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Chemokine associations with blood cerebrospinal fluid (CSF) barrier permeability and delirium

Paul Denver [a,1], Lucas Tortorelli [a,1], Karen Hov [b], Jens Petter Berg [c], Lasse M. Giil [d], Arshed Nazmi [a], Ana Lopez-Rodriguez [a], Daire Healy [a], Carol Murray [a], Robyn Barry [a], Leiv Otto Watne [b,c,1], Colm Cunningham [a,*,1]

[a] School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute & Trinity College Institute of Neuroscience, Trinity College Dublin, Pearse Street, Dublin 2, Ireland
[b] Oslo Delirium Research Group, Department of Geriatric Medicine, Akershus University Hospital, Lørenskog, Norway
[c] Institute of Clinical Medicine, University of Oslo, Norway
[d] Department of Clinical Science, University of Bergen, 5020, Bergen, Norway

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ABSTRACT

Delirium is a highly prevalent neuropsychiatric syndrome characterised by acute and fluctuating impairments in attention and cognition. Mechanisms driving delirium are poorly understood but it has been suggested that blood cytokines and chemokines cross the blood brain barrier during delirium, directly impairing brain function. It is not known whether these molecules reach higher brain levels when the blood cerebrospinal fluid barrier (BCSFB) is impaired. Here, in human hip-fracture patients, we tested the influence of BCSFB integrity on CSF levels of chemokines and assessed their association with delirium. CSF levels of IP-10, eotaxin, eotaxin 3 and TARC showed weak to moderate correlations with BCSFB permeability, as measured by the $Q_{albumin}$ ratio, while MCP1, IL-8, MIP1α and MIP1β showed no significant correlation. Chemokines were not associated with delirium in univariate analysis or when stratified on dementia status, but exploratory analyses showed that elevated Eotaxin (CCL11) and MIP1α (CCL3) were associated with prevalent delirium. Modelling acute systemic inflammation, we used bacterial LPS (250 μg/kg) or sterile laparotomy surgery in mice to demonstrate *de novo* synthesis of chemokines at the choroid plexus (CP) and microvasculature. Gene expression data showed CP-enriched expression of *Il1b, Tnfa, Cxcl1* and *Ccl3* in both models and immunohistochemistry showed cytokine and chemokine synthesis in CP stromal (IL-1β, CCL2/MCP1) or epithelial cells (CXCL10/IP-10) cells and at the microvasculature. Larger studies are required to confirm these human findings on chemokine associations with BCSFB permeability and prevalent delirium. Preclinical studies are warranted to determine whether chemokines might play a role in the pathophysiology of delirium.

1. Introduction

Delirium is a highly prevalent neuropsychiatric syndrome characterised by acute and fluctuating impairments in attention among other cognitive functions (Inouye et al., 2014; Wilson et al., 2020). Despite its importance in acute medicine, causing significant distress, extending hospitalisation and increasing risk for long-term cognitive decline (Fong et al., 2009; Witlox et al., 2010; Davis et al., 2012), our understanding of delirium pathophysiology remains limited. Acute medical illness, surgical procedures or inflammatory trauma are triggers of delirium, but their capacity to produce this disruption of brain function is clearly increased by previous cognitive frailty (Schor et al., 1992; Oh et al., 2015) and this risk increases progressively with worsening underlying neurodegenerative pathology and baseline cognitive function (Davis et al., 2015). Mouse models have indicated a role for neuroinflammation in delirium but although several blood and CSF biomarker studies may support a role for acute inflammation in inducing delirium (Hall et al., 2018), key causal mechanisms for inflammation-induced delirium are not understood.

The interaction between inflammatory mediators in the systemic

* Corresponding author.
E-mail address: colm.cunningham@tcd.ie (C. Cunningham).
[1] These authors contributed equally.

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circulation and the brain in producing acute cognitive dysfunction are key events to understand. The cerebrospinal fluid (CSF) is a key interface between these two compartments and a number of inflammatory mediators are elevated in this brain fluid during systemic inflammation. The cytokines/chemokines IL-8 (MacLullich et al., 2011; Sajjad et al., 2020), IL-1β (Cape et al., 2014), IL-6 and IP-10 (Hirsch et al., 2016) are elevated, or suppressed (Westhoff et al., 2013), following hip fractures and are associated with perioperative delirium. However studies remain small and significant gaps in our knowledge exist as to production of inflammatory molecules in the blood and the brain in these patients.

In particular, cytokines in the CSF, or within the brain tissue, arising from systemic inflammatory events may arise in the blood or might be synthesized by various brain cell types. Possibilities include perivascular macrophages, cells of the brain vasculature, in which junctional proteins between endothelial cells form the blood brain barrier (BBB), and cells within the CP, in which junctional proteins between epithelial cells forms the blood CSF barrier (BCSFB). The CP is of particular interest because it is a highly secretory, epithelial tissue that is suspended within the ventricles of the brain and synthesises and secretes CSF (Damkier et al., 2013). It is richly vascularised, with a permeable endothelium, and harbours multiple immune cell populations. The CP is thus a key part of the neuroimmune network mediating interactions between peripheral and brain immune responses (Cui et al., 2021), sensing circulating inflammatory mediators, responding to them and secreting inflammatory molecules into the CSF (Wolburg and Paulus, 2010). It is established that a loss of integrity of the BCSFB can allow plasma proteins to enter the CSF and that the ratio of CSF albumin to plasma albumin (Q_{alb}) informs on integrity of the BCSFB (Asgari et al., 2017). Despite the fact that the BCSFB and the BBB are structurally and molecularly distinct Qalb may also, to some extent, be regarded as a proxy for BBB impairment since the interstitial fluid of the brain tissue (ISF) exchanges with the CSF (Shetty and Zanirati, 2020). It has been proposed that blood cytokines/chemokines 'leak' into the brain interstitial fluid (ISF) via a compromised BBB, or into the CSF via a compromised BCSFB, and contribute to delirium (Marcantonio, 2012). However it is not known whether chemokines do enter the CSF in this way during delirium.

Recent studies indicate a modest association between elevated barrier permeability and delirium (Hov et al., 2016; Taylor et al., 2022; Devinney et al., 2023). However significant disruption of BCSFB was absent in most cases of delirium in a hip fracture cohort (Hov et al., 2016), while change from baseline in CSF:plasma albumin ratio (CPAR), rather than absolute CPAR, was higher in delirious patients in 2 separate mixed surgical cohorts with n = 8 and n = 26 delirious patients respectively (Taylor et al., 2022; Devinney et al., 2023). Another small study (n = 10) using repeated CSF sampling (Hirsch et al., 2016) suggested correlations between plasma and CSF levels for some chemokines while other data have shown that there was no correlation between serum and CSF IL-6 in hip fracture patients (Neerland et al., 2016). To our knowledge there are no studies that interrogate whether CSF levels of cytokines/chemokines are higher during impaired BCSFB integrity in patients with delirium.

In the current study we used human CSF, from hip-fracture patients, to assess whether the CSF concentrations of several inflammatory chemokines were associated with BCSFB permeability and examined whether the chemokines identified were associated with delirium in those patients. The choice of this panel of chemokines was a continuation of prior studies of pro-inflammatory cytokines in this cohort (Neerland et al., 2016; Sajjad et al., 2020) and was motivated by observed neuromodulatory effects of chemokines (Villeda et al., 2011; Marciniak et al., 2015; Blank et al., 2016) as well as their classical roles in attracting monocytes (CCL2/MCP1) and neutrophils (CXCL8/IL-8), which have been suggested to have roles in acute cognitive dysfunction during acute systemic inflammation (Terrando et al., 2015; Garre et al., 2017; Andonegui et al., 2018).

Since the sources of CSF cytokines/chemokines are difficult to assess in humans we proposed that mice would constitute an complimentary experimental tool in which we could demonstrate the ability of systemic inflammation, whether induced by bacterial endotoxin (lipopolysaccharide; LPS), or by sterile laparotomy surgery, to trigger *de novo* chemokine expression in cells of the BCSFB and BBB which include stromal and epithelial cells of the choroid plexus and cells of the microvasculature). We used quantitative PCR and immunohistochemistry to analyse the expression of IL-1β and multiple chemokines in the CP and cerebral vasculature of mice, to determine the localisation and, in some cases, cell types responsible for inflammatory mediator synthesis. We asked this question agnostic to the occurrence of delirium or otherwise in these mouse models, which were not designed to interrogate delirium *per se*. However, since a number of inflammatory molecules have been shown to be causal in acute cognitive deficits in animal models (Cibelli et al., 2010; Barrientos et al., 2012; Griffin et al., 2013; Blank et al., 2016; Skelly et al., 2019a) it is necessary to perform comparative studies between mice and humans where these are possible and within the acknowledged constraints of comparison across species.

Thus in the current study we assess levels of chemokines present in the CSF after hip fracture in humans, address the extent to which levels are correlated with impaired BCSFB integrity and associated with delirium and then assess sterile injury and LPS for their ability to induce similar chemokines in the choroid plexus in mice. In so doing we aimed to contribute information about pathways by which systemic inflammation may elevate levels of cytokines and chemokines in the CSF. Some constraints and limitations arise from human versus mouse comparisons, but the data add to our knowledge on the relationship between BCSFB integrity and CSF chemokine concentrations during delirium in humans.

2. Methods

2.1. Ethics

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

2.2. Sampling of human CSF and assessment of delirium and dementia

Patients were recruited from the Oslo Orthogeriatric Trial (OOT), a randomized controlled trial evaluating the effect of an orthogeriatric service on delirium associated with long-term cognitive decline. OOT included patients with a hip fracture admitted to Oslo University Hospital from 2009 to 2012. A secondary aim of that study was to investigate delirium pathophysiology. For this purpose, CSF was collected at the onset of spinal anaesthesia. All CSF samples available from OOT (n = 116) were included in the current study without prior calculations of statistical power. For further details see the previous publications (Wyller et al., 2012; Watne et al., 2014b). Delirium was assessed in every patient at the bedside upon admission to hospital and daily thereafter, until the fifth postoperative day or discharge, using the Confusion Assessment Method (CAM (Inouye et al., 1990);). Many patients had delirium before surgery (prevalent, n = 41), some developed it after surgery (incident, n = 17), while almost half of all patients did not develop delirium at all (n = 52). Four patients with delirium were not designated as prevalent or incident. These were included in delirium Y/N analyses but omitted from prevalent delirium vs no delirium analyses. Collection of CSF before surgery allowed analyses of association of current chemokine levels with prevalent delirium, which is useful in that it allows analysis of delirium in the absence of any influence of surgery or anaesthesia. Pre-fracture dementia status was determined by expert consensus, based on all available information, including the Informant

Questionnaire on Cognitive Decline in the Elderly (IQ-CODE) (Jorm, 2004), as described previously (Watne et al., 2014a). CSF was collected immediately before surgical repair of the hip fracture, at the onset of spinal anaesthesia, and stored at −80 °C in polypropylene tubes, pending biochemical analyses.

2.3. Determination of chemokines in human CSF

CSF concentrations of the inflammatory chemokines IL-8, MCP1, Eotaxin, Eotaxin 3, MIP1 alpha, MIP1 beta, TARC, IP-10, MDC and MCP4 were analysed using a Mesoscale Discovery (MSD) human chemokines V-plex immunoassay (K15047D-1). All analyses were performed according to manufacturer's instructions by researchers blinded to clinical information. MDC and MCP4 were detected in <30% of samples and were not pursued further, MIP1α was detected in 69% of samples and all other chemokines were detected in >89% of samples. 2 samples in which ≥4 of the analysed chemokines were undetectable, were excluded from further analysis. Thus data are reported for 114 patients in total. Where specific chemokines were undetectable, in samples that showed otherwise robust levels of most chemokines, a concentration of 50% of the lowest detected value was imputed according to conservative practice for dealing with measurements below the detection limit (Giskeodegard and Lydersen, 2022). Qalb values were obtained directly from prior published work on this cohort, in which these were calculated from blood and CSF albumin determinations (Hov et al., 2016). The full method is included in supplementary methods. Blood samples and CSF samples were not typically collected at the same time and may, in some cases, be > 24 h apart. This is a limitation of the study. However the original study demonstrates that the CSF albumin concentration is the major determinant of the Qalb value and that there is no statistically significant relationship between serum albumin concentration and the time gap between serum and CSF collection (suppl. files 1 and 2 in Hov et al., 2016).

2.4. Animals

All animal experiments were performed under licence from the Irish national regulatory body for experimentation with live animals (Health Products Regulatory Authority) after ethical review by the TCD Animal research ethics committee. Experimental design and conduct is in accordance with the ARRIVE guidelines. For LPS experiments we used female C57BL6J mice (3–6 months) since the human patient cohort was predominantly (73%) female. Animals were housed in groups of 4 or 5, at 21 ± 2 °C on 12h light/dark cycle with food and water ad libitum. Power calculations using G*Power software determined that n = 7 is sufficient to demonstrate an effect size of 0.8 with >80% power in these chemokine analyses. Animals were challenged with LPS (*Salmonella equine abortus*, Cat.#L5886; Sigma) at 250 μg/kg intraperitoneally (i.p.) and, 3 h later, hippocampi, choroid plexus (CP; from lateral and 4th ventricles) and whole blood was collected direct from the right atrium before rapid perfusion with ice cold heparinised saline. This mouse paradigm was not intended to represent a model of delirium. Rather it provides a standardised systemic inflammatory stimulus, the dose and timing of which produce robust elevations of blood cytokines and chemokines and a transient production of neuroinflammatory transcripts (Skelly et al., 2013). Further animals were similarly challenged but perfused with 10% neutral-buffered formalin at 3 h post-LPS before post-fixing overnight and paraffin wax embedding.

To provide an animal model relevant to inflammatory trauma we performed laparotomy surgery in an additional cohort of C57BL6 mice, aged 2–5 months. In line with the growing expectation of performing sex-balanced studies we included both sexes in our sterile surgery model. These mice were anaesthetised using ketamine:xylazine (100 mg/kg:10 mg/kg i.p.). One group were subjected to a laparotomy (Lap), whereby they were placed in a supine position on a sterile surgical drape, with a heating pad maintained at 37 °C throughout the procedure. The lower abdomen was shaved and lidocaine ointment applied to the skin. A midline skin incision was made with sharp fine scissors and extended to a length of 1–2 cm, without perforating the underlying peritoneal membrane. A subcutaneous cavity was made by inserting closed scissors between the skin and peritoneal membrane and opening the scissors and fine forceps were used to tent the peritoneal membrane allowing another incision to be made along the fascia of the linea alba. The cecum was exteriorized and then carefully returned to the abdominal cavity, before incisions in the peritoneal cavity and skin were closed with 4-0 silk sutures and surgical adhesive and lidocaine ointment were applied to the incision site and the mouse placed in a heated chamber for recovery. No surgery controls (NS) were anaesthetised and then allowed to recover without any surgical procedure. All mice received post-operative buprenorphine (0.05 mg/kg) for analgesia and 500 μl of warmed saline (0.9% s.c.) for fluid resuscitation. These animals were euthanised at 24 h and brain tissue and choroid plexus were removed for RNA isolation and PCR analysis.

2.5. RNA isolation, cDNA synthesis and quantitative PCR

We performed PCR on RNA isolated from CP and parenchymal tissue from the hippocampus. Total RNA was isolated using the RNeasy Plus Mini method (Qiagen, Limburg, Netherlands) following the manufacturer's instructions. To ensure complete DNA elimination from the column-bound RNA, an on-column DNase step was performed. The RNA yield and quality of each sample were quantified based on Optical Density (OD) using the 'NanoDrop' ND-1000 UV–vis spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). Primer and probe sets were designed using Applied Biosystems Primer Express software and amplified a single sequence of the correct amplicon size, as verified by SDS-PAGE. Where no probe sequence is shown, the DNA binding dye SYBR green was used in its place. Pre-made primers were also used and run following the manufacturer's instructions (Thermo Fisher Scientific). Primer pair/probe sequences are shown in Table 1.

Samples for RT-PCR were run in duplicate using FAM/SYBR (Roche) in a StepOne Real-Time PCR system (Applied Biosystems, Warrington, UK) under the cycling conditions: 95 °C for 10 min followed by 95 °C for 10 s and 60 °C for 30 s for 40–45 cycles. Quantification was achieved by exploiting the relative quantitation method, using cDNA from LPS-injected mouse brain as a standard expressing all genes of interest and serial 1 in 4 dilutions of this cDNA to construct a linear standard curve relating cycle threshold (CT) values to relative concentrations. Gene expression data were normalized to the housekeeping (reference) gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold-increases with respect to WT saline-treated values for that specific tissue. GAPDH has been an acceptable reference gene, even under neuroinflammatory conditions, in prior studies (Supplementary Fig. 1).

2.6. Immunohistochemistry

We performed immunohistochemistry in fixed tissue for cytokines and chemokines at brain epithelial and endothelial surfaces. Sections were cut using a Leica rotary microtome (Laboratory Instruments and Supplies (LIS; Ashbourne) and dried overnight at 37 °C. Thereafter they were rehydrated and were labelled with antibodies against CCL2 (1:200; part 840288 of DY479 (R&D Systems), IL-1β (1:50; Peprotech 500-P51), CXCL1 (1:50; R&D AF-453-NA) and CXCL10 (1:10000; Peprotech P129). Briefly, all sections were quenched for 20 min with 1% H_2O_2/methanol, subjected to antigen retrieval using microwaving in 0.01M citrate buffer, pH 6 for 2 × 5 min, blocked for non-specific secondary antibody labelling using normal appropriate normal sera and then incubated overnight at 4 °C with the primary antibody at the dilutions indicated above. The next day, sections were incubated for 1 h with biotinylated secondary antibody (1:100, Vector). After several washes in phosphate

Table 1
PCR *Primer sequences*. Bold/Italics denote the gene name in the revised chemokine nomenclature, while the non-bold text represents older names for these chemokines. Where no probe sequence is shown, SYBR green dye was used. For pre-made primers, assay ID is provided. Forward (F), reverse (R), probe (P).

Gene	Access. num.	BP	Sequence
Ccl2 MCP1	NM_011333	81	F: 5′GTTGGCTCAGCCAGATGCA3′ R: 5′AGCCTACTCATTGGGATCATCTTG3′ P: 5′TAACGCCCCACTCACCTGCTGCTACT3′
Ccl3 MIP1α	NM_011337.2	103	F: 5′CCCAGCCAGGTGTCATTTTCC3′ R: 5′GCATTCAGTTCCAGGTCAGTG3′
Ccl4 MIP1β	NM_013652.2	65	F: 5′CTCAGCCCTGATGCTTCTCAC3’ R: 5′AGAGGGGCAGGAAATCTGAAC3′
Ccl5 RANTES	NM_013653	75	F: 5′GCAGTCGTGTTTGTCACTCGAA3′ R: 5′GATGTATTCTTGAACCCACTTCTTCTC3′
Ccl11 Eotaxin	NM_011330.3	136	F: 5′GCCCAGAGGCTGAGATCCAAGCAGT3′ R: 5′TGGGATGGAGCCTGGGTGAGCC3′
Ccl17 TARC	NM_011332.3	219	F: 5′CAAGCTCATCTGTGCAGACC3′ R: 5′CGCCTGTAGTGCATAAGAGTCC3′
Cxcl1 CINC-1/KC	NM_008176	82	F: 5′CACCCAAACCGAAGTCATAGC3′ R:5′AATTTTCTGAACCAAGGGAGCTT3′
Cxcl10 IP-10	M33266.1	127	F: 5′GCCGTCATTTTCTGCCTCAT3′ R: 5′GCTTCCCTATGGCCCTCATT3′ P: 5′TCTCGCAAGGACGGTCCGCTG3′
Il1b IL-1β	M15131	69	F: 5′GCACACCCACCCTGCA3′ R: 5′ACCGCTTTTCCATCTTCTTCTT3′ P: 5′TGGAGAGTCTGGATCCCAAGCAATACCC3′
Tnfa TNFα	M11731	149	F: 5′CTCCAGGCGGTGCCTATG3′ R: 5′GGGCCATAGAACTGATGAGAGG3′ P: 5′TCAGCCTCTTCTCATTCCTGCTTGTGG3′

buffer saline (PBS), the ABC method was used (Vectastain Kit, PK6100 Vector) and the activity of peroxidase was revealed using H_2O_2 as substrate and 3,3’ diaminobenzidine as chromogen (Sigma Aldrich). Slides were counterstained using Haemotoxylin (VWR International Ltd, Dublin, Ireland), dehydrated, coverslipped, examined and photographed using an Olympus DP25 camera (Mason) mounted on a Leica DM3000 microscope (LIS, Ashbourne).

2.7. Statistics

All statistical analyses of human data were conducted using IBM SPSS Statistics version 29.0.2.0. Correlations between CSF chemokines and Qalb were performed using Spearman rho’s as both chemokines and Qalb followed non-normal distributions. We considered all samples by imputing samples below the minimum detectable concentration at 50% of the lowest quantifiable value. Using QQ-plots we found that both Qalb and chemokines followed log-normal distrubtions and we thus transformed the to normality using log10-transforations. Subsequently, normality was confirmed through visual inspection of Q-Q plots and histograms. Following log transformation, variables were standardized by converting them into z-scores so that the mean is zero and one standard deviations equals one. A series of linear regression models were employed to investigate the associations between each log-transformed chemokine (dependent variable) and Q-albumin (independent variable) individually. Each model was adjusted for potential confounders that were identified *a priori*, specifically age, sex, pre-fracture dementia status, blood CRP at admission and APACHE (Knaus et al., 1985) at admission. Given the multiple comparisons inherent in evaluating several chemokines, the Benjamini-Hochberg procedure was utilized to control the false discovery rate (FDR). This adjustment aimed to minimize the likelihood of type I errors across the multiple regression analyses performed. We used an FDR of 0.05 and identified the significance threshold by simple calculus. First, we sorted the p-values from highest through lowest (1–8). Second, we divided the FDR by the m number of tests (ie FDR at 0.05 was divided by 1, 2, 3 and so forth until 8). P-values were considered significant after correction if the p-values were lower than the FDR rate divided by the m number of ranked tests.

In our analyses of delirium versus chemokine levels, delirium status was defined as a binary variable (delirium anytime during hospital stay: yes/no). Most human CSF data were non-parametric and were analysed by Mann-Whitney U tests. In Univariate analysis pairwise tests were 2-tailed and were adjusted for multiple comparisons using the *Holm-Šídák correction method in order to mitigate for the possibility of type 1 errors*. As is normal practice in delirium biomarker studies, the ‘no delirium’ group included patients with subsyndromal delirium (SSD, defined as fulfilling at least two, but not all, required CAM criteria for the full syndrome). However since there is a clear basis to regard subsyndromal patients as not showing normal cognition, we also performed an exploratory analysis, which omitted patients with SSD, in order to test the hypothesis (Boettger et al., 2018) that patients with full syndromal delirium at the time of CSF sampling (i.e. prevalent delirium) would show elevated CSF chemokines with respect to patients who never devoloped any signs of delirium. Those data were analysed by Mann-Whitney U tests and not corrected for multiple comparisons. Data on mouse chemokine expression were analysed by 2-way ANOVA using treatment and brain region as independent factors, followed by Bonferroni post-hoc test for selected pairwise comparisons. All animal group sizes were established by power calculations performed in G*Power software statistical using α-error probability of 0.05 and statistical power of 80%.

3. Results

3.1. Influence of BCSFB integrity on human CSF chemokine levels

We addressed the extent to which a compromised BCSFB, defined by the appearance of blood albumin in the CSF (as measured by the Qalb), predicts the magnitude of CSF chemokine concentration. This analysis was performed with no attention to delirium status and no attention to dementia status and was performed solely to assess whether CSF concentrations in the entire patient cohort were dependent on BCSFB integrity. Historically BCSFB leakiness has been inferred from a Qalb value > 10.2 (Blennow et al., 1993), however the reference ranges for Qalb are now recognized to differ by age (Frolich et al., 1991; Skillback et al., 2017; Castellazzi et al., 2020). Therefore, we considered Qalb as a continuous variable and plotted correlations of CSF chemokine concentration with Qalb. Spearman’s rho values are shown and data are interpolated where significant associations ($p<0.05$) were found (Fig. 1). However since both Qalb and chemokine concentrations may be influenced by multiple confounders, we then performed regression

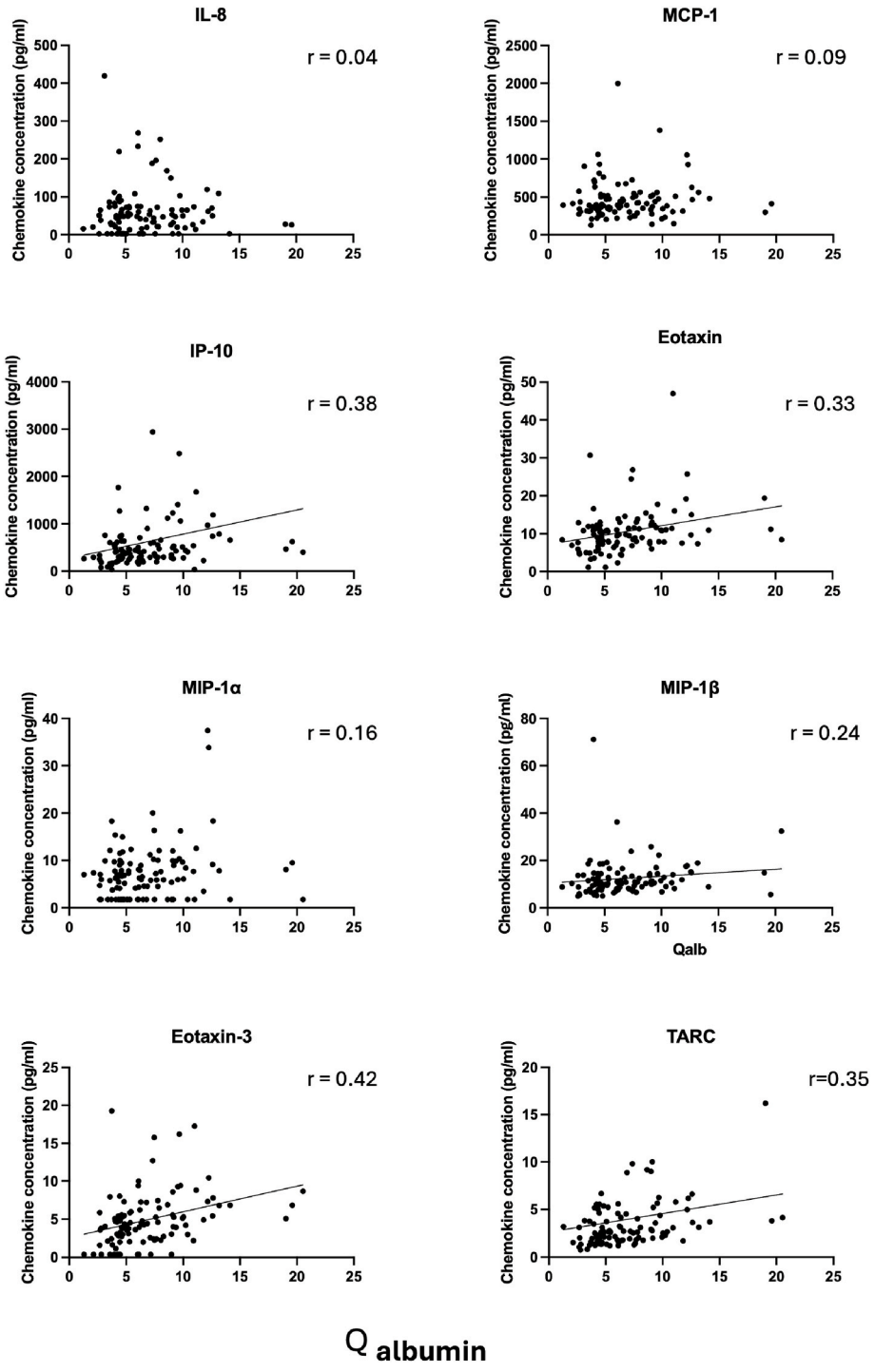

Fig. 1. Correlation plots for the relationship between Qalb and CSF chemokines. Correlations between CSF chemokine levels and the Qalb value (to assess the impact of BCSFB permeability) were analysed by regression analysis and Spearman's correlations are shown. Data are interpolated only where correlation was statistically significant. There were 3 samples non-detected for Eotaxin, 19 non-detected for IL-8, 31 non-detected for MIP-1α, 14 non-detected for eotaxin 3, 2 non-detected for IP-10 and none missing for the remaining chemokines (total n = 114). In both statistical analysis and graphical depiction, non-detected samples were imputed at 50% of the lowest detected value for that chemokine.

analysis, adjusting for the *a priori* potential confounders age, sex, pre-fracture dementia status, blood CRP at admission and APACHE score at admission (Knaus et al., 1985). The Benjamini-Hochberg procedure was utilized to control the false discovery rate (FDR) given the multiple comparisons performed in evaluating several chemokines. These regression analyses are shown in Table 2 and supported no significant correlation between Qalb and IL-8, MCP1, MIP1α, or MIP1β and weak to moderate correlations for IP-10, TARC, and eotaxin and eotaxin 3. Eotaxin 3, eotaxin, TARC and IP10 were all significant, even after considering multiple comparisons (as they were below the FDR threshold).

Therefore, CSF chemokine levels of patients experiencing hip fracture-induced acute systemic inflammation show some weak to moderate correlations with impaired integrity of the BCSFB but others do not. These relationships were trivially different when analysed using a univariate analysis of chemokine concentration in those above or

Table 2
A series of linear regression models were employed to investigate the associations between Q-albumin (independent variable) and, individually, each log-transformed chemokine (dependent variable). Each model was adjusted for potential confounders, specifically age, sex, pre-fracture dementia status, blood CRP at admission and APACHE at admission. * Eotaxin 3, Eotaxin, TARC and IP 10 remained significant following adjustment for confounders and after correction for multiple comparisons using the Benjamini Hochberg correction. FDR: false discovery rate.

Chemokine	Standardized β	95 % Confidence interval for β	p-value	FDR threshold (Benjamini-Hochberg correction)
IL-8	0.11	−0.1–0.33	0.287	0.05
MIP1α	0.13	−0.08–0.35	0.224	0.025
MCP1	0.14	−0.06–0.36	0.168	0.017
MIP1β	0.18	−0.02–0.42	0.067	0.013
Eotaxin 3	0.32	0.12–0.52	0.002*	0.01
Eotaxin	0.34	0.14–0.52	<0.001*	0.008
TARC	0.35	0.18–0.59	<0.001*	0.007
IP10	0.36	0.17–0.60	<0.001*	0.006

below the widely used Qalb threshold of 10.2 (Blennow et al., 1993). Those analyses are shown in supplementary data (**Suppl. table 1).**

3.2. Association between CSF chemokines and delirium in the OOT hip fracture cohort

The significance of CSF chemokine concentrations in delirium was assessed in the same patients. Demographic and chemokine data are shown in table 3. Including prevalent and incident cases, delirium occurred in 54.4% of hip fracture patients (n = 114, median age 85, (IQR 80–89). As is typical in aged hip fracture admissions, prevalent dementia was high (78%) among those experiencing delirium, but present in only 19% of those without delirium. We hypothesised that elevated chemokines would be associated with delirium. These analyses considered subsyndromal delirium as 'no delirium' in line with most biomarker studies of delirium. Associations of pro-inflammatory cytokines (IL-1β, IL-6, IL-8) with delirium in this cohort have previously been published (Neerland et al., 2016; Sajjad et al., 2020) so they were excluded from our hypotheses here. However since we assessed 7 chemokines for association with delirium we adjusted for multiple comparisons. Although there was a general trend for chemokines to be slightly higher in delirium cases (6 out of 7 chemokines), univariate analyses, adjusted for multiple comparisons, showed no significant associations between CSF chemokine concentrations and delirium (Table 3).

Dementia is a major risk factor for delirium in hip fracture patients, explaining the very high incidence of delirium among those with dementia in the current cohort. However, when those with and without delirium were stratified on presence or absence of dementia there were no significant differences for any chemokines between patients with and without delirium after analyses were adjusted for multiple comparisons (Table 4).

3.3. Exploratory analysis of CSF chemokine levels of chemokines in prevalent delirium versus cognitively normal patients in the OOT hip fracture cohort

Subsyndromal delirium (SSD) is a confounder in biomarker studies of delirium and there is a new impetus to sub-phenotype patients where possible (Bowman et al., 2023). Since SSD patients do not meet criteria for delirium they are typically included with the 'no delirium' group (as was the case in primary analyses in the current study). However, since those patients are not cognitively normal (i.e. they meet some criteria for delirium but not all) there is a strong biological rationale to perform an exploratory analysis in which the 'no-delirium' group excludes SSD and includes only cases that are cognitively normal. Indeed, chemokine expression in normal, SSD, prevalent delirium and incident delirium (shown for illustrative purposes in Suppl. Fig. 2, do suggest that levels of some chemokines, including Eotaxin, IP-10 and MIP1α are already somewhat elevated in sub-syndromal patients. Thus SSD patients are not equivalent to 'no delirium' patients and this justifies performing an exploratory comparison of prevalent delirium with no delirium (i.e. excluding SSD). Therefore, we tested the hypothesis that levels of chemokines in patients at the time of delirium (i.e. in prevalent delirium), would be significantly higher than levels in those who never showed any signs of delirium. Using Mann Whitney U tests for these non-parametric distributions, we found significantly higher concentrations of Eotaxin (CCL11, p = 0.017) and MIP1α (CCL3, p = 0.03) at the time of delirium (Fig. 2) compared to patients with no signs of delirium. All other chemokines showed no association between prevalent delirium and elevated chemokines (Supplementary Table 2).

3.4. Systemic inflammation triggers chemokine transcription in mouse choroid plexus

Since the concentration of some chemokines, post-inflammatory trauma (hip fracture), was not affected by BCSFB leakiness and, given the impracticality of assessing the cellular sources of CSF chemokines in human hip fracture patients, we assessed a similar panel of chemokines at the BCSFB or in the brain parenchyma of mice during systemic inflammation. Thus we challenged mice with bacterial LPS to model the acute response to a pathogen associated molecular pattern or subjected them to laparotomy surgery to produce an acute inflammatory trauma.

Table 3
Demographic data and univariate analysis of chemokines in delirium anytime versus no delirium (including subsyndromal delirium). C-Reactive protein (CRP) was missing in 5 patients (3 delirium, 2 no delirium). APACHE score at admission was missing in 1 patient (no delirium). Analyses were performed by Mann-Whitney test, 2-tailed p values are reported. P values were subsequently adjusted for multiple comparisons determined using the Holm-Šídák multiple comparisons correction method.

	Delirium n = 62	No Delirium n = 52	Unadjusted p	Adjusted p
Age, median (IQR)	85 (81–89)	84 (74.3–89)	0.136	
Sex, male (%)	20 (32.3)	11(21.2)	0.21	
Dementia, number (%)	48 (77.4)	10 (19.2)	**<0.001**	
CRP, preoperatively	8 (3–69)	4 (1–21)	0.08	
APACHE at admission	9 (8–10)	8 (7–10)	**0.021**	
Chemokines	**Median (IQR)**	**Median (IQR)**		
CSF MCP1 (pg/ml)	414 (303–522)	379 (316–509)	0.656	0.877
CSF Eotaxin, (pg/mL)	10.1 (7.3–13.0)	9.4 (6.5–11.3)	0.17	0.603
CSF IP10, (pg/mL),	392 (275–677)	397 (261–602)	0.642	0.877
CSF MIP1α, (pg/mL)	6.9 (1.7–10.6)	6.0 (3.6–9.3)	0.502	0.877
CSF MIP1β, (pg/mL)	11.2 (9.2–15.6)	10.5 (7.9–13.3)	0.042	0.259
CSF TARC, (pg/mL)	2.71 (1.98–5.08)	2.69 (1.72–4.27)	0.298	0.768
CSF Eotaxin3, (pg/mL)	4.56 (2.86–6.98)	3.99 (1.27–5.49)	0.088	0.420

Table 4
Chemokine concentration associations with delirium, stratified on baseline dementia status, as assessed by IQCODE. All analyses adjusted for multiple comparisons using the Holm-Sidak correction.

Analyte (pg/ml)	No dementia		Mann-Whitney *U* test	Dementia		Mann-Whitney *U* test
	No delirium n = 42 median ± IQR	Delirium n = 14 median ± IQR	Adjusted For multiple comparisons	No Delirium n = 10 median ± IQR	Delirium n = 48 median ± IQR	Adjusted For multiple comparisons
Eotaxin	9.182 (6.74–9.18)	9.28 (7.39–17.38)	0.865	10.18 (4.82–12.21)	10.51 (7.29–12.99)	0.529
MCP1	393 (343–555)	468 (296–777)	0.757	292 (263–348)	382 (301–514)	0.156
IP10	397.4 (264–616.1)	298.1 (193–490.4)	0.939	382.1 (226–533.2)	404.9 (270.2–733.5)	0.73
MIP1a	5.83 (1.74–8.98)	6.79 (1.75–10.63)	0.541	7.845 (5.55–9.908)	6.86 (1.74–11.01)	0.957
MIP1b	10.54 (7.99–13.41)	12.64 (10.55–19.39)	0.174	10.02 (7.76–12.03)	10.5 (8.87–15.13)	0.755
TARC	2.64 (1.77–4.59)	2.94 (1.75–6.63)	0.911	2.69 (1.98–3.14)	2.64 (2.0–4.82)	0.707
Eotaxin 3	4.15 (1.16–5.43)	3.67 (2.72–10.66)	0.777	3.85 (1.91–5.64)	4.79 (2.9–5.82)	0.773

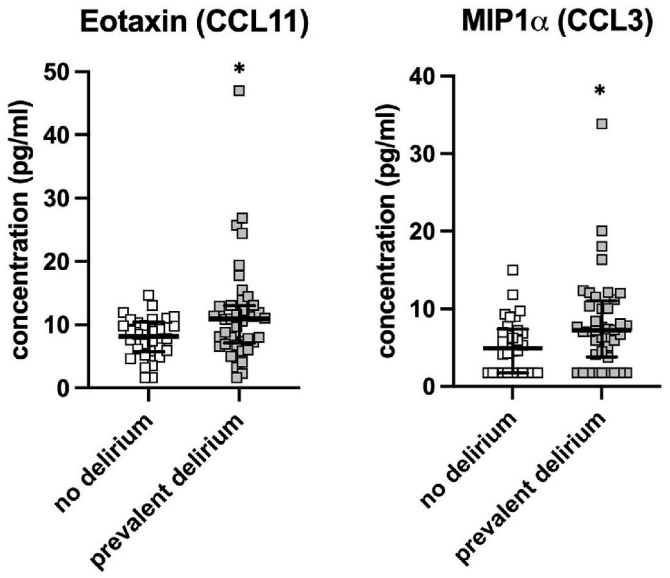

Fig. 2. Human CSF levels of Eotaxin (CCL11) and MIP1α (CCL3) in patients with no delirium versus those with prevalent delirium after hip-fracture. N = 33, 41 for no delirium and prevalent delirium respectively (information on SSD/prevalent delirium was missing on 4 patients). Data are represented by median ± IQR and all non-detects were imputed at 50% of LOD. Assay analyte names are shown while those names in parentheses indicate new chemokine nomenclature for the same analytes. Differences between no delirium and prevalent delirium were analysed by 2-tailed Mann-Whitney analyses, not adjusted for multiple comparisons in this exploratory analysis. Associations between chemokine and delirium are represented by * for $p < 0.05$.

We do not propose either of these settings as models of delirium. Rather they are deployed simply to demonstrate that cells of the BCSFB can transcribe chemokine genes in response to acute systemic inflammation, triggered by molecular patterns present during infection and sterile injury.

3.4.1. LPS

Challenging with LPS at 250 μg/kg i.p. we show the transcription of *Il1b* and a number of CC and CXC chemokines in hippocampi and choroid plexus (CP) dissected from these animals (Fig. 3). All expression levels were normalized to *Gapdh* expression in the same samples (validated in Suppl. Fig. 2) and thus corrected for the considerable difference in the amount of tissue used to prepare CP and hippocampal RNA. Thus all LPS-induced levels are expressed as fold-increase with respect to their saline-treated levels in that specific tissue. All cytokine/chemokines examined were significantly induced in the CP (*Il1b, Cxcl1, Cxcl10, Ccl2, Ccl5, Ccl11, Ccl3, Ccl4, Tarc)* and all except *Ccl11, Ccl5* and *Tarc* were also significantly induced in the hippocampus. In 2 way ANOVA analysis, using LPS treatment and region as independent factors, there was a main effect of LPS on all mediators measured ($F_{1,26} \geq 6.23$, $p \leq 0.019$). In general, LPS-induced expression was higher in the CP than in the hippocampus although *Cxcl10* was expressed more robustly in the hippocampus than in CP. These enriched expression levels in the CP are supported by 2 way ANOVA interactions between LPS and region for all but *Ccl11* and *Cxcl1*. Some interactions were modest (*Il1b*: $F_{1,26} = 5.374$, $p = 0.0286$; *Tarc*: $F_{1,26} = 5.29$, $p = 0.0297$), while those for *Ccl2, Ccl3, Ccl4* and *Ccl5* were very robust ($F_{1,26} \geq 19.37$, $p < 0.001$). Significant Bonferroni post-hoc comparisons are shown in Fig. 3. Therefore, the brain transcribes many chemokines in response to systemic LPS and this is generally enriched in the choroid plexus compared to that in hippocampal tissue. Comparisons between these regions come with the caveat that since a greater proportion of the cells in the CP may be responsive to systemic inflammation than in the hippocampus as a whole, so fold differences versus saline-treated mice might be expected to be larger. Therefore these divergent fold differences cannot reliably show which compartment has more responsive cells, only that as a proportion to the total number of cells present, the CP transcribes higher levels of chemokines than the hippocampus.

3.4.2. Sterile injury: laparotomy

Chemokine expression analysis was also examined in the CP and hippocampus of mice on whom sterile laparotomy surgery was performed. Animals were euthanised 24h post-surgery to reflect a post-trauma time more similar to that in the current hip fracture cohort. *Il1b, Tnfa, Cxcl1* and *Ccl3* were significantly elevated in the CP of laparotomy subjects compared to non-operated controls. In the CP, there were main effects of laparotomy for *Tnfa* ($F_{1,50} = 5.79$, $p = 0.0199$), *Cxcl1* ($F_{1,50} = 6.87$, $p = 0.0116$) and *Ccl3* ($F_{1,50} = 6.77$, $p = 0.0121$), while there was an interaction of laparotomy and region for *Il1b* ($F_{1,50} = 5.26$, $p = 0.026$). These increases in the CP were confirmed by post-hoc pairwise comparisons between laparotomy and non-operated controls with Bonferroni correction for multiple comparisons (post-hoc tests are shown on Fig. 4). Levels of these cytokines and chemokines were typically very low in the hippocampus compared to the CP of the same animals (main effect of brain region for all mediators: $F_{1,51} \geq 5.14$, $p \leq 0.0276$) and only *Cxcl1* and *Ccl2* showed hippocampal increases with respect to non-operated controls, and even these were not statistically significant in 2 way ANOVA analyses.

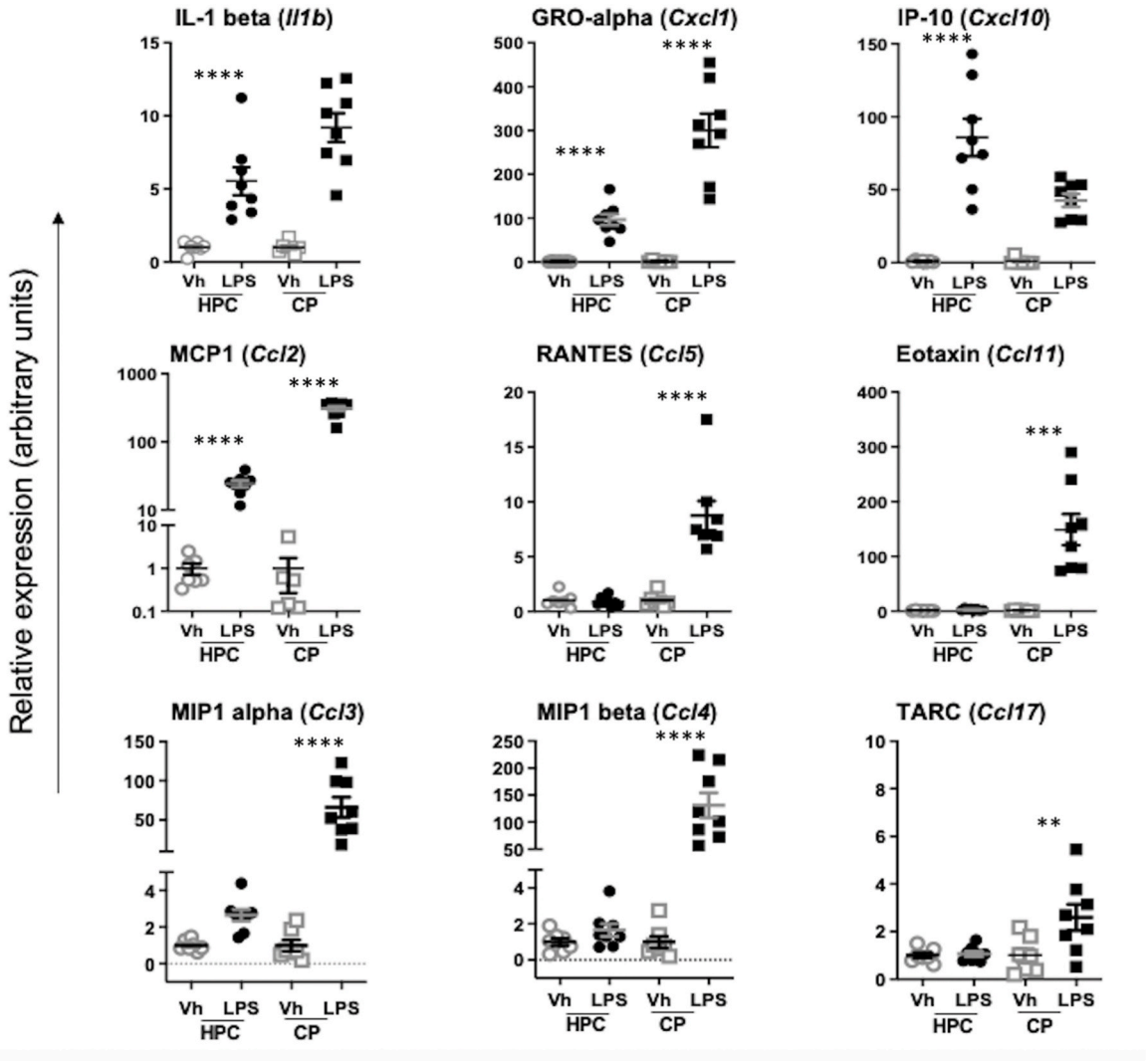

Fig. 3. Systemic LPS injection induced transcription of *Il1b* and chemokines in hippocampus and choroid plexus of mice. Graphs showing transcription of *Il1b, Cxcl1, Cxcl10, Ccl2, Ccl5, Ccl11, Mip1a, Mip1b* and *Tarc* respectively in the hippocampi (HPC) and choroid plexus (CP) of mice treated with LPS (250 μg/kg) compared to vehicle-treated mice (Vh). Fold increases in the transcripts were compared using 2-way ANOVA with region and treatment as between subjects factors. Statistically significant differences by post-hoc pairwise comparisons are denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p<0.0001$). All data points are displayed in addition to the mean ± SEM for n = 7, Vh and n = 8 LPS.

3.4.3. Localisation of chemokines in choroid plexus and brain microvasculature

We investigated the expression of some cytokines and chemokines in the CP and in brain microvessels of mice 3 h after an acute LPS injection. Reliable immunolabelling for cytokines and chemokines is not trivial and here we include all that were successful in our hands: IL-1β, CCL2 (MCP1) and CXCL10 (IP-10). In the CP IL-1β positive cells were found, after LPS challenge, to reside in the stromal space (Fig. 5A). Epithelial cells from CP showed low level, homogenous labelling with no evidence of subcellular localisation, whether treated with saline or LPS. CCL2 (MCP1) labelling showed a similar pattern to IL-1β in stromal cells in the CP and no obvious differences in the epithelial layer (Fig. 5B, upper CCL2 panel). However, in some areas along the ependymal layer we found CCL2-positive flat cells (Fig. 5B, lower CCL2 panel). CXCL10 shows a different pattern in CP with no obvious stromal cell labelling but with distinct sub-cellular structures, occurring predominantly in the apical area of choroidal epithelial cells (Fig. 5C CXCL10), suggesting a possible vesicular secretory mechanism to the CSF (i.e. putative secretory vesicles). The same granular pattern was also observed in flat cells along the glia limitans that borders the ventricular space that joins the lateral and third ventricles (i.e. that which occupies the space between the ventral hippocampus and thalamus; Fig. 5C lower panel). The endothelial IL-1β expression in the vascular units of the hippocampus (Fig. 5D) showed a clear induction by LPS, but labelling was not apparent in the parenchyma at this time or in saline-treated animals in any region. CCL2 (MCP1) and CXCL10 (IP-10) showed a similar induction by systemic LPS (Fig. 5E and **F**), changing from completely absent to a granular pattern of labelling. Thus systemic inflammation induced by LPS induces new chemokine transcription and translation of at least these 3 cytokines/chemokines at important immunological gateways, the BBB and the BCSFB. We do not suggest that these are the 3 key inflammatory mediators made at these borders, nor that those found to be transcribed at these borders are not synthesized at protein level. The immunohistochemistry images provide proof of principle that important inflammatory mediators are made at these borders during systemic inflammation.

4. Discussion

In the current study, using CSF chemokine analysis in a human hip-fracture cohort we show that CSF levels of chemokines, following inflammatory trauma, are modestly influenced by the integrity of the

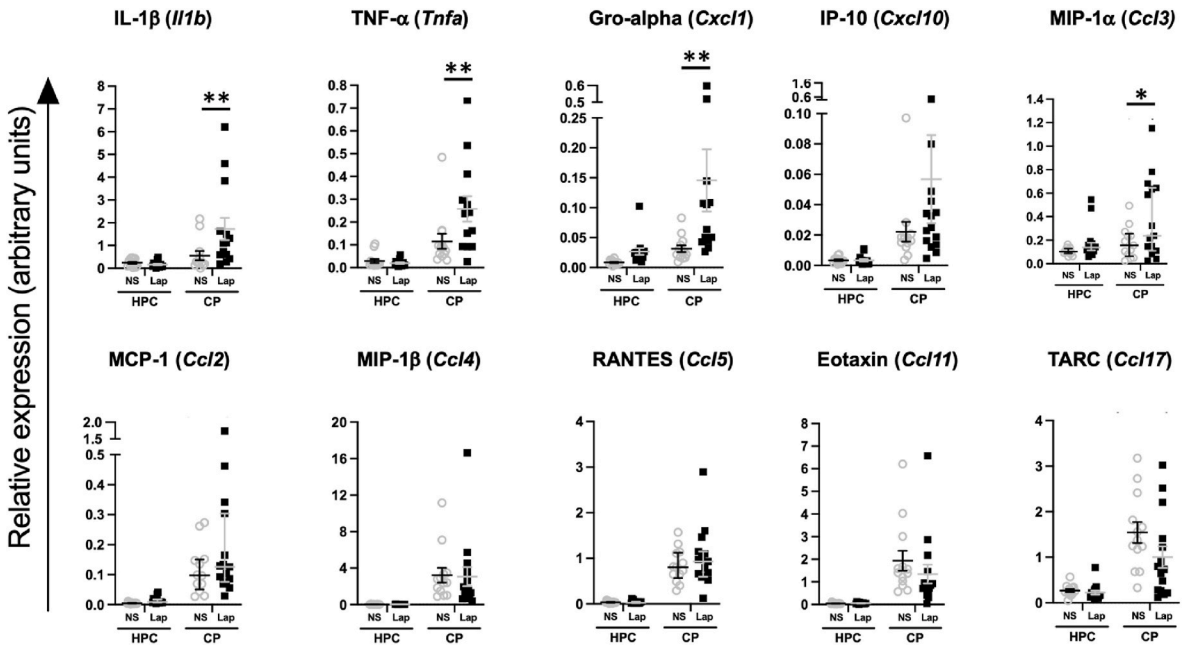

Fig. 4. Transcription of chemokines and cytokines in the hippocampus and choroid plexus 24 h following laparotomy in mice. Graphs depicting relative expression of transcripts for *Il1b, Tnfa*, *Cxcl1*, *Cxcl10, Ccl3*, *Ccl2*, *Ccl4*, *Ccl5*, *Ccl11* and *Ccl17* in the hippocampus (HPC) and choroid plexus (CP) of male and female mice subjected to a surgical laparotomy, compared to those that received anaesthesia but no surgical procedure (NS). Bars represent as means ± SEM or medians ± IQR n = 13 for NS controls and n = 13 for laparotomy. To maximally exploit the factorial design, groups were analysed by 2 way ANOVA and contingent on significant main effects or interaction, Bonferroni post-hoc comparisons of NS and laparotomy were performed. *P < 0.05, **P < 0.001.

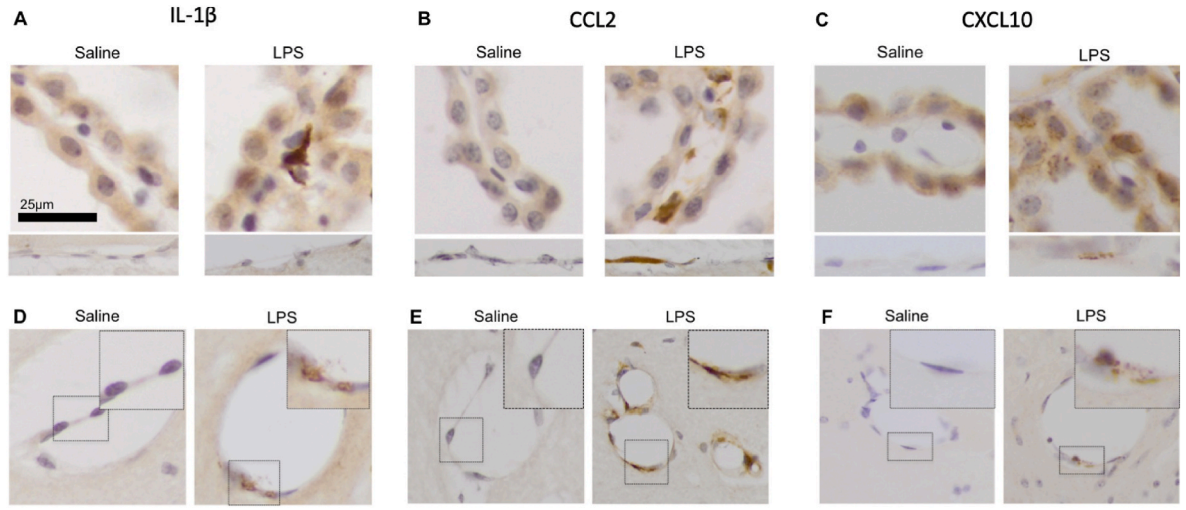

Fig. 5. IL-1β and chemokine immunohistochemistry in the choroid plexus, epithelial, endothelial and ependymal cells. C57BL6J mice were intraperitoneally challenged with LPS (100 μg/kg) and 3 h later the animals were transcardially perfused with 10% formalin and paraffin-embedded. The brains were sectioned in a microtome (10 μm) and stained for A,D) IL-1β (1:50, Peprotech 500-P51, B,E) CCL2 (1:100, R&D ELISA kit) and C,F) CXCL10 (1:10000, Peprotech P129). Photomicrographs are shown as follows: A-C) Choroid plexus epithelial and stromal cells as well as ependymal border cells and D-F) blood vessels in the hippocampus. Photographs were obtained at ×40 magnification. Scale bar = 25 μm.

BCSFB. Increased 'leakiness', as measured by Q_{alb}, correlated moderately with increased levels of eotaxin, eotaxin 3, IP-10 and TARC while IL-8, MCP-1, MIP-1α and MIP1β were not significantly influenced by BCSFB permeability. There were not strong associations between chemokine levels and delirium but in exploratory analyses, CSF levels of Eotaxin (CCL11) and MIP1α (CCL3) were associated with prevalent delirium. In mice we showed that systemic inflammation 24 h after sterile injury also induced transcription of *Il1b, Tnfa, Ccl3* and *Cxcl1* at the CP and LPS induced several additional chemokines indicating local *de novo* synthesis under those conditions (i.e. within the ventricles). Immunohistochemistry confirmed that both CP and brain vasculature expressed protein for a number of these mediators. The findings come with the caveats that there are significant differences between humans and mice and that the peripheral insults are not identical in the human and mouse studies. However, working within those constraints, the findings should nonetheless motivate detailed study of sources and actions of chemokines with respect to delirium and other brain consequences of systemic inflammation.

4.1. Contributions of peripheral and local synthesis of chemokines

There is ample evidence that BBB and BCSFB permeability can be

altered by systemic inflammation (Varatharaj and Galea, 2017; Wang et al., 2020) and that several cytokines can enter the brain actively or passively (Erickson and Banks, 2018). Though widely presumed that inflammatory mediators enter the brain from the blood and subsequently contribute to delirium direct evidence for this blood to brain transit during delirium is lacking. An association of compromised BCSFB with delirium was shown in one study (Hov et al., 2016) and another showed a correlation between Qalb and severity in 11 patients (Taylor et al., 2022). Two other studies showed that change from baseline in CSF:plasma albumin ratio (CPAR = Qalb), rather than absolute CPAR, was higher in delirious patients in 2 separate mixed surgical cohorts with n = 8 and n = 26 delirious patients respectively (Taylor et al., 2022; Devinney et al., 2023).

Here we have shown that IL-8, MCP1, MIP1α and MIP1β were not higher in those with impaired BCSFB but Eotaxin, Eotaxin 3, IP-10, and TARC were moderately higher when the BCSFB showed higher permeability. Elevation of chemokines in the CSF after hip fracture should not be assumed to originate in the blood. During systemic inflammation it is clear that there are elevated levels of chemokines in both blood and CSF (Hirsch et al., 2016) and determining contribution of different compartments to CSF concentrations is relatively complex. High peripheral levels may cross brain barriers but the ability of cells at the barrier to secrete chemokines either into the blood or into the CSF also complicates interpretations. It would be beneficial to calculate CSF:blood ratios of the concentrations of these chemokines to gain further insight into the likely contribution of blood chemokines to levels appearing in the CSF. Therefore the lack of availability of bloods for this cohort due to their prior use in published studies is a limitation in the current study. However previous small studies addressing this question showed mixed results. There was limited or no correlation between blood and CSF cytokines in surgical and hip-fracture patients (Bromander et al., 2012; Neerland et al., 2016; Fertleman et al., 2022) but using repeat CSF sampling in the first 24 h after elective surgery (n = 10 patients) showed some significant plasma-CSF correlations in chemokine expression (IL-8, MIP1α and MIP1β) (Hirsch et al., 2016). It is notable that IL-8 levels were actually higher in the CSF than in the plasma in the latter study and IL-8 concentrations were found, in the current study, not to be even weakly associated with impaired BCSFB integrity. Hirsch and colleagues found that most chemokines were increased in both plasma and CSF. Although ratios between these levels are not supplied therein, clearly levels rising in both compartments will tend to produce correlations but this would not, of itself, indicate that CSF levels of any chemokine are driven by concentrations in the blood: a systemic stimulus does induce levels of at least some chemokines both in the blood and at brain barriers to similar extents.

This is relevant to our findings on correlations between Qalb and CSF chemokine concentrations. Chemokines are small proteins (~8–15 kDa) and it is not intuitive that some chemokines would cross compromised barriers while others would not, especially when the permeability of those barriers to albumin, which is a much larger protein (67 kDa), is raised. One possible explanation for the differential correlations with Qalb is that while it may be possible for any or all chemokines to cross a permeabilised BCSFB, the correlation may be weaker when local synthesis, in cells at the barrier, contributes to the overall local concentration. We do not have access to human brain tissues that might address this question. However in the current study, in mice, both sterile injury and bacterial LPS were sufficient to induce *de novo* synthesis of multiple chemokines in the CP. Although it is not novel to show chemokine synthesis at brain barriers following sterile injury (Balusu et al., 2016; Blank et al., 2016), the data do emphasise the important point that brain barriers can synthesise these molecules *de novo* and that this likely contributes to CSF levels observed after systemic inflammatory insults. Given the rather modest associations between some chemokines and BCSFB permeability shown here, and the lack of any correlation for others, one might speculate that both peripheral and local sources contribute to levels of these mediators in patients with delirium during systemic inflammation.

The expression of chemokines in the CP in both mouse models of systemic inflammation here is suggestive of this being a source of cytokines/chemokines in response to circulating pathogen-associated molecules like LPS, damage-associated molecules like HMGB1 or indeed to pro-inflammatory cytokines such as IL-1 (Marques et al., 2009; Liu et al., 2019). Our immunohistochemical approaches show that stromal cell (IL-1, CCL2) and epithelial cell (CXCL10) populations of the CP synthesise these inflammatory mediators upon systemic inflammatory challenge, consistent with prior studies showing that CP stromal macrophages produce IL-1β and CP stromal and epithelial cells make CCL2 and CXCL1 (Liu et al., 2019; Shimada and Hasegawa-Ishii, 2021). CP IL-1RI expression is necessary for IL-1β-induced CCL2 expression and monocyte infiltration (Liu et al., 2019) and is chiefly expressed in endothelial and epithelial cells (Dani et al., 2021). Choroidal epithelial cells are secretory: recent imaging studies show calcium dynamics and vesicular secretion into the CSF (Shipley et al., 2020) and illustrate the formation and release of exosomes from CP epithelium into the CSF in response to systemic inflammation (Balusu et al., 2016). Among the inflammatory molecules in the secretome of CP explants from amyloid-β oligomer-treated animals are MCP1 (CCL2) and CXCL1 (Vandendriessche et al., 2021). Thus the CP can secrete these chemokines into the CSF.

The BBB and the BCSFB are not equivalent at molecular or structural levels and interstitial fluid and CSF are not identical, but the significant exchange between these 2 brain fluids (Shetty and Zanirati, 2020) suggests that elevated chemokines in the CSF may also permeate the brain ISF and both endothelial and choroidal sources may contribute to this. We also demonstrate mouse brain endothelial expression of IL-1β, CCL2 and CXCL10 (Fig. 5) and this may contribute to brain responses to systemic inflammation. It is well established that the brain endothelium can transduce inflammatory signals into areas of the brain that the ventricular system cannot reach, that it has pivotal roles in the fever and sickness behaviour responses via secretion of prostaglandins (Dantzer et al., 2008) and that it can secrete cytokines and chemokines influencing cognitive function and CNS metabolic adaptation to systemic inflammation (Blank et al., 2016; Le Thuc et al., 2016; Skelly et al., 2019b).

Thus, neuroactive mediators likely emerge from the blood, the brain endothelium and the CP and examples from the mouse literature illustrate contributions of different compartments to brain levels and actions of chemokines: eotaxin (CCL11) can enter the brain from the circulation and directly impact on neuronal function (Villeda et al., 2011), while endothelial and epithelial cells synthesise brain IP-10 (CXCL10), which can disrupt hippocampal neuronal function (Blank et al., 2016). Which mediators, from which compartments, mediate the brain effects of systemic inflammation when superimposed on underlying evolving brain pathology (Cunningham and Maclullich, 2013; Wang et al., 2020) are now important questions to investigate in detail.

4.2. *Might chemokines contribute to delirium?*

It was not an aim of the current study to identify a diagnostic biomarker of delirium and the statistical power was likely insufficient to identify one, should it exist. It is clear that chemokines were readily detectable in the CSF of all hip fracture patients in the current study, regardless of delirium status, and we propose that levels of these mediators are more likely indicative of the severity of inflammation experienced by the brain during delirium, rather than an indicator of delirium *per se*. However, chemokines have important biological effects, as chemoattractants and as neuromodulators in brain tissue and could be important in disruption of brain function in these patients. Exploratory analysis found that 2 chemokines, MIP-1α and eotaxin were elevated during prevalent delirium.

MIP-1α (CCL3) is a monocyte, neutrophil and lymphocyte chemoattractant but i.c.v. injection of CCL3 also impairs spatial and long-term

memory and these effects are inhibited by a specific antagonist of the CCR5 receptor, which recognises CCL3, CCL4 and CCL5 (Marciniak et al., 2015). MIP-1α levels are typically low but increase with time, exclusively in the CSF, in the first 24 h after elective surgery (Hirsch et al., 2016) and were associated with prevalent delirium in the current study. The CNS transcription of *Ccl3* in the choroid plexus of mice after both LPS and sterile surgery and the reported expression of *Ccl3* in microglia in mouse models of Alzheimer's disease (Grubman et al., 2021) do show that the brain can produce CCL3 *de novo*, but we have not yet been able to optimise immunohistochemistry for CCL3. It is important to determine whether CCL3 originates in the brain during delirium and whether it might contribute to symptoms observed.

CCL11 (Eotaxin) is chemoattractant for eosinophils and T cells, but has also been shown to contribute to cognitive dysfunction and impaired neurogenesis in mice (Villeda et al., 2011). It is of interest that that study demonstrated, in both mice and humans, that blood CCL11 can penetrate the blood brain barrier. Consistent with this, eotaxin was well correlated with extent of compromised BCSFB in the current study. It is of note that CCL11/eotaxin and CCL26/eotaxin 3 share the CCR3 receptor (Beck et al., 2006) and can be either chemotactic or immunomodulatory (Petkovic et al., 2004).

In their classical role, chemokines might contribute to delirium via chemoattraction of inflammatory lymphocytes and leukocytes to the brain. Infiltration of monocytes has been shown to contribute to short and long-term cognitive effects of both infection and fracture in animal models (Terrando et al., 2015; Garre et al., 2017; Andonegui et al., 2018) and, with the associations observed in the current study, it is of interest to test whether the immune cell populations for which the chemokines noted in the current study are chemoattractant, may also contribute to acute cognitive deficits in mice or delirium in humans.

4.3. Study limitations and priorities for future investigations

The current work is exploratory and has several limitations. The sample size was relatively small, the elevations of chemokines in patients with delirium were modest and simultaneous blood samples were not available. Also it would be preferable to calculate Qalb values and chemokine ratios using CSF and blood samples collected at the same time. There were few dementia patients that did not experience delirium and few patients that were cognitively normal at baseline that then experienced delirium. Since dementia is a strong risk factor for delirium this is expected, but it nevertheless significantly limits the power of analyses stratified on dementia status. Such analyses may be important since the stronger associations with elevated chemokines tended to be in patients who experienced delirium despite not having dementia as a risk factor and this requires more study.

Therefore, firstly, it will be important to validate the findings in larger human studies. The exploratory findings on eotaxin (CCL11) and MIP1α (CCL3) association with prevalent delirium provide hypotheses for such follow-up studies. In the course of future biomarker studies it will also be important to consider the status of subsyndromal patients (Bowman et al., 2023). That is, since some chemokines in this study were elevated in SSD, inclusion of SSD patients in the 'no-delirium' group in subsequent studies will likely weaken associations between delirium and putative biomarkers in the CSF.

Secondly, it is important to further interrogate the relationship between BCSFB permeability, CSF chemokines and delirium. The Q_{alb} approach taken here provides a proxy for whether there was BCSFB leakiness at the time the CSF chemokine levels were assessed, and for some chemokines this permeability was not associated with higher CSF levels, while for others it was. We speculate that the current data might suggest a mixed contribution of local and circulating sources. Blood:CSF chemokine ratio calculations, calculated from blood and CSF sampled at the same time point, would be very useful in future studies. However this ratio would not, alone, resolve the question: given that brain barrier cells could, in principle, secrete chemokines into the CSF or into the blood, and that chemokines levels could be equally induced by peripheral and CNS compartments, ratios could remain stable even if there was robust synthesis at brain barriers or movement between compartments. Moreover even brain parenchymal production of chemokines may contribute to CSF levels so this remains a complex issue to resolve.

Likewise, it is premature to suggest that BCSFB barrier permeability causes or contributes to the development of delirium. Since most patients that develop delirium do not show robustly compromised BCSFB (Hov et al., 2016; Pan et al., 2019) and more recent studies suggest that change from baseline Qalb, rather than overt impairment *per se* are associated with short and long-term outcomes (Taylor et al., 2022; Devinney et al., 2023) it becomes important to know whether permeability to molecules smaller than albumin, such as cytokines or chemokines may have impacts on brain function even when Qalb values remain relatively low (ie showing limited permeability).

The associations between elevated chemokines and delirium shown in the current study also cannot infer causation. It is now necessary to assess whether individual chemokines actually cause acute cognitive deficits and, if so, whether this is mediated by neuro-modulatory effects or by chemokine-induced immune cell infiltration. Animal models for delirium represent a significant scientific challenge but behavioural and neurophysiological analysis show acute and fluctuating cognitive dysfunction in cognitive domains relevant to delirium, that are not better explained by the underlying dementia-like brain disease (Davis et al., 2015; Wang et al., 2020; Sultan et al., 2021). Therefore, we propose that the models that we and others have developed (Vasunilashorn et al., 2023) are appropriate to the scientific aim of interrogating particular inflammatory pathways in the induction of delirium-like deficits. The approaches discussed above will help to elucidate whether and how CSF chemokines may contribute to delirium and will add essential detail to a 'neuroinflammatory hypothesis of delirium' that is still quite vague on numerous details.

CRediT authorship contribution statement

Paul Denver: Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Lucas Tortorelli:** Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Karen Hov:** Data curation, Methodology. **Jens Petter Berg:** Data curation, Formal analysis, Methodology. **Lasse M. Giil:** Formal analysis. **Arshed Nazmi:** Formal analysis, Investigation, Methodology. **Ana Lopez-Rodriguez:** Formal analysis, Investigation, Methodology. **Daire Healy:** Formal analysis, Investigation, Methodology. **Carol Murray:** Formal analysis, Investigation, Methodology. **C. Robyn Barry:** Investigation, Methodology. **Leiv Otto Watne:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Colm Cunningham:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Data sharing

The data supporting the findings are available from the corresponding author upon reasonable request. Availability of the human data is dependent on approval from the Regional Committee for Medical and Health Research Ethics in Norway.

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Declaration of competing interest

The authors declare that they have are no financial or other conflicts of interest relevant to the data presented in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100920.

Data availability

Data will be made available on request.

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