also excluded from the NFL correlation-analysis due to high levels presumably caused by other injury mechanisms. Brevican and the inflammatory data for this patient did not stand out, except for the baseline measurement of MCP-1/ CCL2 where the levels were elevated.

Correlation analysis

Visual comparison of ECM biomarkers with the longitudinal changes in biomarker levels from the previous study revealed a similarity between longitudinal changes in brevican and soluble amyloid PCI, GAP-43 was still elevated with 24% compared to baseline. The same pattern was seen for patients with metastases, where the increase was 45%, compared to baseline.
Fig. 1 Measurements of cerebrospinal fluid markers for extracellular matrix and neuronal plasticity are presented over time for patients with and without metastases following prophylactic cranial irradiation (PCI). Measured markers are brevican (a), neurocan (b), growth-associated protein 43 (GAP-43) (c), synaptosomal-associated protein 25 (SNAP-25) (d-e), neurogranin (f), synaptotagmin-1 (SYT-1). *P < .05, SCLC = small-cell lung cancer. ♦ Represent the patient with a minor stroke. Baseline (BL)
precursor protein \(\alpha\) (sAPP\(\alpha\)) levels, as well as between GAP-43 and NFL. To assess this, data on sAPP\(\alpha\) and NFL levels, as published previously [8], were included in the repeated-measures correlation analysis.

Repeated-measures correlation analysis revealed a strong common intra-individual correlation between levels of brevican and sAPP\(\alpha\) (\(r = 0.92, P < 0.001\), Fig. 3a) as well as between brevican and neurocan (\(r = 0.88, P < 0.001\), Fig. 3b). A significant correlation was also seen between GAP-43 and NFL (\(r = 0.76, P = 0.018\), Fig. 3c). A weak negative correlation was seen between brevican and GAP-43 (\(r = -0.62, P = 0.044\), Fig. 3d).

Discussion

The aim of this study was to investigate how inflammatory signalling, synaptic plasticity and ECM-related biomarkers in CSF were affected by PCI in patients with SCLC. The major findings of this study were that: (i) The investigated extracellular matrix proteins, brevican and neurocan, showed a progressive decline following PCI. (ii) The plasticity marker GAP-43 was increased by cranial radiotherapy, and a similar trend was seen for SNAP-25. (iii) Inflammatory signalling was elevated 3 months following PCI. (iv) There is a strong correlation between the progressive decline of sAPP\(\alpha\) and brevican levels in CSF. These findings can help us to get a better understanding of the mechanisms behind cognitive decline following radiotherapy to the brain.

Our group has previously investigated markers of neuronal degradation in CSF following PCI. In that study, both NFL and tau were highly elevated 3 months following PCI [8]. Both NFL and tau are well-established CSF markers for axonal injury. In the current study, a similar increase was also seen for the presynaptic protein GAP-43 [41]. GAP-43 plays an important role in axonal plasticity [42]. The increase in GAP-43 presumably reflects an attempt of regenerative axonal sprouting at a time when axonal damage is still present (corresponding
to an increase in NFL and tau). The concentrations of GAP-43 are elevated during a time when the normal tissue in the brain is assumed to be recovering from the radiotherapy. Two other synaptic markers reflecting plasticity, SNAP-25 and SYT1, were also assessed. SNAP-25 and SYT1 are

Fig. 3 Correlation analyses were performed between neuronal plasticity, neuronal injury and ECM markers. (a) Correlation between brevican and sAPPα, (b) correlation between brevican and neurocan, (c) correlation between GAP-43 and NFL, (d) correlation between GAP-43 and brevican. P and r values are presented in the figure.

Fig. 4 Irradiation-induced changes in neuronal markers are depending on cellular localization. Presynaptic (GAP-43 and SNAP-25) and axonal (NFL and tau) markers increased in CSF following prophylactic radiotherapy. Proteins involved in the structure of extracellular matrix (ECM) and perineuronal nets (PNN) decrease.
part of or interact with the SNARE complex [23] and play a key role in protein and membrane trafficking, hormone secretion and neurotransmitter release. SNAP-25 has been shown to be involved in spine maturity [24, 43]. For example, Tomasoni et al. [43] have shown that an acute reduction in SNAP-25 expression leads to an immature phenotypic of dendritic spines that are less functional. In this study, we investigated both SNAP-25tot and SNAP-25aa40. SNAP-25tot measures all soluble forms of SNAP-25, whereas SNAP-25aa40 only measures the longer soluble forms (including at least amino acid 32–40). SNAP-25tot did not show any change over time, whereas SNAP-25aa40 followed the same pattern as GAP-43, NFL and tau, but it was not significantly altered. This could be due to that SNAP-25 is expressed in both presynaptic and postsynaptic parts of the neuron, and therefore, the pattern is not as clear in CSF [24]. Nevertheless, considering the function of SNAP-25aa40 and the trend towards an increase, our data suggest a possible role in recovery after cranial irradiation. Interestingly, the postsynaptic marker neurogranin showed an entirely different pattern. It has been shown that the dendritic morphology is negatively affected, for example through disoriented sprouting and less dendritic spines, by irradiation [44–46]. Our data suggest that CSF concentrations of axonal and presynaptic markers are highly affected following PCI. Further investigations are needed to clarify the full temporal pattern of the changes.

Neuroinflammation following cranial irradiation has been thoroughly investigated in preclinical models. We have previously shown that markers for both microglial and astrocytic activation are increased following PCI [8]. In this study, we investigated the inflammatory signalling by measuring different chemokines and cytokines. IL-15 was transiently upregulated following PCI. IL-15 is pleiotropic, meaning it has proinflammatory effects [47], but it can also be anti-apoptotic and neurotropic [48]. Further, IL-16 was elevated in patients with metastases, but the same trend was seen for patients without metastases. IL-16 also has proinflammatory properties and has lately been associated with the progression of a number of different cancers [49]. For the proinflammatory chemokine CCL2/MCP-1, there was a trend towards an increase. Several studies have shown an increase in MCP1/CCL2 following cranial irradiation in young and adult rodents [50–53]. In the current study, the levels of CCL2 were measured 3 months following PCI, whereas in preclinical studies the increase has been acute and transient, indicating that the time-point in this study presumably was too late. Interestingly, it has been shown that blocking the CCL2 signalling after irradiation could attenuate the chronic microglia activation and allowed recovery of neurogenesis following irradiation [54]. This is an interesting strategy to moderate the long-term defects in neural stem cell function following cranial radiotherapy. To the best of our knowledge, this is the first attempt to measure inflammatory signalling in humans and our data support the preclinical findings.

In this study, two of the four main chondroitin sulphate proteoglycans, brevican (also called brain link protein, Bral-1) and neurocan (also called brain link protein-2, Bral-2), were investigated. Both brevican and neurocan seem to be specific to the central nervous system and have an important role in neuroplasticity and neuronal repair processes [20, 55]. They are not only key linking components of the PNNs, but they are also localized on the neuronal cell surface. Both proteins are highly expressed in the node of Ranvier [56–58]. The levels of brevican and neurocan have been observed to increase in CNS injury [59, 60] and schizophrenia [61, 62]. They might also play an important role in Alzheimer’s disease. The aggregation of amyloid beta, which is the main component of abnormal structures in Alzheimer’s disease called amyloid plaques, was observed to alter brevican’s structure and its proteolytic cleavage [63] as well as to upregulate the neurocan expression [64]. Brevican was also observed to be highly upregulated in glioma [65, 66]. Other proteins related to ECM, such as fibronectin and MMPs, have been elevated in CSF after pathological conditions in the brain. Fibronectin is a high molecular weight glycoprotein that is important for cell movement through ECM. During epilepsy, the levels of fibronectin are elevated in CSF and this has been interpreted as a sign for neurodegeneration and damage of the ECM [67]. Further, in cerebral adrenoleukodystrophy the levels of MMP increase in CSF [68]. Interestingly, several studies have illustrated an upregulation of MMP in the brain following cranial irradiation [69]. Increase in mRNA expression levels of MMP-2 and MMP-9 has been shown after whole-brain irradiation in rodents [70]. Several MMPs, including MMP-2, degrade recombinant brevican and neurocan [71, 72]. However, no or negligible digestion was observed with MMP-9 [72]. Our previous study...
showed an elevation of YKL-40, a potential marker of microglial and astrocytic activation, as well as glial fibrillary acidic protein (GFAP), which could suggest a radiation-induced activation of astrocytes, possibly leading to an increased secretion of MMP-2 and other MMPs. Radiation-induced upregulation of MMPs and subsequent degradation of ECM proteins could possibly explain the observed decreasing levels of brevican and neurocan following cranial radiotherapy. However, this transient injury response does not explain the long-term decrease in brevican and neurocan levels in our data 1 year after radiotherapy.

In this cohort of patients, we observed a progressive decline of both brevican and sAPPα and there was a strong correlation between the two markers (Fig. 3a). APP has many suggested biological functions and is commonly discussed in Alzheimer’s disease, but there is growing evidence that APP plays a role in synaptogenesis and synaptic plasticity [10, 73]. The nonamyloidogenic soluble form of APP, sAPPα, has been shown to be important for memory. One study showed improvement of hippocampal-dependent learning in old rats after administration of exogenous sAPPα [74]. Another study found increase brevican levels in hippocampus following a spatial memory task that is hippocampal-dependent [75]. In our studies, the levels of brevican and sAPPα were continuously decreasing in CSF after irradiation, which follows the clinical situation with cognitive decline and memory dysfunction. It has been shown from several groups that the hippocampus is severely injured by irradiation which makes the correlation between brevican and hippocampal memory dysfunction very interesting [12, 76]. After irradiation to the developing brain, the structure and orientation of newly formed neurons in the neurogenic niche in hippocampus were disrupted [46]. Those findings in combination with the decreased levels of brevican and sAPPα suggest that the ECM and plasticity are negatively affected by irradiation. It should be noted, however, that sAPPα correlates strongly with sAPPβ [8, 77]; most of the correlations for sAPPα were also seen for sAPPβ, although less strongly. As mentioned in the introduction, these peptides belong to different pathways, but the decline indicates that there could be an accumulation of full-length APP in the brain tissue, hence less peptides in the CSF. Another explanation for the decline could be that the clearance of the peptides from CSF has been altered. It is known that Aβ is eliminated from the brain by numerous mechanisms, such as enzymatic degradation, absorption into the blood and perivascular lymphatic drainage [78]. The blood–brain barrier, measured as albumin ratio between blood and CSF, is not affected in this cohort of patients [8]. However, one cannot exclude that the lymphatic clearance of APP, brevican and neurocan from CSF may have been affected following PCI. The concentrations of the other investigated markers, for example, GAP-43, did, however, dropped back to the baseline levels 1 year after PCI, which would argue against a chronic change in CSF clearance. Nevertheless, further investigations are needed to clarify why there is a progressive decline in brevican and neurocan following PCI.

In summary, brevican and neurocan downregulation suggests long-term structural remodelling of the ECM after radiotherapy to the adult human brain. This would be consistent with our previous findings regarding axonal degradation, glial activation and decrease in sAPPα. Further investigations are needed to evaluate the use of GAP-43, NFL, or tau, as biomarkers for radiation-induced brain injury and how they are related to tumour activity and treatment effects.

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Conflict of interest statement

KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. The other authors declare that they have no conflict of interest.

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Correspondence: Marie Kalm, PhD, The Sahlgrenska Academy at the University of Gothenburg, Institute of Neuroscience and Physiology, Department of Pharmacology, S-405 30, Gothenburg, Sweden. (fax: +4631821795; e-mail: marie.kalm@gu.se).

Supporting Information
Additional supplemental material may be found online in the Supporting Information section at the end of the article.

Data S1. In house SNAP-25/Synaptotagmin-1 IP-MS assay.

Table S1. Protein levels in CSF during longitudinal follow-up (inflammation).