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## Minimally invasive serial collection of cerebrospinal fluid reveals sex-dependent differences in neuroinflammation in a rat model of mild traumatic brain injury

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### ABSTRACT

Traumatic brain injuries (TBI) are the seventh leading cause of disability globally with 48.99 million prevalent cases and 7.08 million years lived with disability. Approximately 80 % of TBI patients are diagnosed with mild TBI (mTBI), or concussion, caused by nonpenetrating mechanical trauma to the head or body along with sudden rotational motion of the head. Studies investigating the temporal dynamics of neuroinflammation after mTBI are greatly needed. Without longitudinal studies, translating preclinical studies to clinical studies remains challenging as the difference in timing remains poorly understood. In this study, we describe a method of minimally invasive serial cerebrospinal fluid (CSF) collection that enables longitudinal investigation of CSF inflammation. The method described in this study can easily be adapted by any laboratory prepared for animal studies. Multiplex immunoassay of serially collected and singly collected CSF samples show collection frequency does not alter protein expression in the CSF. Further, sex-dependent differences in TBI have been reported, but remain poorly understood. This study establishes a framework for assessing sex difference in neuroinflammation after a concussion. We showed that results vary based on the framing of the statistical test. However, it is evident that males experience a more robust inflammatory response to a single concussion than females.

### 1. Introduction

Traumatic brain injuries (TBI) are the seventh leading cause of disability globally with 48.99 million prevalent cases and 7.08 million years lived with disability (Guan et al., 2023). Approximately 80 % of TBI patients are diagnosed with mild TBI (mTBI), or concussion, caused by nonpenetrating mechanical trauma to the head or body along with sudden rotational motion of the head (Capizzi et al., 2020; Levy et al., 2023; Grovola et al., 2023). mTBIs commonly occur in falls, motor vehicle crashes, contact sports, military duty, and incidences of domestic violence (Capizzi et al., 2020; Neale et al., 2023; Fraunberger et al., 2020; Feigin et al., 2019). While the typical prognosis of a single concussion is positive, with full recovery within 3 months (Levy et al., 2023), approximately 15 percent of patients also experience post-

concussion symptoms (PCS) that are exacerbated with repeated mTBIs (Levy et al., 2023; Fraunberger et al., 2020; Gold et al., 2018; Gard et al., 2023).

The pathophysiology of mTBI remains poorly understood, primarily due to heterogeneity in presentation and outcome. mTBI is associated with structural abnormalities, excitotoxicity, metabolic stress, and increased inflammation (Visser et al., 2023; Giza and Hovda, 2014; Howell and Southard, 2021). However, much of our understanding of mTBI pathophysiology stems from more severe injuries. Major distinctions between mTBI and more severe TBI include the absence of intracranial lesions, hemorrhages, and skull fractures in mTBI (Neale et al., 2023; Fraunberger et al., 2020; Gold et al., 2018; Shultz et al., 2017). Further, there are differences in the inflammatory response based on injury severity. In a study analyzing whole blood from pediatric TBI

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patients, severe TBI caused greater concentrations of TNF $\alpha$ , IL-6, IL-8, and IL-10 compared to mTBI. However, mTBI patients experience a greater concentration of IFN $\gamma$  and IL-17A (Ryan et al., 2022). While some overlap of the neuroinflammatory response across TBI severities is expected, studies investigating the temporal dynamics of neuroinflammation after mTBI are still needed to uncover the mechanisms behind PCS, as well as to identify potential biomarkers that can be leveraged to develop more reliable point-of-care diagnostics and therapeutic strategies. Furthermore, identified mechanisms and biomarkers will need validation in both sexes.

There have been many reported sex-dependent differences in mTBI (Neale et al., 2023; Fraunberger et al., 2020; Gard et al., 2023; Villapol et al., 2017; Daisy et al., 2022). Interrogating sex differences in mTBI is important simply because TBI outcomes exhibit significant sex differences with women experiencing lower incidences of morbidity and mortality after TBI than age-matched men (Brotfain et al., 2016). In a recent publication, Liu et al. reported that males, but not females, treated with minocycline after a repeat mTBI (rmTBI) experienced an increased cold thermal threshold and decreased anxiety-like behavior. There was also sex differences in the microglial response to rmTBI as both males and females exhibited increased microglia and brain-derived neurotrophic factor (BDNF) mRNA in the anterior cingulate cortex, but only males saw an increase in BDNF in the nucleus accumbens (Liu (Steve) et al., 2023). Another sex difference noted in the literature is the effect of regulatory T cells (Tregs) on microglial activation. In a hypoxia-ischemia model of brain injury, Beckmann et al. found increased numbers of Tregs and decreased microglial activation in female brains, but in male brains they found Tregs promoted vascular injury (Beckmann et al., 2022). While this study was not in TBI, it highlights the sex-differences in the innate and adaptive immune responses, which are hypothesized to be affected by sex hormones. Estrogen and progesterone have been shown to be neuroprotective after TBI, reducing mortality and improving neurological outcomes, by reducing neuronal apoptosis, inhibiting microglial and astrocytic activation, and reducing brain edema (Brotfain et al., 2016; Chakrabarti et al., 2016; Wang et al., 2021; Zlotnik et al., 2012). To fully understand the inflammatory response to TBI, it is vital to assay cerebrospinal fluid (CSF), as it has been shown that CSF biomarkers such as MAP-2 enhance prognosis capabilities (Wang et al., 2018). The literature analyzing the CSF proteome over time after TBI is very limited, primarily due to the lack of a CSF collection method that is minimally invasive and enables serial collections (Liu et al., 2010; Yao et al., 2009; Zoltewicz et al., 2013; Han et al., 2023).

This study has three primary goals: 1) establish a method of minimally invasive serial CSF collection that can be easily utilized by any laboratory equipped for animal studies without any specialized equipment, 2) determine if serial CSF collection alters CSF protein detection, and 3) provide a framework for interrogation of sex differences in CSF proteomics after neurotrauma.

## 2. Methods

### 2.1. Animal care and concussion

All experiments, including animal housing conditions, surgical procedures, and postoperative care, were in accordance with the Institutional Animal Care and Use Committee guidelines at the University of California–Irvine (UCI). Eighteen male and eighteen female Long-Evans rats (200–225 g, Charles River Laboratories, San Diego, CA) were randomly divided into six experimental groups: female concussion with single CSF withdrawal, female concussion with serial CSF withdrawal, female sham with serial CSF withdrawal. Male concussion with single CSF withdrawal, male concussion with serial CSF withdrawal, and male sham with serial CSF withdrawal. Each group had  $n = 6$  rats.

A pneumatic controlled cortical impact device (TBI-0310 Head Impactor, Precision Systems and Instrumentation, LLC, Fairfax Station,

VA) was used for concussion. A foam bed modified from “Marmarou foam” (Type E Bed, Foam to Size, Inc., Ashland, VA) was modified to fit on the stage of the CCI device, and a trench was cut out to position tubing and a nose cone to keep the animal under anesthesia for the duration of the procedure. All animals spent 10 min under anesthesia, including shams; 5 min induction at 3.5 % isoflurane, and 2 min to mark the tail, shave the head, and apply ophthalmic ointment. The animal was placed onto the modified Marmarou foam and positioned to breathe 3.5 % isoflurane from a nose cone. Lab tape was lightly applied to the ears and fixed to the foam to position the head level and prevent movement during breathing. The rat was at the CCI station for 3 min until a 5-mm diameter probe tip was used to deliver an impact with speed 5.0 m/s, 1.0 mm depth, and 50 ms dwell time. In total, rats are under anesthesia for 10 min from knockdown to TBI impact. The isoflurane percentages were designed to minimize head movement from respiration while zeroing the piston and during impact while still keeping the animal unresponsive to a toe pinch reflex. Following impact, animals were moved to a recovery area where righting time was recorded. Sham animals underwent the same 10-min procedure with anesthesia but were not impacted.

### 2.2. Cerebrospinal fluid sample collection and storage

This protocol is adapted from Han et al. making the procedure easy to adapt for any laboratory without the use of custom built equipment or specialized tools (Han et al., 2023). To prep for CSF collection, an isoflurane nose cone was taped to the short side of a rectangular brick (can be any raised platform such as a pipette tip box) so the rat’s head could be positioned vertically while breathing in isoflurane.

Before CSF collection, Long-Evans rats were anesthetized using an isoflurane induction chamber with 3.5 % isoflurane and shaved. For serial collections, the shaved area was sterilized with ethanol and betadine. After the rat is placed on the brick with the nose in the nose cone, the rat’s head was positioned vertically. The rat skin is then pulled back to ensure the area around the foramen magnum is tight, and tape is placed over the shoulder blades.

To start the CSF collection, a sterile hemostat was used to bend the needle of the 27G winged butterfly needle approximately 90 degrees so the bent part is about 4 mm (Fig. 2A). Before insertion of the needle, the foramen magnum needs to be located. The external occipital protuberance was used as a landmark. This should feel like a ridge on the skull. About 6 mm past the ridge is the foramen magnum (Fig. 2B). The bent 27G winged needle was inserted into the foramen magnum (an extremely soft spot) with no resistance. Once inserted, the syringe was pulled to 0.1 mL, and clear CSF fluid flowed into the tubing of the needle (Fig. 2C). Once CSF stopped flowing, the needle was removed. There were no visible signs of damage besides the insertion point (Fig. 2D). The CSF was then transferred from the tubing into a microcentrifuge tube on ice (Fig. 2E). CSF samples were measured using a pipette then supplemented with 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies, #5872). CSF was stored at  $-80^{\circ}\text{C}$ . Final numbers of CSF

**Table 1**

Sample numbers: Table shows total sample number for each experimental group at each timepoint. Each group had 6 total animals, except for the male and female concussion groups which include an additional 6 animals for the single CSF collection cohorts at the 7 dpi timepoint for a potential total of 12 samples. Samples were excluded if they became contaminated with blood and clear CSF could not be collected.

Number of CSF Samples Group	Timepoints				
	Pre-injury	1 dpi	3 dpi	7 dpi	14 dpi
Male Sham (MS)	6	6	4	6	6
Male Concussion (MC)	5	5	5	11	5
Female Sham (FS)	5	5	6	6	6
Female Concussion (FC)	5	4	6	9	6

samples per gender and timepoint are included in [Table 1](#).

### 2.3. Luminex xMAP multiplex immunoassay

CSF Samples were thawed on ice and centrifuged. A bead-based multiplex cytokine kit (LXSARM-17, R&D Systems, Minneapolis, MN, USA) was used to measure CSF levels of CXCL2, CXCL3, ICAM-1, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, L-Selectin, TIMP1, TNF- $\alpha$ , and VEGF. This kit was chosen due to its published use in rat TBI studies ([Baskin et al., 2023](#); [Heyburn et al., 2023](#)). All samples were run in duplicate. Results were analyzed using mean fluorescent intensities as previously reported ([Breen et al., 2016](#); [Breen et al., 2015](#)).

### 2.4. Immunohistochemical labeling of Iba1 and GFAP

Brain tissue sections were immunohistochemically labeled with primary antibodies for Iba1 (1:500, 019-19741, FujiFilm Wako) and GFAP (1:1500, Z033429-2, Agilent). Biotinylated donkey anti-rabbit (1:500, 715-066-152, Jackson ImmunoResearch) secondary antibodies were used, followed by VECTASTAIN® Elite® ABC Kit Peroxidase (PK-6100, Vector Laboratories) and DAB Substrate Kit, Peroxidase with Nickel (SK-4100, Vector Laboratories) according to manufacturer's instructions.

### 2.5. Stereological quantification of microglia and astrocytes

Stereological quantification was performed on every 12th brain tissue section with thickness of 30 $\mu$ m. The corpus colosum was traced at the connection between the left and right cerebral hemisphere. An average of 10 counting frames measuring 80  $\mu$ m  $\times$  80  $\mu$ m was implemented. The systematic random sampling (SRS) grid measured 250  $\mu$ m  $\times$  250  $\mu$ m. The optical dissector height consisted of a guard zone thickness of 1 $\mu$ m and a dissector height of 18 $\mu$ m. The CE (Coefficient Error) was 0.1.

### 2.6. Semi-quantitative assessment of microbleed presence

A categorical scale of 0–3 was implemented to rate the presence of microbleeds at three regions of the brain: cerebral cortex, corpus colosum, and hippocampus. A score of 0 indicates no visible hemosiderin granules. A score of 1 indicates that a few hemosiderin granules were visible. A score of 2 indicates that some hemosiderin granules were visible. A score of 3 indicates that many hemosiderin granules were

present. The scores for each region were summed up for each section. 5 serial sections were used for each animal. The total sum of all 5 sections was used for statistical analysis.

### 2.7. Statistical analysis

For analysis of serially collected samples, statistical analysis was performed on mean fluorescent intensity (MFI) values for each time-point normalized to pre-injury values. A two-way mixed effects (for [Tables 2–5](#)) or a three-way mixed effects analysis with a post-hoc Holm-Šidák's multiple comparisons test was used ([Table 6](#)). For statistical tests that did not require matching samples, an ordinary two-way ANOVA with a post-hoc Holm-Šidák's multiple comparisons test was used.

## 3. Results

### 3.1. CSF was collected serially from rats with no evidence of negative side effects

CSF was collected from a total of 36 rats (18 male and 18 female). 24 rats underwent serial collections 1 day before concussion and then 1 day post injury (dpi), 3 dpi, 7 dpi, and 14 dpi. The remaining 12 rats underwent a single CSF collection at 7 dpi only ([Fig. 1](#)). No rats exhibited signs of pain/distress, weight loss, infection, or swelling. There was no attrition of animals due to mortality. If CSF samples were contaminated with blood upon collection, they were immediately centrifuged for 30 s to separate the CSF from blood, yielding clear CSF samples. Centrifugation has previously been shown not to affect CSF composition ([Batllori et al., 2019](#)). Of the 132 attempts to collect CSF samples, 121 attempts (92 %) resulted in clear CSF ([Fig. 2A–E](#)).

### 3.2. Serial CSF collection did not alter protein detection

Compared to CSF samples that were collected only at 7 dpi. Analysis by two-way ANOVA demonstrated that collection frequency did not alter protein levels in either males ( $F = 2.492$ ;  $p = 0.1135$ ) or females ( $F = 1.022$ ;  $p = 0.3140$ ) across 16 of the 17 total protein analytes. Interestingly, TIMP-1 was significantly increased ( $p = 0.0143$  in females and  $p = 0.0055$  in males) in serially collected CSF samples using a post-hoc Holm-Šidák's multiple comparisons test ([Fig. 2F](#)). TIMP-1 is an inhibitor of matrix metalloproteinases, and has been shown to play a role in alleviating inflammatory pain associated with wound healing ([Knight et al., 2019](#)). Given the role of TIMP-1 role in inflammatory pain and

**Table 2**

Statistical testing comparing injury to sham, ignoring sex. Two-way mixed effects analysis with post-hoc Holm-Sidak multiple comparisons test. Ignoring sex, analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between concussion and sham. The associated  $p$ -value is reported for all instances of statistical significance.

Sex not considered	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons (Concussion vs Sham)			
	Time	Injury	Time $\times$ Injury	1 dpi	3 dpi	7 dpi	14 dpi
Protein							
CXCL3	ns	ns	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns
GM-CSF	ns	ns	ns	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns	ns	ns	ns
IFN $\gamma$	<0.0001	0.027	ns	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns	ns	ns	ns
IL-1b	ns	ns	ns	ns	ns	ns	ns
IL-2	ns	ns	ns	ns	ns	ns	ns
IL-4	ns	ns	ns	ns	ns	ns	ns
IL-6	ns	ns	ns	ns	ns	ns	ns
IL-10	ns	ns	ns	ns	ns	ns	ns
IL-13	ns	ns	ns	ns	ns	ns	ns
IL-18	ns	ns	ns	ns	ns	ns	ns
L-selectin	ns	ns	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns
TNF $\alpha$	ns	ns	ns	ns	ns	ns	ns
VEGF	ns	ns	ns	ns	ns	ns	ns

**Table 3**

Statistical testing comparing injury to sham in females. Two-way mixed effects analysis with post-hoc Holm-Sidak multiple comparisons test comparing concussion vs sham in female CSF samples only. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between concussion and sham. The associated *p*-value for significant interactions is reported.

Females Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons Males (Concussion vs Sham)				
	Protein	Time	Injury	Time × Injury	1 dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	ns	ns	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF	0.0298	ns	ns	ns	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns	ns	ns	ns	ns
IFNγ	0.0065	ns	ns	ns	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns	ns	ns	ns	ns
IL-1b	0.0109	ns	ns	ns	ns	ns	ns	ns
IL-2	ns	ns	ns	ns	ns	ns	ns	ns
IL-4	ns	ns	ns	ns	ns	ns	ns	ns
IL-6	0.0462	ns	ns	ns	ns	ns	ns	ns
IL-10	ns	ns	ns	ns	ns	ns	ns	ns
IL-13	ns	ns	0.0388	ns	ns	ns	ns	ns
IL-18	ns	ns	ns	ns	ns	ns	ns	ns
L-selectin	0.0065	0.0095	ns	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns	ns
TNFα	ns	ns	ns	ns	ns	ns	ns	ns
VEGF	ns	ns	0.0049	ns	ns	ns	ns	ns

**Table 4**

Statistical testing comparing injury to sham in males. Two-way mixed effects analysis with post-hoc Holm-Sidak multiple comparisons test comparing concussion vs sham in male CSF samples only. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between concussion and sham. The associated *p*-value for significant interactions is reported.

Males Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons Males (Concussion vs Sham)				
	Protein	Time	Injury	Time × Injury	1 dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	ns	ns	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF	ns	ns	ns	ns	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns	ns	ns	ns	ns
IFNγ	0.0111	ns	ns	ns	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns	ns	ns	ns	ns
IL-1b	ns	ns	ns	ns	ns	ns	ns	ns
IL-2	0.0005	ns	0.0023	ns	ns	ns	ns	C > S; 0.0051
IL-4	ns	ns	ns	ns	ns	ns	ns	ns
IL-6	ns	ns	ns	ns	ns	ns	ns	ns
IL-10	ns	ns	ns	ns	ns	ns	ns	ns
IL-13	ns	ns	ns	ns	ns	ns	ns	ns
IL-18	0.0022	ns	ns	ns	ns	ns	ns	ns
L-selectin	0.0094	ns	ns	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns	ns
TNFα	0.0067	ns	0.0412	ns	ns	ns	ns	ns
VEGF	0.0073	ns	ns	ns	ns	ns	ns	ns

wound healing, this result is consistent with specific modulation of this pathway. Together, these data suggest that while detection of protein changes over time is possible, repeated CSF collection did not result in a generalized alteration of protein analyte detection.

**3.3. 16 of 17 CSF protein analytes were unaltered by a single mTBI when data were analyzed without consideration of sex**

To interrogate the sex differences in post-concussion CSF protein levels, we took a progressive approach to analyzing the data. First, we compared CSF protein levels between concussion and sham to assess how a single concussion alters the proteome of the CSF without consideration for sex. Several publications presenting clinical concussion data ignore sex, often grouping patients into two groups: concussion vs sham (Gard et al., 2023; Daisy et al., 2022; Giza et al., 2021; Hicks et al., 2023; Vedantam et al., 2021). Using this approach, we compared concussion and sham CSF samples normalized to pre-injury levels using two-way mixed effects analysis and post-hoc Holm-Sidak’s multiple comparisons test (Table 2). A single concussion had no significant effect on any of the 17 analytes, except for interferon-γ (IFNγ). For IFNγ

(Fig. 3), both time (F = 14.46; *p* < 0.0001) and injury (F = 5.408; *p* = 0.0270) had significant effects, however the interaction (F = 1.023; *p* = 0.3891) of these two factors had no significant effect.

**3.4. Males exhibit a greater inflammatory response to a single concussion than females**

We next separated samples by sex, comparing concussion and sham animals within each. Using the same statistical approach as before, females showed significant responses to injury in multiple pro-resolving/anti-inflammatory proteins, whereas males showed significant responses to injury in several pro-inflammatory proteins. For females, two-way Mixed Effects analysis (Table 3) identified a significant interaction effect for time and injury on IL-13 (F = 3.066, *p* = 0.0388, Fig. 4A), however, neither time (F = 0.06031, *p* = 0.9103) nor injury (F = 0.001776, *p* = 0.9666) alone had a significant effect on IL-13 levels. Similarly, VEGF (Fig. 4C) showed comparable results, where the interaction of time and injury (F = 5.436, *p* = 0.0049) had a significant effect on VEGF levels, but neither time (F = 2.164, *p* = 0.1352) nor injury (F = 0.006961, *p* = 0.9348) alone had significant effects on VEGF levels.

**Table 5**

Statistical testing comparing injured males to injured females. Two-way mixed effects analysis with post-hoc Holm-Sidak multiple comparisons test comparing male vs female in concussion CSF samples only. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between male and female. The associated *p*-value for significant interactions is reported.

Concussions Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons				
	Protein	Time	Sex	Time × Sex	Concussion (Male vs Female)			
					1 dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	0.0019	ns	ns	ns	ns	ns	
CXCL2	ns	ns	ns	ns	ns	ns	ns	
GM-CSF	ns	0.0085	ns	ns	ns	ns	ns	
ICAM-1	ns	0.0211	ns	ns	ns	ns	ns	
IFN $\gamma$	0.0012	ns	ns	ns	ns	ns	ns	
IL-1a	ns	ns	ns	ns	ns	ns	ns	
IL-1b	ns	0.0108	0.03	ns	ns	ns	ns	
IL-2	ns	ns	0.0191	ns	ns	ns	ns	
IL-4	ns	ns	ns	ns	ns	ns	ns	
IL-6	ns	ns	ns	ns	ns	M > F; 0.0200	ns	
IL-10	ns	0.0186	ns	ns	ns	ns	ns	
IL-13	ns	ns	ns	ns	ns	ns	ns	
IL-18	0.0225	0.0285	ns	ns	ns	M > F; 0.0410	ns	
L-selectin	0.0192	0.0303	ns	ns	ns	ns	ns	
TIMP-1	ns	ns	ns	ns	ns	ns	ns	
TNF $\alpha$	ns	0.0496	ns	ns	M < F; 0.0275	ns	ns	
VEGF	ns	0.0437	0.0014	ns	ns	M > F; 0.0113	ns	

**Table 6**

Statistical testing comparing all four experimental groups. A three-way mixed effects analysis was used to perform statistical analysis on all four experimental groups over time with time, sex, and injury being the three variables tested. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between groups. Calculated *p*-values for all significant interactions are listed in the table.

Protein	Three-way Mixed Effects Analysis						
	Time	Sex	Injury	Time × Sex	Time × Injury	Sex × Injury	Time × Sex × Injury
CXCL3	ns	<0.0001	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns
GM-CSF	ns	0.0196	ns	ns	ns	ns	ns
ICAM-1	0.0115	0.0171	ns	ns	ns	ns	ns
IFN $\gamma$	<0.0001	0.0309	0.0248	ns	ns	ns	ns
IL-1a	0.0134	ns	ns	ns	ns	ns	ns
IL-1b	0.0153	0.0003	ns	0.0144	ns	ns	ns
IL-2	0.0099	ns	ns	ns	ns	ns	0.0194
IL-4	ns	ns	ns	ns	ns	ns	ns
IL-6	ns	ns	ns	ns	ns	ns	ns
IL-10	ns	0.0081	ns	ns	ns	ns	ns
IL-13	ns	ns	ns	ns	ns	ns	ns
IL-18	<0.0001	0.0029	ns	ns	ns	ns	ns
L-selectin	<0.0001	ns	ns	ns	ns	0.0161	ns
TIMP-1	ns	0.0386	ns	ns	ns	ns	ns
TNF $\alpha$	0.0079	0.0114	ns	ns	ns	ns	ns
VEGF	0.0057	0.0012	ns	0.0224	ns	ns	0.0018

Third, time ( $F = 8.068, p = 0.0065$ ) and injury ( $F = 7.458, p = 0.0095$ ) both affected the levels of soluble L-selectin, an adhesion protein found in leukocytes, (Fig. 4B) in injured female rats but not the interaction of time and injury ( $F = 1.267, p = 0.2995$ ).

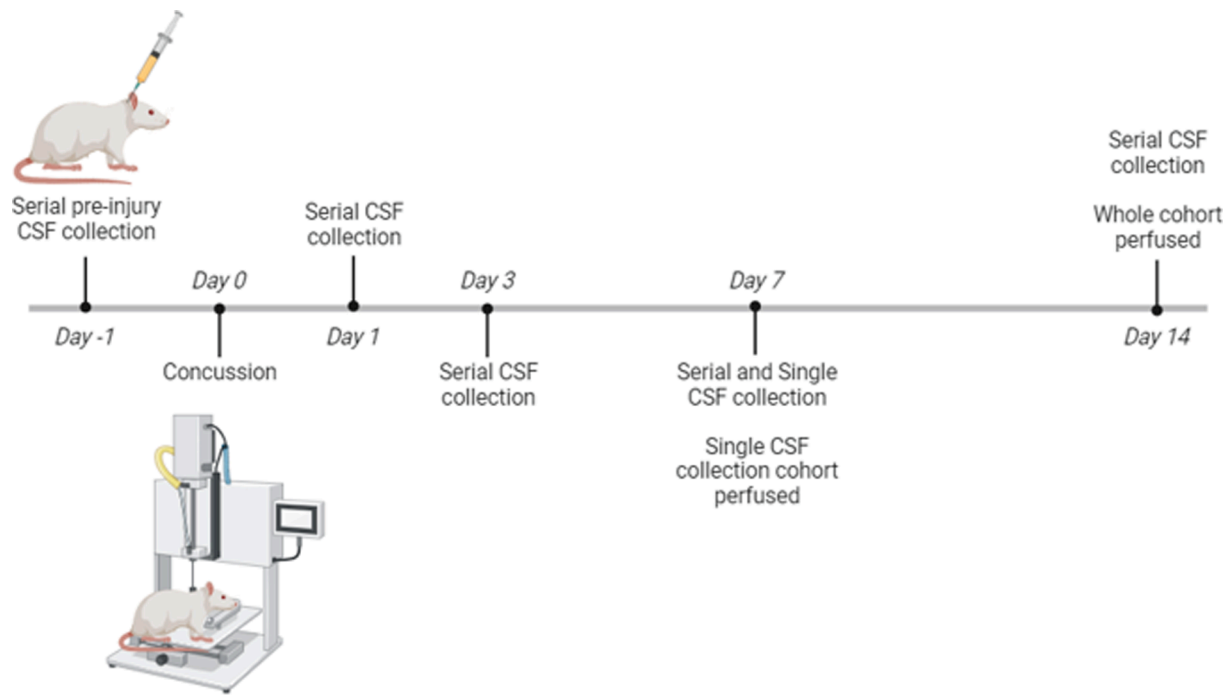
For males, two pro-inflammatory proteins exhibited clear modulation (Table 4). First, a two-way Mixed Effects analysis reported time ( $F = 8.598; p = 0.0005$ ) and the interaction of time and injury ( $F = 5.732; p = 0.0023$ ) to be significant factors affecting interleukin-2 (IL-2, Fig. 4D) levels with a significant increase in IL-2 levels after concussion at 14 dpi ( $p = 0.0051$ ) in male rats. Injury alone was not shown to be a significant factor affecting IL-2 levels over time ( $F = 2.955; p = 0.0933$ ). Second, a two-way Mixed Effects analysis reported time ( $F = 5.549; p = 0.0067$ ) and the interaction of time and injury ( $F = 3.185; p = 0.0412$ ) to be significant factors affecting tumor necrosis factor- $\alpha$  (TNF $\alpha$ , Fig. 4E) levels. Injury alone was not shown to be a significant factor affecting TNF $\alpha$  levels ( $F = 0.07991; p = 0.7813$ ), and there were no significant pairwise comparisons between sham and concussion at any specific timepoints.

Interestingly, both males and females had several proteins give time

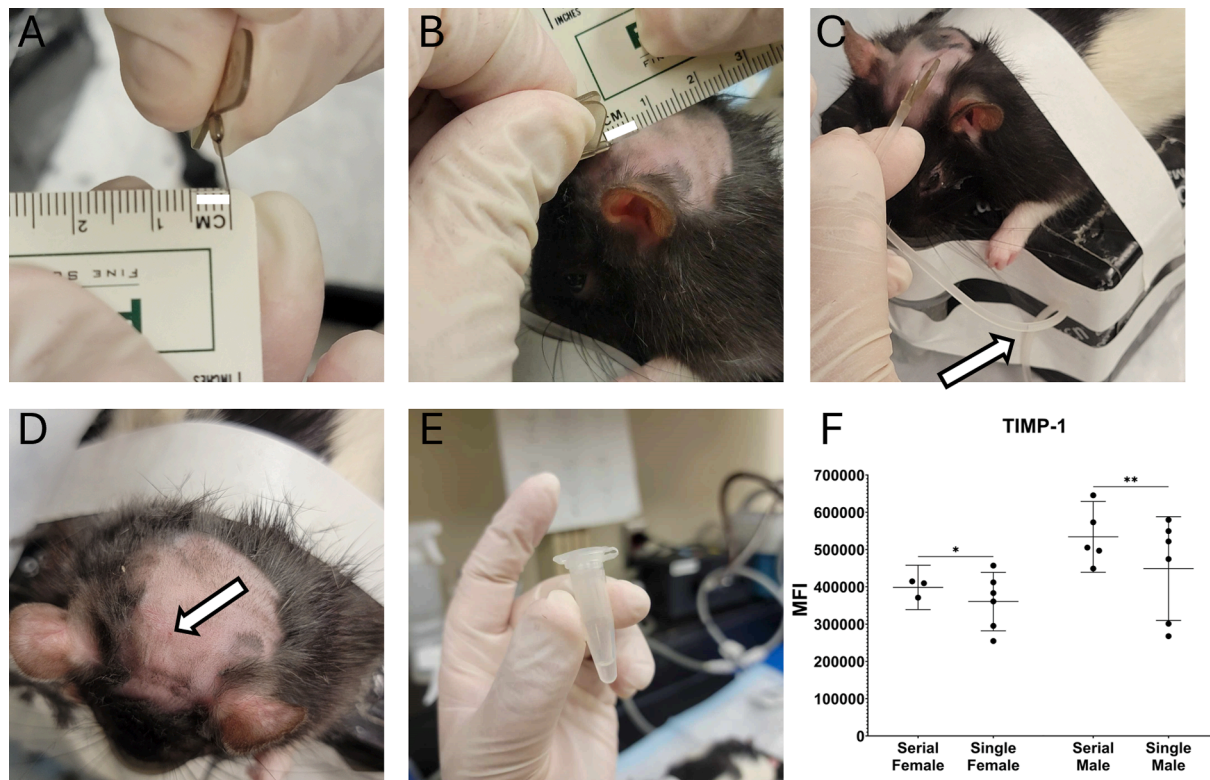
alone as a significant factor affecting protein levels. In females (Table 3), time was significant for GM-CSF ( $F = 3.939, p = 0.0298$ ), IFN $\gamma$  ( $F = 7.900, p = 0.0065$ ), IL-1 $\beta$  ( $F = 5.653, p = 0.0109$ ), and IL-6 ( $F = 3.765, p = 0.0462$ ). L-Selectin ( $F = 6.145, p = 0.0056$ ). In males (Table 4), time was significant for IFN $\gamma$  ( $F = 7.555, p = 0.0111$ ), IL-18 ( $F = 9.450, p = 0.0022$ ), L-selectin ( $F = 7.434, p = 0.0094$ ), and VEGF ( $F = 3.733, p = 0.0413$ ).

**3.5. Sex is a significant factor in CSF protein levels after a single concussion, with males showing significantly greater levels of pro-inflammatory proteins up to 7 dpi**

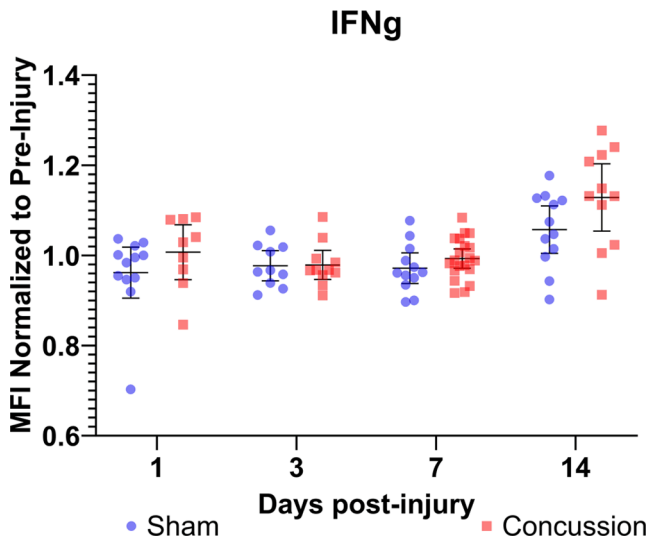
Third, we compared the CSF protein levels of males and females in response to a single concussion (Table 5). Sex was found to be a significant factor affecting the levels of 9 proteins: CXCL3 ( $F = 11.05, p = 0.0019$ , Fig. 5A), GM-CSF ( $F = 7.604; p = 0.0085$ , Fig. 5B), ICAM-1 ( $F = 6.451, p = 0.0211$ , Fig. 5C), IL-1 $\beta$  ( $F = 8.072, p = 0.0108$ , Fig. 5D), IL-10 ( $F = 6.006; p = 0.0186$ , Fig. 5G), IL-18 ( $F = 5.137; p = 0.0285$ , Fig. 5H), L-selectin ( $F = 5.585, p = 0.0303$ , Fig. 5I), TNF $\alpha$  ( $F = 4.083, p = 0.0496$ ,



**Fig. 1.** Experimental timeline schematic. Briefly, pre-injury CSF samples were collected 1 day before injury, except from the single collection CSF group. On day 0, all groups of rats were given either a concussion or were shams. Serial collections of CSF were taken at 1 day, 3 days, 7 days, and 14 days post-injury. At 7 dpi, a group of rats with injury underwent a single CSF collection and were sacrificed. All other groups were sacrificed at 14 dpi.



**Fig. 2.** CSF withdrawal procedure. First, a 27G winged needle tip is bent approximately 90° with a 4 mm tip (emphasized by the white rectangle) (A). The needle is inserted into the foramen magnum, an extremely soft spot approximately 6 mm (emphasized by the white rectangle) past the external occipital protuberance of the skull (B). Clear CSF (white arrow) should flow into the tubing of the winged needle (C). After removal of the needle, there should not be any visible signs of damage on the skin, aside from the insertion point (white arrow) (D). On average, 90–100  $\mu$ L of clear CSF can be collected (E). Our statistics show that collection frequency does not affect the expression of any analyte on the panel, except TIMP1, in males and females (F).



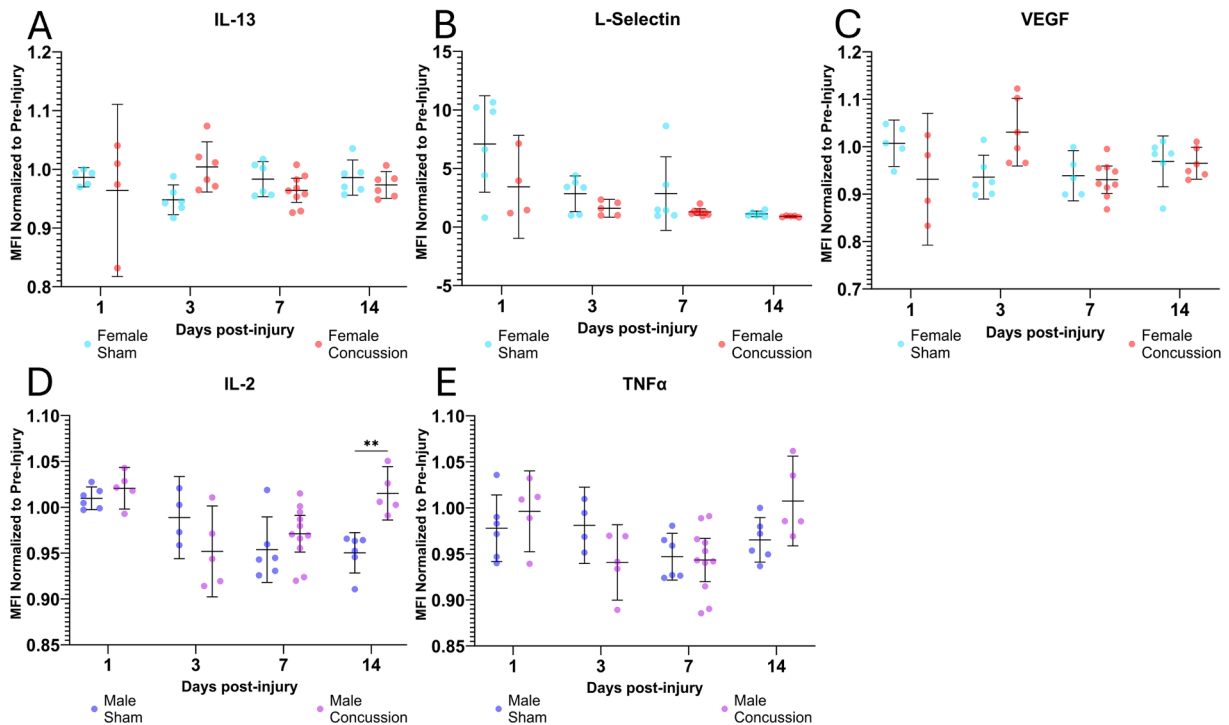
**Fig. 3.** IFN $\gamma$  increases slightly over time after concussion. Graph shows the mean fluorescent intensity (MFI) at each timepoint normalized to pre-injury. Mean with 95 % CI is plotted with individual points representing individual rats.

Fig. 5J), and VEGF ( $F = 4.704, p = 0.0437$ , Fig. 5K). Additionally, time was also found to be a significant factor affecting the levels of IFN $\gamma$  ( $F = 10.45, p = 0.0012$ ), IL-18 ( $F = 5.164, p = 0.0225$ ), and L-selectin ( $F = 6.101, p = 0.0192$ ). Further, the interaction of sex and time was shown to significantly affect the levels of an additional three proteins: IL-1 $\beta$  ( $F = 3.531; p = 0.0300$ , Fig. 5D), IL-2 ( $F = 3.683; p = 0.0191$ , Fig. 5E), and VEGF ( $F = 7.095; p = 0.0014$ , Fig. 5K). Post-hoc multiple comparisons testing comparing CSF protein levels after a single concussion in males vs females at specific timepoints showed no significant differences at 1 dpi and 14 dpi. However, at 3 dpi females had significantly higher TNF $\alpha$

levels than males ( $p = 0.0275$ , Fig. 5J). At 7 dpi, males showed significantly greater levels of three proteins compared to females: IL-6 ( $p = 0.0200$ , Fig. 5F), IL-18 ( $p = 0.0410$ , Fig. 5H), and VEGF ( $p = 0.0113$ , Fig. 5K).

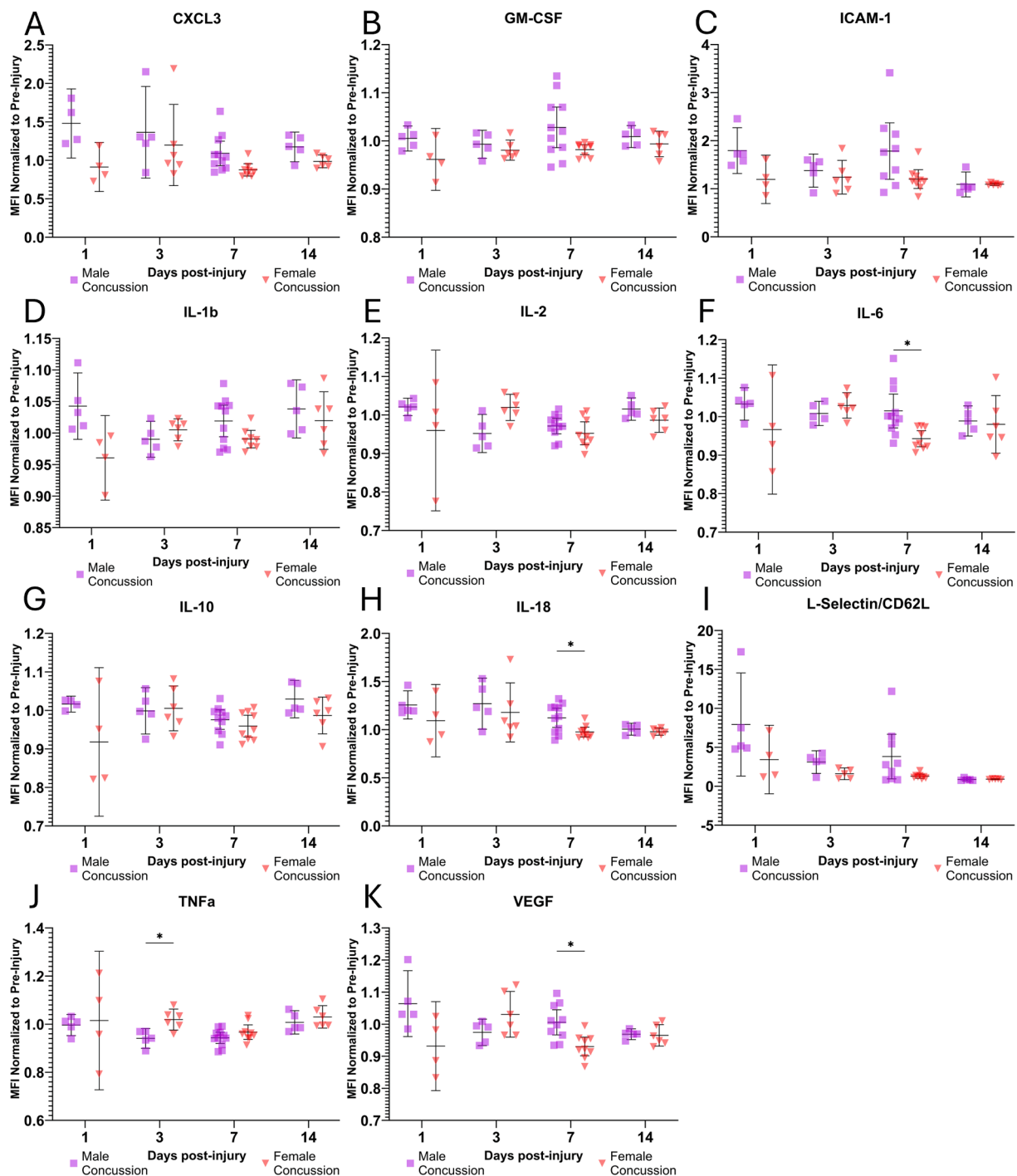
**3.6. Male and female sham animals exhibit baseline differences in CSF protein detection that need to be considered**

The last component of our CSF analysis was comparing all four groups: Male concussion, male sham, female concussion, and female sham in a combined model. To account for an additional variable in this statistical model, a three-way mixed effects analysis (Table 6) was performed, followed by a post-hoc Holm-Sidak multiple comparisons testing (Table 7 and Fig. 6). The three-way mixed effects analysis revealed that sex was a significant factor affecting 10 proteins: CXCL3 ( $F = 37.68, p < 0.0001$ ), GM-CSF ( $F = 5.669, p = 0.0196$ ), ICAM-1 ( $F = 6.461, p = 0.0171$ ), IFN $\gamma$  ( $F = 5.167, p = 0.0309$ ), IL-1 $\beta$  ( $F = 14.34, p = 0.0003$ ), IL-10 ( $F = 8.122, p = 0.0081$ ), IL-18 ( $F = 9.414, p = 0.0029$ ), TIMP-1 ( $F = 4.420, p = 0.0386$ ), TNF $\alpha$  ( $F = 6.700, p = 0.0114$ ), and VEGF ( $F = 12.90, p = 0.0012$ ). Time was a significant factor affecting the levels of 9 proteins: ICAM-1 ( $F = 4.076, p = 0.0115$ ), IFN $\gamma$  ( $F = 14.20, p < 0.0001$ ), IL-1a ( $F = 3.936, p = 0.0134$ ), IL-1 $\beta$  ( $F = 3.688, p = 0.0153$ ), IL-2 ( $F = 4.048, p = 0.0099$ ), IL-18 ( $F = 11.27, p < 0.0001$ ), L-selectin ( $F = 14.72, p < 0.0001$ ), TNF $\alpha$  ( $F = 4.227, p = 0.0079$ ), and VEGF ( $F = 4.713, p = 0.0057$ ). The interaction of time and sex significantly affected the levels of 2 proteins: IL-1 $\beta$  ( $F = 3.737, p = 0.0144$ ) and VEGF ( $F = 3.488, p = 0.0224$ ). Additionally, injury only significantly affected the levels of IFN $\gamma$  ( $F = 5.627, p = 0.0248$ ), but the interaction of sex and injury affected the levels of L-selectin ( $F = 6.588, p = 0.0161$ ). The interaction of time, sex, and injury significantly affects the levels of IL-2 ( $F = 3.492, p = 0.0194$ ) and VEGF ( $F = 5.778, p = 0.0018$ ). The interaction of time and injury did not significantly affect the levels of any protein. In post-hoc multiple comparisons testing, there were several significant pairwise comparisons (Table 7). At 1 dpi, the male



**Fig. 4.** Injury is a significant factor affecting expression in pro-resolving proteins for females and pro-inflammatory proteins for males. The MFI for each sample at each timepoint normalized to pre-injury is reported for IL-13 (A), L-Selectin (B), and VEGF (C) in females, as well as IL-2 (D) and TNF $\alpha$  (E) in males. These proteins were selected based on results from statistical testing reported in Tables 3 and 4. Mean with 95 % CI is plotted with individual points representing individual rats. \*\* $p < 0.01$ .





**Fig. 5.** Males show a delayed inflammatory response to concussion compared to females. The MFI for each sample at each timepoint normalized to pre-injury is reported for CXCL3 (A), GM-CSF (B), ICAM-1 (C), IL-1 $\beta$  (D), IL-2 (E), IL-6 (F), IL-10 (G), IL-18 (H), L-selectin/CD62L (I), TNF $\alpha$  (J), and VEGF (K). These proteins were selected based on results from statistical testing reported in Table 5 showing sex as a significant factor affecting protein expression. Mean with 95 % CI is plotted with individual points representing individual rats. \* $p < 0.05$ .

concussion group had significantly greater levels of IL-1 $\beta$  (Fig. 6F) compared to female sham ( $p = 0.0127$ ) and female concussion ( $p = 0.0320$ ), while the female concussion group had significantly lesser levels of VEGF (Fig. 6M) compared to male sham ( $p = 0.0481$ ) and male concussion ( $p = 0.0303$ ). At 7 dpi, the female concussion group also had significantly lesser levels of CXCL3 (Fig. 6A) compared to male shams ( $p = 0.0460$ ). Furthermore, when comparing how protein levels changed over time within each group, L-selectin (Fig. 6J) dropped significantly from 1 dpi to 14 dpi in both male concussion ( $p = 0.0062$ ) and female

shams ( $p = 0.0172$ ), while VEGF (Fig. 6M) dropped significantly from 3 dpi to 7 dpi in the female concussion group ( $p = 0.0492$ ).

### 3.7. Males exhibit changes in blood–brain-barrier (BBB) permeability and microglia number after a concussion, but not females

In addition to analyzing changes in CSF protein levels, we performed histological characterization of the brain tissue. We quantified the numbers of astrocytes (GFAP+, Fig. S1) and microglia (Iba1+, Fig. 7)

**Table 7**

Post-hoc multiple comparisons testing comparing all four experimental groups. Following a three-way mixed effects analysis, a post-hoc Holm-Sidak multiple comparisons test was used to determine significant pairwise comparisons. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between groups. Calculated *p*-values for all significant interactions are listed in the table. MS = Male Sham. MC = Male Concussion. FS = Female Sham. FC = Female Concussion.

Post-hoc Holm-Sidak Multiple Comparisons Concussion (Sex and Injury Factors)				
Protein	1 dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	ns	(MS > FC; 0.0460)	ns
CXCL2	ns	ns	ns	ns
GM-CSF	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns
IFNg	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns
IL-1b	(MC > FS; 0.0127) (MC > FC; 0.0320)	ns	ns	ns
IL-2	ns	ns	ns	ns
IL-4	ns	ns	ns	ns
IL-6	ns	ns	ns	ns
IL-10	ns	ns	ns	ns
IL-13	ns	ns	ns	ns
IL-18	ns	ns	ns	ns
L-selectin	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns
TNFA	ns	ns	ns	ns
VEGF	(MS > FC; 0.0481) (MC > FC; 0.0303)	ns	ns	ns

Post-hoc Holm-Sidak Multiple Comparisons (Time Factor)				
Protein	Male Sham	Male Concussion	Female Sham	Female Concussion
CXCL3	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns
GM-CSF	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns
IFNg	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns
IL-1b	ns	ns	ns	ns
IL-2	ns	ns	ns	ns
IL-4	ns	ns	ns	ns
IL-6	ns	ns	ns	ns
IL-10	ns	ns	ns	ns
IL-13	ns	ns	ns	ns
IL-18	ns	ns	ns	ns
L-selectin	ns	(1 dpi > 14 dpi; 0.0062)	(1 dpi > 14 dpi; 0.0172)	ns
TIMP-1	ns	ns	ns	ns
TNFA	ns	ns	ns	ns
VEGF	ns	ns	ns	(3 dpi > 7 dpi; 0.0492)

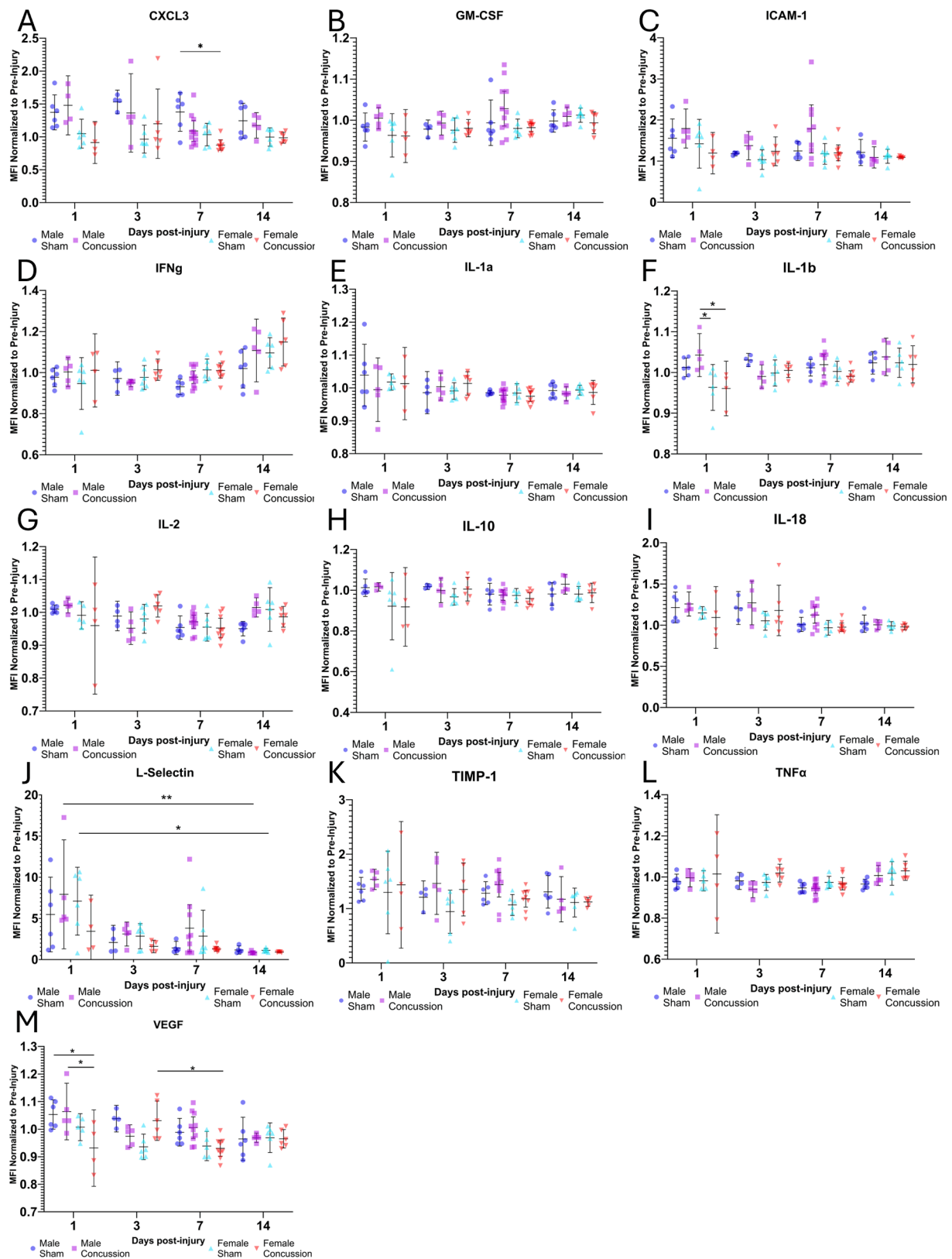
using stereological counting and performed a two-way ANOVA and post-hoc Holm-Šidák's multiple comparisons test on the estimated number of astrocytes. Our two-way ANOVA showed that neither injury ( $p = 0.6664$ ,  $F = 0.4115$ ), sex ( $p = 0.8045$ ,  $F = 0.06237$ ), nor the interaction of the two ( $p = 0.9444$ ,  $F = 0.05730$ ) were significant factors affecting the number of GFAP+ astrocytes. Conversely, our two-way ANOVA showed that injury ( $p = 0.0346$ ,  $F = 3.772$ ) significantly affected the number of Iba1+ microglia. Post-hoc multiple comparisons testing reveals a significant increase ( $p = 0.0151$ ) in Iba1+ microglia from 7 dpi to 14 dpi in males (Fig. 7G). Neither sex ( $p = 0.3142$ ,  $F = 1.048$ ) nor the interaction of sex and injury ( $p = 0.3022$ ,  $F = 1.246$ ) significantly affected the number of microglia. While not significant, there is a slight decrease in the number of microglia at 7 dpi compared to sham in males. There were no significant changes in the number of microglia in females, but at 14 dpi, the number of microglia seems to be diverging between males and females (Fig. 7G).

The trends in this inflammatory cell data correspond well with trends seen in scoring of Prussian Blue staining for microbleeds (Fig. 8). Microbleeds were semi-quantitatively analyzed as previously mentioned. While two-way ANOVA revealed that sex ( $p = 0.0552$ ,  $F = 3.981$ ), injury ( $p = 0.0749$ ,  $F = 2.829$ ), or the interaction of the sex and injury ( $p = 0.0511$ ,  $F = 3.289$ ) did not significantly affect the presence of microbleeds, post-hoc Holm-Šidák's multiple comparisons testing shows

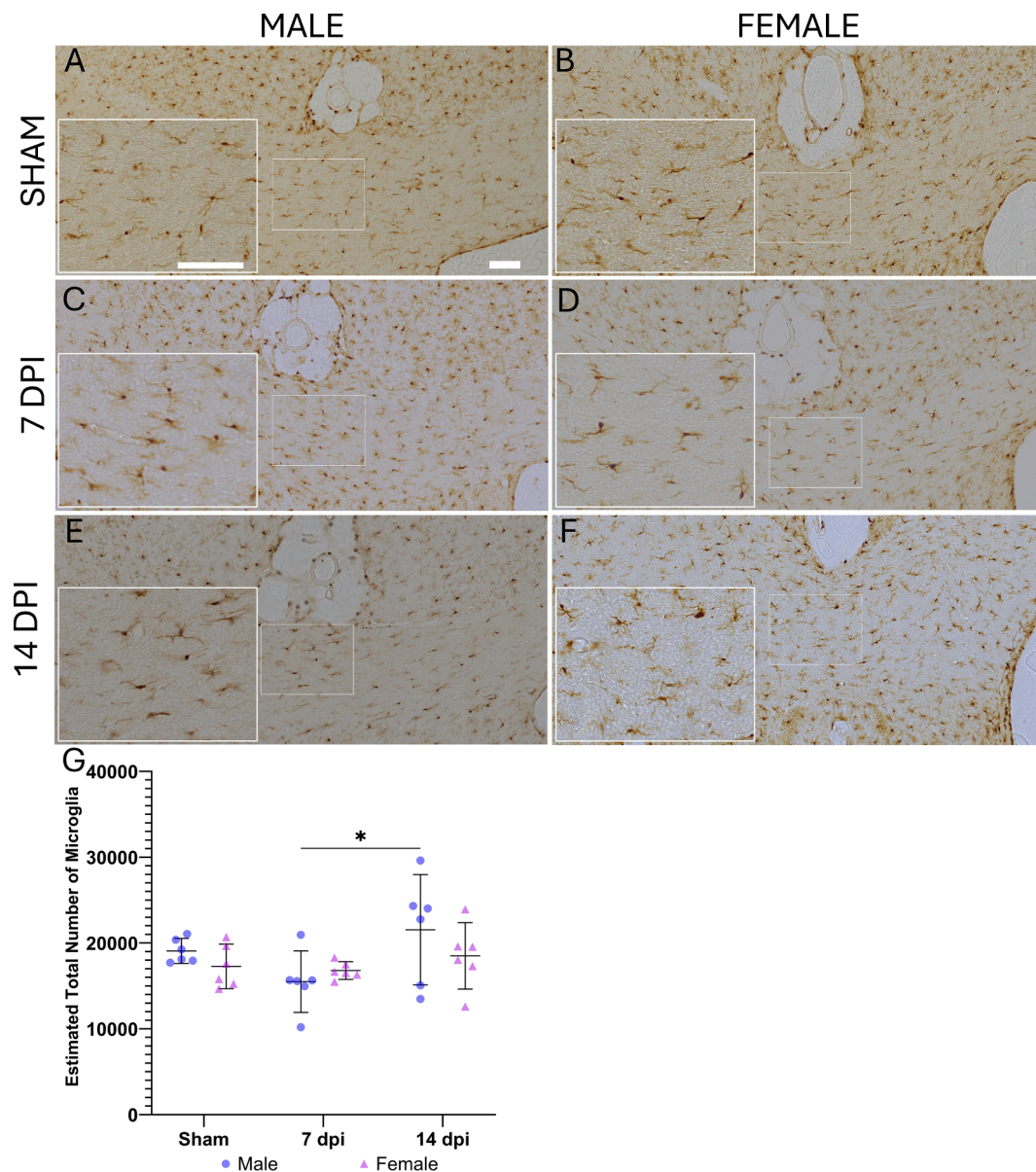
there are significant differences between groups (Fig. 8G). In males, there is a significantly greater presence of microbleeds at 14 dpi compared to sham ( $p = 0.0376$ ) and 7 dpi ( $p = 0.0073$ ). Further, at 14 dpi there is a significantly greater presence of microbleeds in males compared to females ( $p = 0.0029$ ). There are no significant differences in the presence of microbleeds in females. Further, co-staining of Iba1 and Prussian Blue reveal microglial phagocytosis of microbleeds (Fig. 9).

#### 4. Discussion

While mTBI has been widely studied, there are still many aspects of the pathophysiology that remain poorly understood. First, it is unclear if repeated collection of CSF from the same animals will alter the proteome being investigated and confound the results. Second, it has been quite challenging to fully understand the temporal dynamics of the neuro-inflammatory response to mTBI as currently established methods (i.e. stand-alone CSF collection, brain tissue lysates, histology) require large numbers of animals for studies. Third, the majority of mTBI literature fails to investigate sex as a biological variable. This study addressed these limitations.



**Fig. 6.** Males and females have varying levels of analytes in sham groups which can confound analysis. The MFI for each sample at each timepoint normalized to pre-injury is reported for CXCL3 (A), GM-CSF (B), ICAM-1 (C), IFNγ (D), IL-1a (E), IL-1b (F), IL-2 (G), IL-10 (H), IL-18 (I), L-Selectin (J), TIMP-1 (K), TNFα (L), and VEGF (M). These proteins were selected based on results from statistical testing reported in Table 6 where significance was reported. Additionally, significant pairwise comparisons reported in Table 7 are also shown in the corresponding graphs. Mean with 95 % CI is plotted with individual points representing individual rats. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Fig. 7.** Males experience a significant increase in the estimated total number of microglia, but not females, 14 days after a concussion. Stereological quantification of Iba1+ cells was performed on serial sections of brain tissue. Representative images are shown (A-F). Quantification was performed only in the corpus callosum and is reported as the estimated total number of microglia (G). Data is presented as mean with 95 % CI. Statistical analysis was performed using a two-way ANOVA and post-hoc Holm-Sidak multiple comparisons test. \* $p < 0.05$ . Scale bar = 100  $\mu$ m.

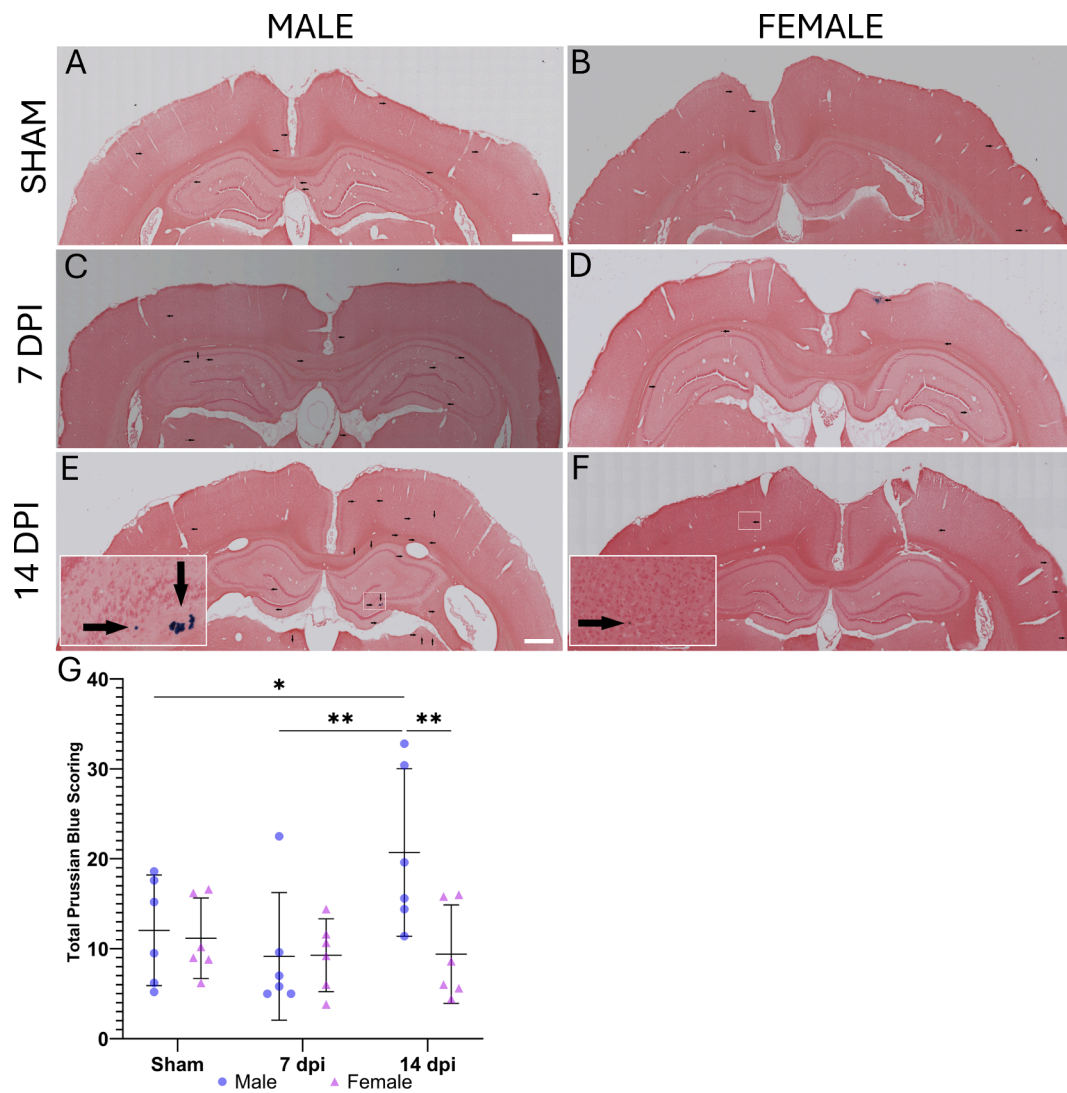
#### 4.1. Serial collection of CSF does not significantly alter CSF inflammation

We first described a method for minimally invasive serial collection of CSF. CSF proteomics are not widely studied in pre-clinical models due to the difficult nature of CSF collection. Previously described methods of CSF collection do not allow for repeated collections, require specialized tools and equipment, and can cause further brain injury or death (Zoltewicz et al., 2013; Han et al., 2023; Amen et al., 2017). The serial collection method used herein did not cause the rats to experience unusually significant weight changes, exhibit evidence of pain/distress (shallow or labored breathing, poor grooming, hunching, or porphyria), show signs of infection or swelling, or die.

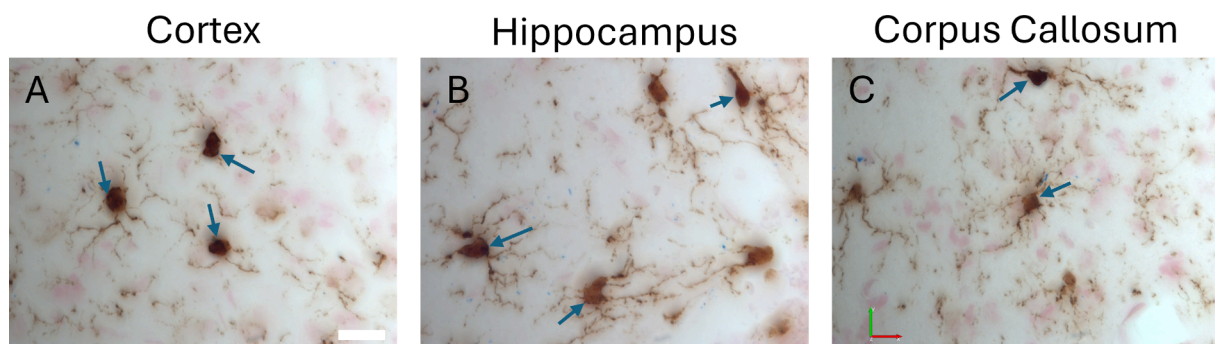
Further, since serial collection of CSF requires multiple punctures with a needle over time, we compared a 7 dpi fresh CSF sample from rats who underwent serial collection at pre-injury, 1 dpi, 3 dpi, 7 dpi, and 14 dpi to a CSF sample from rats who only underwent a single CSF collection at 7 dpi (Fig. 1). A two-way ANOVA revealed that collection

frequency was not a significant factor affecting the proteome results, at least for the 17 analytes we assessed. Interestingly, however, a post-hoc Holm-Šidák's multiple comparisons test revealed that none of the analytes in our panel showed significant differences between serial collection and single collection except TIMP-1 in both males and females (Fig. 2). TIMP-1 is an inhibitor of metalloproteinases that plays a role in alleviating inflammatory pain associated with wound healing (Knight et al., 2019). Given this role for TIMP-1, the elevation in TIMP-1 resulting from repeated collection of CSF makes sense as repeated punctures would cause increased inflammation and prolonged wound healing. This one elevated protein provides confidence in our statistical testing as it shows that the statistical approach has enough power to detect a significant result and supports the assumption that our negative statistical results using similar methods are unlikely to be false negatives.

The establishment of an easily adapted serial CSF collection method is also critical to closing the translational gap as it enables more



**Fig. 8.** Males, but not females, experience a significant increase in the presence of microbleeds 14 days after a concussion. A semi-quantitative scoring system for microbleed presence in the cortex, corpus callosum, and hippocampus was used. Representative images are shown (A-F). Black arrows, added by someone blinded to the experimental groups, signify microbleeds in the images. The total scores of 5 brain sections were summed up for each animal and used for statistical testing (G). Data is presented as mean with 95 % CI. Statistical analysis was performed using a two-way ANOVA and post-hoc Holm-Sidak multiple comparisons test. \* $p < 0.05$ . \*\* $p < 0.01$  Scale bar = 1000  $\mu\text{m}$ . Inset scale bar = 100  $\mu\text{m}$ .



**Fig. 9.** Microglia phagocytose microbleeds. Representative images show colocalized positive staining for Iba1 and microbleeds confirming microglia are responding to BBB permeability in the cortex (A), hippocampus (B), and corpus callosum (C). Scale bar = 20  $\mu\text{m}$ .

longitudinal studies to be performed with a clinically relevant output. Clinically relevant outputs used in longitudinal studies can help address the problem of translating time between rodents and humans. Such a translation of time scale is inherently difficult, as there is no universal conversion factor, presenting a major obstacle in relating preclinical studies to clinical studies.

#### 4.2. In sex-blended analysis, a single concussion only affects the levels of IFN $\gamma$ in the CSF

After showing our method of serial CSF collection is not globally increasing inflammatory protein levels in the CSF, we began to interrogate the effects of a single concussion on inflammation and the potential sex-based differences in analytes from CSF. To do this, we took a stepwise approach to analyzing the levels of proteins in the CSF. First, we asked how CSF protein levels are affected by a single concussion, without considering sex. Many clinical mTBI studies do not consider sex, combining male and female samples, only comparing injured and uninjured groups (Gard et al., 2023; Daisy et al., 2022; Giza et al., 2021; Hicks et al., 2023; Vedantam et al., 2021). This is often due to insufficient numbers of female patient samples. For our statistical test, we combined male and female concussion groups and compared them to combined male and female sham groups, effectively giving us two groups: concussion vs sham. The results from this test were rather interesting, as there were not any significant differences in protein levels over time (Table 2), with the exception of IFN $\gamma$ . IFN $\gamma$  (Fig. 3) was shown to be significantly affected by injury.

IFN $\gamma$  has been widely studied as a biomarker for mTBI, however there is little agreement across studies due to a lack of uniformity in study designs. In sex blended studies, IFN $\gamma$  has been shown to be significantly increased acutely after mTBI in blood samples from children (Ryan et al., 2022) and adults (Vedantam et al., 2021). Further, in a sex blended longitudinal study collecting blood samples from adult mTBI patients at admission to 12 months post injury by Chaban and colleagues, IFN $\gamma$  was shown to be significantly affected by injury in a two-way mixed effects analysis (Chaban et al., 2020). While this result from blood testing directly reflects our finding in this study using CSF, it is important to note that Chaban et al. did not report time as a significant factor influencing IFN $\gamma$  levels. Interestingly, adult patients showed no significant differences in IFN $\gamma$  levels compared to controls in CSF samples taken approximately 17 months after concussion (Gard et al., 2023). It should be noted that the athletes in this sample had a median of 5 concussions prior to CSF collection.

While IFN $\gamma$  was the only protein in our panel that showed significance in our sex blended statistical testing, TNF $\alpha$ , IL-2, IL-6, IL-10, and VEGF are also interesting, as these five proteins have shown significant changes in sex blended human studies. Chaban et al. reported that TNF $\alpha$  expression in adult mTBI patient blood samples was significantly affected by both time and injury (Chaban et al., 2020). The 207 patients in this study sustained a single mild TBI, but the injury was severe enough to result in hospitalization. Vedantam et al. showed that IL-2 and IL-6 are both significantly increased compared to controls at 1 dpi and IL-6 stayed significantly increased up to 6 months post-injury in blood samples from adult mTBI patients, and these changes were associated with more severe symptoms, depression, and/or PTSD. Again, these patients were recruited from injuries serious enough to warrant a trip to the emergency room (Vedantam et al., 2021). Ryan et al. reported that TNF $\alpha$  and IL-10 were significantly decreased, while IL-6 was significantly increased in blood sampled from pediatric mTBI patients within 2 weeks of injury (Ryan et al., 2022). Gard et al. reported that IL-2, TNF $\alpha$ , and VEGF were all significantly increased in the CSF of adult mTBI patients 17 months post-injury (Gard et al., 2023). While our results do not fully reflect what has been published in sex blended clinical studies, it is important to note that while these studies are sex blended, they are still predominantly male patient populations with almost a 2:1 male:female ratio (Gard et al., 2023; Ryan et al., 2022; Vedantam et al., 2021; Chaban

et al., 2020). To fully understand these published results, as well as our results in this study, it is vital to separate the sexes and compare the injury response in each sex.

#### 4.3. A single concussion significantly affects pro-resolving proteins in females and pro-inflammatory proteins in males

The second step we took in our stepwise approach to analyzing this dataset was separating the sexes and comparing the injury response in each sex. This approach gave us two sets of two groups: Concussion vs sham in females (Table 3) and concussion vs sham in males (Table 4). The results from this approach were quite intriguing. In females, injury was a significant factor affecting the levels of soluble L-selectin (Fig. 4B) and the interaction of time and injury was a significant factor affecting the levels of IL-13 (Fig. 4A) and VEGF (Fig. 4C). In males, the interaction of time and injury significantly affected the levels of IL-2 (Fig. 4D) and TNF $\alpha$  (Fig. 4E). IL-13 and VEGF are typically secreted by pro-resolving, anti-inflammatory immune cells, while IL-2 and TNF $\alpha$  are pro-inflammatory proteins (Barreto et al., 2018; Simon et al., 2017; Miao et al., 2020). The trends shown in the data paint an interesting picture. Females experience an acute upregulation of pro-resolving proteins, which have also been shown to be neuroprotective (Miao et al., 2020; Lladó et al., 2013), while males initially experience a decrease in pro-inflammatory proteins that eventually increases at later timepoints, with IL-2 levels being significantly greater in concussed males compared to sham males at 14 dpi, which reflects previously published male-biased sex blended studies (Gard et al., 2023; Vedantam et al., 2021). One possible explanation of this paradigm is the differential presence of estrogen and progesterone, which have been shown to be neuroprotective after TBI, reducing mortality and improving neurological outcomes, by reducing neuronal apoptosis, inhibiting microglial and astrocytic activation, and decreasing brain edema (Brotfain et al., 2016; Chakrabarti et al., 2016; Wang et al., 2021; Zlotnik et al., 2012). To further dissect the sex differences in the inflammatory response after concussion, it is imperative to compare CSF protein levels after concussion across both sexes directly.

#### 4.4. Males experience a biphasic and more prolonged inflammatory response compared to females

The third phase of our stepwise approach to analyzing CSF protein concentration after concussion was comparing the protein levels in both concussion groups, giving us two groups for comparison: male vs female. In this comparison, many sex-based differences were revealed. Sex was a factor significantly affecting the levels of 9 of 17 proteins and the interaction of sex and time was a factor significantly affecting the levels of 3 of these proteins (Table 5). There is a clear trend present in the time course representations of each of these proteins (Fig. 5). While there are only four significant pairwise comparisons in post-hoc multiple comparisons testing, in general, males experience a more rapid inflammatory response, with all proteins being greater in males than females, except TNF $\alpha$ , at 1 dpi. At 3 dpi, females have significantly greater TNF $\alpha$  than males, as well as greater levels of IL-1 $\beta$ , IL-2, IL-6, and VEGF, suggesting that females undergo a delayed inflammatory response. At 7 dpi, males experience a second peak in inflammation with significantly greater levels of IL-6, IL-18, and VEGF, as well as greater levels of CXCL3, GM-CSF, ICAM-1, IL-1 $\beta$ , IL-2, and L-selectin, than females. This persists at 14 dpi, with males exhibiting greater levels of CXCL3, GM-CSF, IL-1 $\beta$ , IL-2, and IL-10, though none of these are significant, than females.

Our results are supported by a previous study evaluating serum and brain cytokine concentrations in mice 4 h after the last injury in a repetitive blast-related mTBI model (Baskin et al., 2023). Baskin et al. reported that female mice experience greater anti-inflammatory signaling acutely after mTBI, with significantly higher concentrations of IL-10 and G-CSF and significantly lower concentrations of IL-9, IL-12,

and IP-10 in brain tissue homogenates, compared to male mice (Baskin et al., 2023). While few studies, especially longitudinal ones, have compared protein levels in biofluids sampled from mTBI patients across sexes, it is critical to analyze these sex differences more holistically, as biomarker bioactivity may vary between sexes. For example, Di Battista et al. reported no significant differences in plasma concentrations of IFN $\gamma$  or TNF $\alpha$  between male and female mTBI patients (Di Battista et al., 2020). However, in males, they found that higher IFN $\gamma$  concentrations in blood were significantly correlated with more severe concussion symptoms, whereas in females, higher concentrations of IFN $\gamma$  and TNF $\alpha$  were significantly correlated with less severe symptoms (Di Battista et al., 2020). This data suggests there are underlying differences in the mechanisms regulating how males and females respond to inflammation. Studies comparing male and female injury responses to mTBI are valuable for bridging the sex-based knowledge gap, and more studies investigating these sex-based differences are clearly needed. With that said, directly comparing the injuries between sexes is not the complete picture. It is imperative to understand uninjured baseline differences between sexes as well, especially in longitudinal biomarker studies such as the present one.

#### 4.5. Statistical analysis of sham and injured groups for both sexes reveals that sex is a significant variable affecting inflammatory protein expression

The last phase in our stepwise approach to analyzing this dataset incorporates the sham groups into the comparisons. By including sham groups into our statistical model, we now have three variables being tested: time, sex, and injury. Including a third variable increases the number of comparisons, requiring a different statistical approach to reduce Type I errors. We employed a three-way mixed effects analysis followed by a post-hoc Holm-Sidak multiple comparisons test to account for the potential increase in Type I errors. A major point to note with this level of statistical testing is that by increasing the number of groups, the number of comparisons also increases, which decreases the statistical power of post-hoc multiple comparisons testing (Lee and Lee, 2018). This is critical to keep in mind, as it can result in a comparison being reported as negative, even though with fewer comparisons, it may have been positive. It is possible that this phenomenon is occurring in this study as none of the reported significant pairwise comparisons in the previous statistical models are reported in the three-way mixed effects analysis and post-hoc testing. While this statistical model does report significant pairwise comparisons, it fails to confirm the significant pairwise comparisons reported in less stringent statistical models (Table 7).

A three-way mixed effects analysis is the most conservative approach to investigating the sex-dependent differences in inflammatory protein expression in the CSF after a concussion; it is also the most appropriate. Despite it being conservative in terms of statistical power, our results revealed that sex or the interaction of sex with time and/or injury significantly affected the levels of 12 of the 17 proteins in the Luminex panel (Table 6). Additionally, injury or the interaction of injury with sex or sex and time significantly affected the levels of 4 of the 17 proteins in the panel. Injury only affecting 4 of the 17 proteins does not seem unreasonable as a concussion is a mild TBI. In a more severe TBI, we would expect to have much greater changes in the inflammatory response after concussion. However, sex affecting the levels of 12 proteins in a mild concussion highlights the need for investigating sex-dependent differences in concussions in order to understand the complete pathophysiology of the injury, while also including the shams in comparing the injury responses, as this data shows baseline differences that could be confounding the results here. For example, at 1 dpi, the male concussion group experienced significantly greater levels of IL-1 $\beta$  (Fig. 6F) compared to both female sham and female concussion. In comparison, the female concussion group experienced significantly lesser levels of VEGF (Fig. 6M) compared to both the male sham and male concussion groups. Shams having results that vary significantly from the injured

groups of the opposite sex makes it unclear if the concussion group differences are due to injury or simply due to inherent sex-based baseline differences. Nonetheless, investigating sex-dependent differences in post-concussion inflammation over time using a three-way mixed effects analysis or three-way repeated measures ANOVA is the most appropriate to account for all major variables in such an approach. Further, while this study is small, the 12 proteins that were significantly affected by sex, and the 4 proteins that were significantly affected by injury, warrant further investigation in future studies for their role in neuro-inflammatory signaling after concussion.

#### 4.6. Presence of microbleeds corresponds with increase in Iba1+ microglia number

Despite a concussion being a mild traumatic brain injury, there are still noticeable differences in brain pathology between sexes after a concussion. We performed histological characterization using a quantitative and semi-quantitative methodology to assess the presence of Iba1+ microglia (Fig. 7) and GFAP+ astrocytes (Fig. S1), as well as microbleeds (Fig. 8), respectively. Stereological quantification of Iba1+ microglia, in the corpus callosum shows a significant increase in the estimated total number of microglia in the corpus callosum of males at 14 dpi compared to 7 dpi (Fig. 7G). Stereological quantification of GFAP+ astrocytes in the corpus callosum showed no significant differences between any groups. The overall trend of this data corresponds well with the semi-quantitative scoring of microbleed presence, where each instance of a microbleed is graded on a scale of 0–3 (0 = none, 1 = a few granules, 2 = several granules, 3 = a lot of granules). This scoring reveals that males experience a significantly greater presence of microbleeds at 14 dpi compared to male sham, males at 7 dpi, and females at 14 dpi (Fig. 8G). This correlation makes sense, as microglia are known to phagocytose blood products after hemorrhage (Yu et al., 2022; Chen et al., 2022). To confirm this response, co-staining of Iba1 and Prussian Blue was performed and showed multiple Iba1+ microglia phagocytosed microbled byproducts (Fig. 9). This data suggests the possibility that the pro-inflammatory CSF protein expression seen in males after a concussion could be driven by an increase in pro-inflammatory microglia polarization due to an increase in BBB permeability. Future studies should investigate variability in microglial phagocytosis between sexes as a possible mechanism underlying the sex-dependent differences in neuroinflammation after concussion. Detection of leakage or microbleeds could also utilize fluorescent dextrans of varying molecular weights, or IHC for Von Willebrand factor, CD-31 or PECAM-1 (Sahyouni et al., 2017).

#### 4.7. Limitations

This study was designed with three goals. First, to establish a method of minimally invasive serial CSF collection that could be used by any laboratory without specialized tools to enable future longitudinal studies utilizing clinically relevant outputs. Second, to determine if the serial collection of CSF altered the CSF proteome in the absence of an experimental condition. Third, to establish a framework for interrogating sex differences in levels of biomarkers for mTBI in rats. While this study accomplishes all three goals, it is not without its limitations. First, the sample size in the present study is small. Each group at each time-point has  $n = 4–6$  rats. While group numbers are sufficient for statistical analysis, groups of 10–12 rats would be ideal and would allow for testing of behavioral outcomes in future interventional studies. Second, it is extremely likely that there are other proteins that play a significant role in mTBI inflammation that are not addressed in this study. This is mainly due to the lack of analytes commercially available in multiplex immunoassay kits for rats. As more and more kits designed for rats come to market, studies like this will be able to be expanded. Third, this study was focused on CSF inflammation. However, future studies would benefit from comparing inflammation in the CSF to systemic

inflammation using blood-based biomarkers, given the increased clinical relevancy of blood testing. This would allow for easier translation of any biomarker discoveries. Last, a limitation of this study is the absence of behavioral data. Future studies should aim to correlate clinically relevant biomarkers over time with functional outputs like behavior to maximize the amount of data that can be generated with the least number of animals.

## 5. Conclusion

This study was designed to accomplish three goals. First, to describe a method of minimally invasive serial CSF collection. The method described in this study can easily be adapted by any laboratory prepared for animal studies. There is no need for special tools or custom-built equipment. Second, to confirm that serial collection of the CSF does not alter CSF protein levels. Of the 17 analytes we tested, 16 showed no difference between serial collection and single collection. TIMP-1 was significantly increased which is expected as TIMP-1 is involved in alleviating inflammatory pain related to wound healing. Third, to establish a framework for assessing sex differences in neuroinflammation after a concussion. We showed that results vary based on the framing of the statistical test. However, it is evident that males experience a more robust inflammatory response to a single concussion than females, possibly caused by increased BBB permeability and microglial polarization. Future studies should adopt this method of serial CSF collection to perform longitudinal studies to better understand the temporal dynamics of neuroinflammation not just after neurotrauma, but any neurological disorder.

## CRediT authorship contribution statement

**Josh Karam:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nimrah Ashfaq:** Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Cynthia Benitez:** Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Victor Morales:** Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Elizabeth Partida:** Project administration, Methodology, Data curation, Conceptualization. **Michelle Hernandez:** Writing – original draft, Visualization, Formal analysis, Data curation. **Jordan Yokoyama:** Writing – original draft, Visualization, Formal analysis, Data curation. **Alyssa Villegas:** Visualization, Formal analysis, Data curation. **Brielle Brown:** Writing – original draft, Visualization, Formal analysis, Data curation. **Pooja Sakthivel:** Visualization, Formal analysis, Data curation. **Aileen J. Anderson:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Brian J. Cummings:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Ethics declaration

All experiments, including animal housing conditions, surgical procedures, and postoperative care, were in accordance with the Institutional Animal Care and Use Committee guidelines at the University of California–Irvine (UCI).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2024.11.015>.

## Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## References

- Amen, E.M., Brecheisen, M., Sach-Peltason, L., Bergadano, A., 2017. Refinement of a model of repeated cerebrospinal fluid collection in conscious rats. *Lab Anim.* 51, 44–53. [https://doi.org/10.1177/0023677216646069/ASSET/IMAGES/LARGE/10.1177\\_0023677216646069-FIG2.JPEG](https://doi.org/10.1177/0023677216646069/ASSET/IMAGES/LARGE/10.1177_0023677216646069-FIG2.JPEG).
- Barreto, G.E., Thelin, E.P., Zhang, Y., Madathil, S.K., Wilfred, B.S., Urankar, S.E., et al., 2018. Early microglial activation following closed-head concussive injury is dominated by pro-inflammatory M-1 type. *Front. Neurol.* 9, 414936. <https://doi.org/10.3389/FNEUR.2018.00964>.
- Baskin, B.M., Logsdon, A.F., Janet Lee, S., Foresi, B.D., Peskind, E., Banks, W.A., et al., 2023. Timing matters: sex differences in inflammatory and behavioral outcomes following repetitive blast mild traumatic brain injury. *Brain Behav. Immun.* 110, 222–236. <https://doi.org/10.1016/j.bbi.2023.03.003>.
- Battlori, M., Casado, M., Sierra, C., del C. Salgado, M., Marti-Sanchez, L., Maynou, J., et al., 2019. Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins. *Fluids Barriers CNS* 16, 1–9. <https://doi.org/10.1186/S12987-019-0154-5/TABLES/2>.
- Beckmann, L., Obst, S., Labusek, N., Abberger, H., Köster, C., Klein-Hitpass, L., et al., 2022. Regulatory T cells contribute to sexual dimorphism in neonatal hypoxic-ischemic brain injury. *Stroke* 53, 381–390. <https://doi.org/10.1161/STROKEAHA.121.037537/FORMAT/EPUB>.
- Breen, E.J., Polaskova, V., Khan, A., 2015. Bead-based multiplex immuno-assays for cytokines, chemokines, growth factors and other analytes: median fluorescence intensities versus their derived absolute concentration values for statistical analysis. *Cytokine* 71, 188–198. <https://doi.org/10.1016/J.CYTO.2014.10.030>.
- Breen, E.J., Tan, W., Khan, A., 2016. The statistical value of raw fluorescence signal in luminex xMAP based multiplex immunoassays. *Sci. Rep.* 6. <https://doi.org/10.1038/SREP26996>.
- Brotfain, E., Gruenbaum, S.E., Boyko, M., Kutz, R., Zlotnik, A., Klein, M., 2016. Neuroprotection by estrogen and progesterone in traumatic brain injury and spinal cord injury. *Curr. Neuropharmacol.* 14, 641. <https://doi.org/10.2174/1570159X14666160309123554>.
- Capizzi, A., Woo, J., Verduzco-Gutierrez, M., 2020. Traumatic brain injury an overview of epidemiology, pathophysiology, and medical management. *Med. Clin. N. Am.* 104, 213–238. <https://doi.org/10.1016/j.mcna.2019.11.001>.
- Chaban, V., Clarke, G.J.B., Skandsen, T., Islam, R., Einarsen, C.E., Vik, A., et al., 2020. Systemic inflammation persists the first year after mild traumatic brain injury: results from the prospective trondheim mild traumatic brain injury study. *J. Neurotrauma* 37, 2120–2130. <https://doi.org/10.1089/neu.2019.6963>.
- Chakrabarti, M., Das, A., Samantaray, S., Smith, J.A., Banik, N.L., Haque, A., et al., 2016. Molecular mechanisms of estrogen for neuroprotection in spinal cord injury and traumatic brain injury. *Rev. Neurosci.* 27, 271–281. <https://doi.org/10.1515/revneuro-2015-0032>.
- Chen, W., Zhang, Y., Zhai, X., Xie, L., Guo, Y., Chen, C., et al., 2022. Microglial phagocytosis and regulatory mechanisms after stroke. *J. Cereb. Blood Flow Metab.* 42, 1579–1596. <https://doi.org/10.1177/0271678X221098841>.
- Daisy, C.C., Varinos, S., Howell, D.R., Kaplan, K., Mannix, R., Meehan, W.P., et al., 2022. Proteomic discovery of noninvasive biomarkers associated with sport-related concussions. *Neurology* 98, E186–E198. <https://doi.org/10.1212/WNL.00000000000013001>.



- Di Battista, A.P., Churchill, N., Rhind, S.G., Richards, D., Hutchison, M.G., 2020. The relationship between symptom burden and systemic inflammation differs between male and female athletes following concussion. *BMC Immunol.* 21. <https://doi.org/10.1186/s12865-020-0339-3>.
- Feigin, V.L., Nichols, E., Alam, T., Bannick, M.S., Beghi, E., Blake, N., et al., 2019. Global, regional, and national burden of neurological disorders, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol.* 18, 459–480.
- Fraunberger, E.A., Dejesus, P., Zanier, E.R., Shutt, T.E., Esser, M.J., 2020. Acute and persistent alterations of cerebellar inflammatory networks and glial activation in a rat model of pediatric mild traumatic brain injury. *J. Neurotrauma* 37, 1315–1330. <https://doi.org/10.1089/neu.2019.6714>.
- Gard, A., Vedung, F., Piehl, F., Khademi, M., Wernersson, M.P., Rorsman, I., et al., 2023. Cerebrospinal fluid levels of neuroinflammatory biomarkers are increased in athletes with persistent post-concussive symptoms following sports-related concussion. *J. Neuroinflamm.* 20, 1–10. <https://doi.org/10.1186/s12974-023-02864-0/FIGURES/2>.
- Giza, C.C., Hovda, D.A., 2014. The new neurometabolic cascade of concussion. *Neurosurgery* 75, S24–S33. <https://doi.org/10.1227/NEU.0000000000000505>.
- Giza, C.C., McCreary, M., Huber, D., Cameron, K.L., Houston, M.N., Jackson, J.C., et al., 2021. Assessment of blood biomarker profile after acute concussion during combative training among US military cadets: a prospective study from the ncaa and us department of defense CARE consortium. *JAMA Netw. Open* 4, E2037731. <https://doi.org/10.1001/JAMANETWORKOPEN.2020.37731>.
- Gold, E.M., Vasilevko, V., Hasselmann, J., Tiefenthaler, C., Hoa, D., Ranawaka, K., et al., 2018. Repeated mild closed head injuries induce long-term white matter pathology and neuronal loss that are correlated with behavioral deficits. *ASN Neuro* 10. [https://doi.org/10.1177/1759091418781921/ASSET/IMAGES/LARGE/10.1177\\_1759091418781921-FIG2.JPEG](https://doi.org/10.1177/1759091418781921/ASSET/IMAGES/LARGE/10.1177_1759091418781921-FIG2.JPEG).
- Grovala, M.R., Von Reyn, C., Loane, D.J., Cullen, D.K., 2023. Understanding microglial responses in large animal models of traumatic brain injury: an underutilized resource for preclinical and translational research. *J. Neuroinflamm.* 20, 67. <https://doi.org/10.1186/s12974-023-02730-z>.
- Guan, B., Anderson, D.B., Chen, L., Feng, S., Zhou, H., 2023. Global, regional and national burden of traumatic brain injury and spinal cord injury, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *BMJ Open* 13. <https://doi.org/10.1136/BMJOPEN-2023-075049>.
- Han, J.R., Yang, Y., Wu, T.W., Shi, T.T., Li, W., Zou, Y., 2023. A minimally-invasive method for serial cerebrospinal fluid collection and injection in rodents with high survival rates. *Biomedicines* 11. <https://doi.org/10.3390/biomedicines11061609>.
- Heyburn, L., Batuure, A., Wilder, D., Long, J., Sajja, V.S., 2023. Neuroinflammation profiling of brain cytokines following repeated blast exposure. *Int. J. Mol. Sci.* 24, 12564. <https://doi.org/10.3390/IJMS241612564>.
- Hicks, S.D., Onks, C., Kim, R.Y., Zhen, K.J., Loeffert, J., Loeffert, A.C., et al., 2023. Refinement of saliva microRNA biomarkers for sports-related concussion. *J Sport Heal. Sci.* 12, 369–378. <https://doi.org/10.1016/j.jshs.2021.08.003>.
- Howell, D.R., Southard, J., 2021. The molecular pathophysiology of concussion. *Clin. Sports Med.* 40, 39–51. <https://doi.org/10.1016/J.CSM.2020.08.001>.
- Knight, B.E., Kozlowski, N., Havelin, J., King, T., Crocker, S.J., Young, E.E., et al., 2019. TIMP-1 attenuates the development of inflammatory pain through MMP-dependent and receptor-mediated cell signaling mechanisms. *Front. Mol. Neurosci.* 12. <https://doi.org/10.3389/FNMOL.2019.00220/FULL>.
- Lee, S., Lee, D.K., 2018. What is the proper way to apply the multiple comparison test? *Korean J. Anesthesiol.* 71, 353–360. <https://doi.org/10.4097/kja.d.18.00242>.
- Levy, A.M., Saling, M.M., Anderson, J.F.I., 2023. Frequency and extent of cognitive complaint following adult civilian mild traumatic brain injury: a systematic review and meta-analysis. *Brain Impair* 309–332. <https://doi.org/10.1017/BrImp.2022.19>.
- Liu (Steve), S., Pickens, S., Barta, Z., Rice, M., Dagher, M., Lebens, R., et al., 2023. Neuroinflammation drives sex-dependent effects on pain and negative affect in a murine model of repeated mild traumatic brain injury. *Pain.* <https://doi.org/10.1097/j.pain.0000000000003084>.
- Liu, M.C., Akinyi, L., Scharf, D., Mo, J., Larner, S.F., Muller, U., et al., 2010. Ubiquitin C-terminal hydrolase-L1 as a biomarker for ischemic and traumatic brain injury in rats. *Eur. J. Neurosci.* 31, 722–732. <https://doi.org/10.1111/j.1460-9568.2010.07097.x>.
- Lladó, J., Tolosa, L., Olmos, G., 2013. Cellular and molecular mechanisms involved in the neuroprotective effects of VEGF on motoneurons. *Front. Cell. Neurosci.* 7, 64456. <https://doi.org/10.3389/FNCEL.2013.00181/BIBTEX>.
- Miao, W., Zhao, Y., Huang, Y., Chen, D., Luo, C., Su, W., et al., 2020. IL-13 ameliorates neuroinflammation and promotes functional recovery after traumatic brain injury. *J. Immunol.* 204, 1486–1498. <https://doi.org/10.4049/jimmunol.1900909>.
- Neale, K.J., Reid, H.M.O., Sousa, B., McDonagh, E., Morrison, J., Shultz, S., et al., 2023. Repeated mild traumatic brain injury causes sex-specific increases in cell proliferation and inflammation in juvenile rats. *J. Neuroinflamm.* 20. <https://doi.org/10.1186/s12974-023-02916-5>.
- Ryan, E., Kelly, L., Stacey, C., Huggard, D., Duff, E., McCollum, D., et al., 2022. Mild-to-severe traumatic brain injury in children: altered cytokines reflect severity. *J. Neuroinflammation* 19, 36. <https://doi.org/10.1186/s12974-022-02390-5>.
- Sahyouni, R., Gutierrez, P., Gold, E., Robertson, R.T., Cummings, B.J., 2017. Effects of concussion on the blood–brain barrier in humans and rodents. *J. Concussion* 1. <https://doi.org/10.1177/2059700216684518>.
- Shultz, S.R., McDonald, S.J., Haar, C.V., Meconi, A., Vink, R., Van Donkelaar, P., et al., 2017. The potential for animal models to provide insight into mild traumatic brain injury: translational challenges and strategies. *Neurosci. Biobehav. Rev.* 76, 396–414. <https://doi.org/10.1016/j.neubiorev.2016.09.014>.
- Simon, D.W., McGeachy, M.J., Baylr, H., Clark, R.S.B., Loane, D.J., Kochanek, P.M., 2017. The far-reaching scope of neuroinflammation after traumatic brain injury. *Nat. Rev. Neurol.* 171–191. <https://doi.org/10.1038/nrneurol.2017.13>.
- Vedantam, A., Brennan, J., Levin, H.S., McCarthy, J.J., Dash, P.K., Redell, J.B., et al., 2021. Early versus late profiles of inflammatory cytokines after mild traumatic brain injury and their association with neuropsychological outcomes. *J. Neurotrauma* 38, 53–62. <https://doi.org/10.1089/neu.2019.6979>.
- Villapol, S., Loane, D.J., Burns, M.P., 2017. Sexual dimorphism in the inflammatory response to traumatic brain injury. *Glia* 65, 1423–1438. <https://doi.org/10.1002/glia.23171>.
- Visser, K., de Koning, M.E., Ciubotariu, D., Kok, M.G.J., Sibeijn-Kuiper, A.J., Bourgonje, A.R., et al., 2023. An exploratory study on the association between blood-based biomarkers and subacute neurometabolic changes following mild traumatic brain injury. *J. Neurol.* <https://doi.org/10.1007/s00415-023-12146-7>.
- Wang, J., Hou, Y., Zhang, L., Liu, M., Zhao, J., Zhang, Z., et al., 2021. Estrogen attenuates traumatic brain injury by inhibiting the activation of microglia and astrocyte-mediated neuroinflammatory responses. *Mol. Neurobiol.* 58, 1052–1061. <https://doi.org/10.1007/s12035-020-02171-2>.
- Wang, K.K., Yang, Z., Zhu, T., Shi, Y., Rubenstein, R., Tyndall, J.A., et al., 2018. An update on diagnostic and prognostic biomarkers for traumatic brain injury. *Expert Rev. Mol. Diagn.* 165–180. <https://doi.org/10.1080/14737159.2018.1428089>.
- Yao, C., Williams, A.J., Ottens, A.K., Lu, X.C.M., Liu, M.C., Hayes, R.L., et al., 2009. P43/pro-EMAPII: a potential biomarker for discriminating traumatic versus ischemic brain injury. *J. Neurotrauma* 26, 1295–1305. <https://doi.org/10.1089/neu.2008.0811>.
- Yu, F., Wang, Y., Stetler, A.R., Leak, R.K., Hu, X., Chen, J., 2022. Phagocytic microglia and macrophages in brain injury and repair. *CNS Neurosci. Ther.* 28, 1279–1293. <https://doi.org/10.1111/cns.13899>.
- Zlotnik, A., Leibowitz, A., Gurevich, B., Ohayon, S., Boyko, M., Klein, M., et al., 2012. Effect of estrogens on blood glutamate levels in relation to neurological outcome after TBI in male rats. *Intensive Care Med.* 38, 137–144. <https://doi.org/10.1007/S00134-011-2401-3>.
- Zoltewicz, J.S., Mondello, S., Yang, B., Newsom, K.J., Kobeissy, F., Yao, C., et al., 2013. Biomarkers track damage after graded injury severity in a rat model of penetrating brain injury. *J. Neurotrauma* 30, 1161–1169. <https://doi.org/10.1089/NEU.2012.2762/ASSET/IMAGES/LARGE/FIGURE5.JPEG>.