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 diability. 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 diability[1]. 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[2–4]. mTBIs commonly occur in falls, motor vehicle crashes, contact sports, military duty, and incidences of domestic violence[2,5–7]. While the typical prognosis of a single concussion is positive, with full recovery within 3 months[3], approximately 15 percent of patients also experience post-concussion symptoms (PCS) that are exacerbated with repeated mTBIs[3,6,8,9]

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[10–12]. 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[5,6,8,13]. Further, there are differences in the inflammatory response based on injury severity. In a study analyzing whole blood from pediatric TBI patients, severe TBI caused greater concentrations of TNFα, IL-6, IL-8, and IL-10 compared to mTBI. However, mTBI patients experience a greater concentration of IFNγ and IL-17A[14]. 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[5,6,9,15,16]. 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[17]. 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 cingular cortex, but only males saw an increase in BDNF in the nucleus accumbens[18]. 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[19]. 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[17,20–22]. 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[23]. 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[24–27].

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 (200-225g, 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

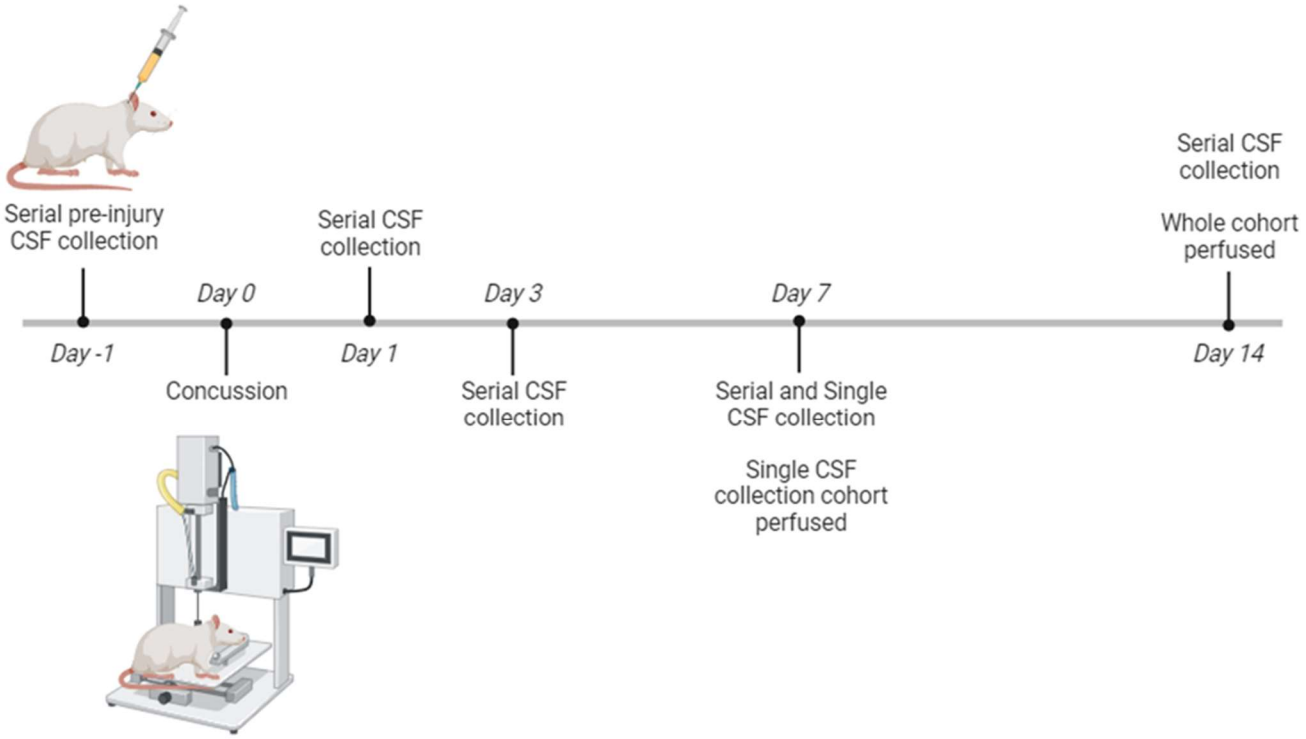


Figure 1: Experimental timeline schematic. Briefly, pre-injury CSF samples were collected 1 day before injury, except from the single collection CSF group. On day 0, all groups of rats were given either given a concussion or were shams. Serial collections of CSF were taken at 1 day, 3 days, 7 days, and 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.

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[27]. To prep for CSF collection, an isofluorane 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 isofluorane.

Before CSF collection, Long-Evans rats were anesthetized using an isofluorane induction chamber with 3.5% isofluorane 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.

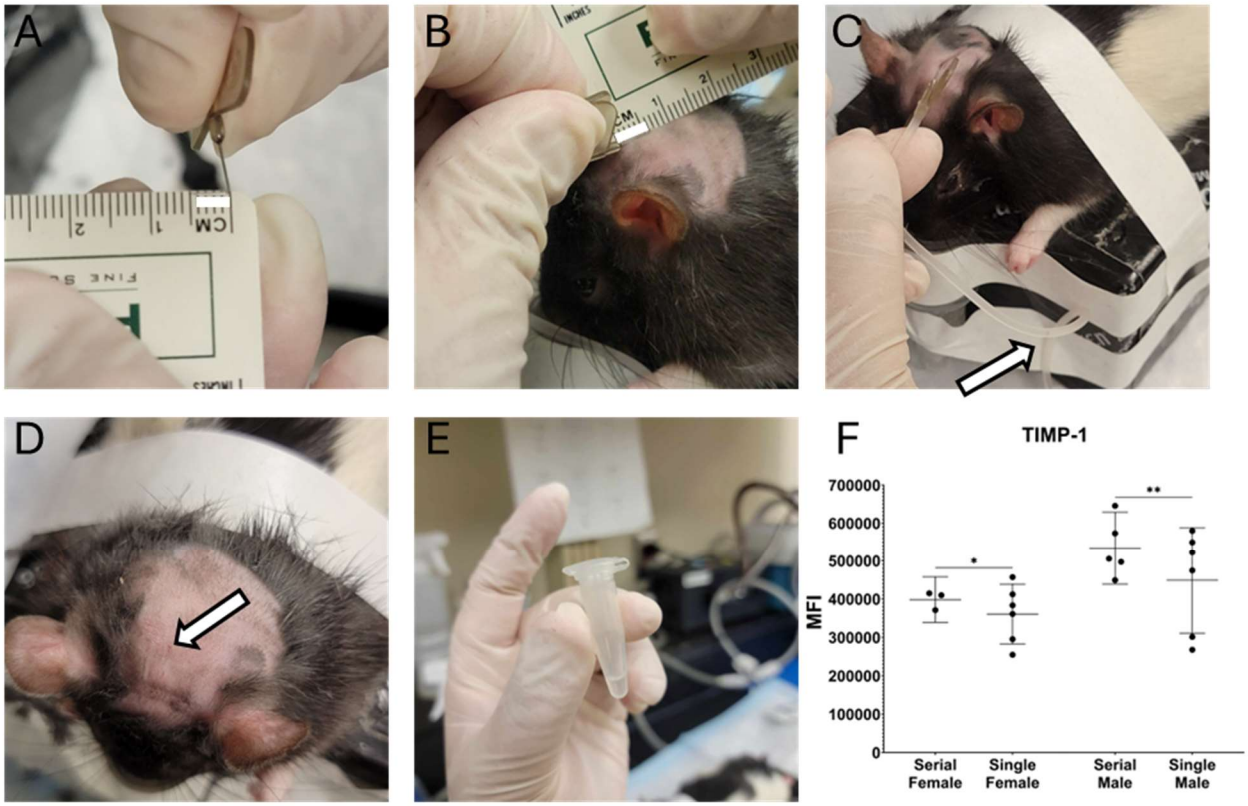


Figure 2: CSF withdrawal procedure. First, a 27G winged needle tip is bent approximately 90° with a 4 mm tip (emphasized by the white rectangle) (A). The needle is inserted into the foramen magnum, an extremely soft spot approximately 6 mm (emphasized by the white rectangle) past the external occipital proturberance 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 µL 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).

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 4mm (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 6mm 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

122 samples were measured using a pipette then supplemented with 1X protease/phosphatase
123 inhibitor cocktail (Cell Signaling Technologies, #5872). CSF was stored at -80℃.

124 *2.3. Luminex xMAP multiplex immunoassay*

125 CSF Samples were thawed on ice and centrifuged. A bead-based multiplex cytokine kit (LXSARM-
126 17, R&D Systems, Minneapolis, MN, USA) was used to measure CSF levels of CXCL2, CXCL3,
127 ICAM-1, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-18, L-Selectin, TIMP1, TNF-α, and
128 VEGF. This kit was chosen due to its published use in rat TBI studies[28,29]. All samples were run in
129 duplicate. Results were analyzed using mean fluorescent intensities as previously reported[30,31].

130 *2.4. Immunohistochemical labeling of Iba1 and GFAP*

131 Brain tissue sections were immunohistochemically labeled with primary antibodies for Iba1 (1:500,
132 019-19741, FujiFilm Wako) and GFAP (1:1500, Z033429-2, Agilent). Biotinylated donkey anti-
133 rabbit (1:500, 715-066-152, Jackson ImmunoResearch) secondary antibodies were used,
134 followed by VECTASTAIN ® Elite® ABC Kit Peroxidase (PK-6100, Vector Laboratories) and DAB
135 Substrate Kit, Peroxidase with Nickel (SK-4100, Vector Laboratories) according to manufacturer's
136 instructions.

137 *2.5. Stereological quantification of microglia and astrocytes*

138 Stereological quantification was performed on every 12th brain tissue section with thickness of
139 30um. The corpus collosum was traced at the connection between the left and right cerebral
140 hemisphere. An average of 10 counting frames measuring 80 μm x 80 μm was implemented. The
141 systematic random sampling (SRS) grid measured 250 μm x 250 μm. The optical dissector height
142 consisted of a guard zone thickness of 1um and a dissector height of 18um. The CE (Coefficient
143 Error) was 0.1.

144 *2.6. Semi-quantitative assessment of microbleed presence*

145 A categorical scale of 0-3 was implemented to rate the presence of microbleeds at three regions
146 of the brain: cerebral cortex, corpus collosum, and hippocampus. A score of 0 indicates no visible
147 hemosiderin granules. A score of 1 indicates that a few hemosiderin granules were visible. A score
148 of 2 indicates that some hemosiderin granules were visible. A score of 3 indicates that many
149 hemosiderin granules were present. The scores for each region were summed up for each
150 section. 5 serial sections were used for each animal. The total sum of all 5 sections was used for
151 statistical analysis.

152 *2.7. Statistical analysis*

153 For analysis of serially collected samples, a two-way mixed effects analysis with a post-hoc Holm-
154 Šídák's multiple comparisons test was used. For statistical tests that did not require matching
155 samples, an ordinary two-way ANOVA with a post-hoc Holm-Šídák's multiple comparisons test
156 was used.

157 **3. Results**

158 *3.1. CSF was collected serially from rats with no evidence of negative side effects.*

159 CSF was collected from a total of 36 rats (18 male and 18 female). 24 rats underwent serial
160 collections 1 day before concussion and then 1 day post injury (dpi), 3 dpi, 7 dpi, and 14 dpi. The
161 remaining 12 rats underwent a single CSF collection at 7 dpi only (Figure 1). No rats exhibited
162 signs of pain/distress, weight loss, infection, or swelling. There was no attrition of animals due to
163 mortality. If CSF samples were contaminated with blood upon collection, they were immediately
164 centrifuged for 30 seconds to separate the CSF from blood, yielding clear CSF samples.
165 Centrifugation has previously been shown not to affect CSF composition[32]. Of the 132 attempts
166 to collect CSF samples, 121 attempts (92%) resulted in clear CSF (Figure 2 A-E).

167 *3.2. Serial CSF collection did not alter protein detection.*

Sex not considered	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons (Concussion vs Sham)			
Protein	Time	Injury	Time x Injury	1dpi	3 dpi	7 dpi	14 dpi
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
IFNg	****	*	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
TNFa	ns	ns	ns	ns	ns	ns	ns
VEGF	ns	ns	ns	ns	ns	ns	ns

Table 1: 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. *p<0.05; ****p<0.0001

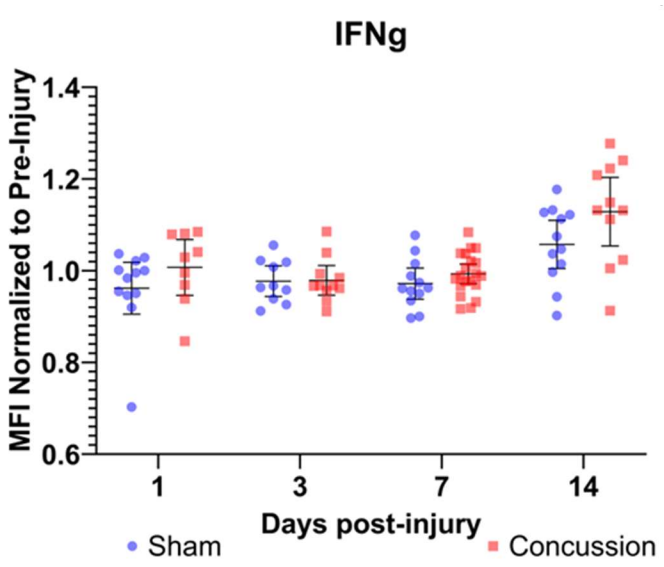


Figure 3: IFNg increases slightly over time after concussion. Graph shows the mean fluorescent intensity (MFI) at each timepoint normalized to pre-injury. Mean with 95% CI is plotted with individual points representing individual rats.

Serially collected 7dpi CSF samples were 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-Šídák's multiple comparisons test (Figure 2F). TIMP-1 is an inhibitor of matrix metalloproteinases, and has been shown to play a role in alleviating

181 inflammatory pain associated with wound healing[33]. Given the role of TIMP-1 role in inflammatory
182 pain and wound healing, this result is consistent with specific modulation of this pathway.
183 Together, these data suggest that while detection of protein changes over time is possible,
184 repeated CSF collection did not result in a generalized alteration of protein analyte detection.

185 *3.3. 16 of 17 CSF protein analytes were unaltered by a single mTBI when data were analyzed*
186 *without consideration of sex*

187 To interrogate the sex differences in post-concussion CSF protein levels, we took a progressive
188 approach to analyzing the data. First, we compared CSF protein levels between concussion and
189 sham to assess how a single concussion alters the proteome of the CSF without consideration
190 for sex. Several publications presenting clinical concussion data ignore sex, often grouping
191 patients into two groups: concussion vs sham[9,16,34–36]. Using this approach, we compared
192 concussion and sham CSF samples normalized to pre-injury levels using two-way mixed effects
193 analysis and post-hoc Holm-Šídák's multiple comparisons test (Table 1). A single concussion had
194 no significant effect on any of the 17 analytes, except for interferon-γ (IFNγ). For IFNγ (Figure 3),
195 both time ($F=14.46$; $p<0.0001$) and injury ($F=5.408$; $p=0.0270$) had significant effects, however
196 the interaction ($F=1.023$; $p=0.3891$) of these two factors had no significant effect.

197 *3.4. Males exhibit a greater inflammatory response to a single concussion than females*

198 We next separated samples by sex, comparing concussion and sham animals within each. Using
199 the same statistical approach as before, females showed significant responses to injury in multiple
200 pro-resolving/anti-inflammatory proteins, whereas males showed significant responses to injury
201 in several pro-inflammatory proteins. For females, two-way Mixed Effects analysis (Table 2)
202 identified a significant interaction effect for time and injury on IL-13 ($F=3.066$, $p=0.0388$, Figure
203 4A), however, neither time ($F=0.06031$, $p=0.9103$) nor injury ($F=0.001776$, $p=0.9666$) alone had
204 a significant effect on IL-13 levels. Similarly, VEGF (Figure 4C) showed comparable results, where

Females Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons Males (Concussion vs Sham)			
Protein	Time	Injury	Time x Injury	1dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	ns	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns
GM-CSF	*	ns	ns	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns	ns	ns	ns
IFNg	**	ns	ns	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns	ns	ns	ns
IL-1b	*	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
IL-10	ns	ns	ns	ns	ns	ns	ns
IL-13	ns	ns	*	ns	ns	ns	ns
IL-18	ns	ns	ns	ns	ns	ns	ns
L-selectin	**	**	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns
TNFa	ns	ns	ns	ns	ns	ns	ns
VEGF	ns	ns	**	ns	ns	ns	ns

Table 2: 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. *$p<0.05$; **$p<0.01$

Males Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons Males (Concussion vs Sham)			
Protein	Time	Injury	Time x Injury	1dpi	3 dpi	7 dpi	14 dpi
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
IFNg	*	ns	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	** (S < C)
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
L-selectin	**	ns	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns
TNFa	**	ns	*	ns	ns	ns	ns
VEGF	**	ns	ns	ns	ns	ns	ns

Table 3: 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. *$p<0.05$; **$p<0.01$; ***$p<0.001$

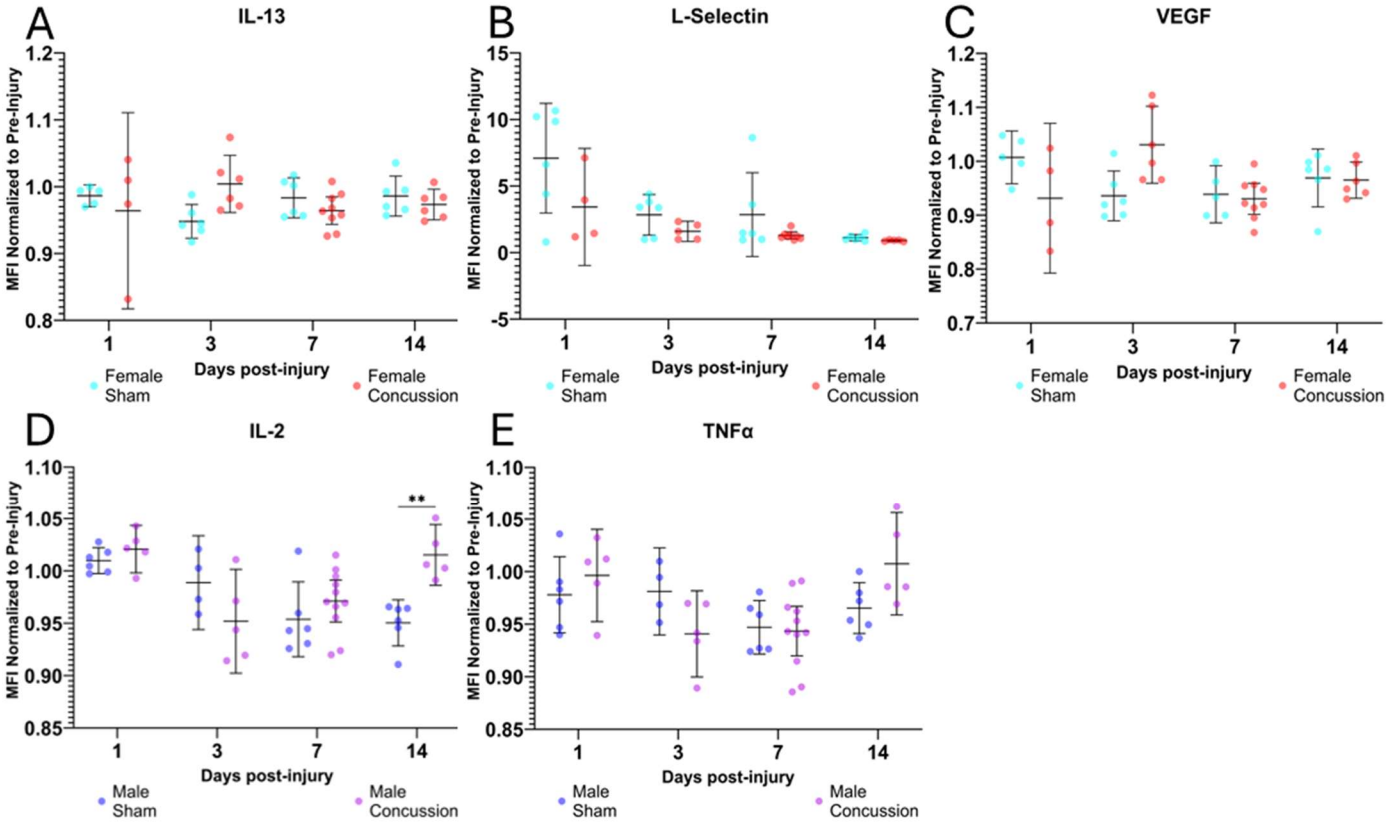


Figure 4: Injury is a significant factor affecting expression in pro-resolving proteins for females and pro-inflammatory proteins for males. The MFI for each sample at each timepoint normalized to pre-injury is reported for IL-13 (A), L-Selectin (B), and VEGF (C) in females, as well as IL-2 (D) and TNFα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

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. 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, (Figure 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 3). 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, Figure 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-α (TNFα, Figure 4E) levels. Injury alone was not shown to be a significant factor affecting TNFα 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 2), time was significant for GM-CSF (F=3.939, *p*=0.0298), IFNγ (F=7.900, *p*=0.0065), IL-1β (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 3), time was significant for IFNγ (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 4). Sex was found to be a significant factor affecting the levels of 9 proteins: CXCL3 (F=11.05, *p*=0.0019, Figure 5A), GM-CSF (F=7.604; *p*=0.0085, Figure 5B), ICAM-1 (F=6.451, *p*=0.0211, Figure 5C), IL-1β (F=8.072, *p*=0.0108, Figure 5D), IL-10 (F=6.006; *p*=0.0186, Figure 5G), IL-18 (F=5.137; *p*=0.0285, Figure 5H), L-selectin (F=5.585, *p*=0.0303, Figure 5I), TNFα (F=4.083, *p*=0.0496, Figure 5J), and VEGF (F=4.704, *p*=0.0437, Figure 5K). Additionally, time was also found to be a significant factor affecting the levels of IFNγ (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-1b (F=3.531; *p*=0.0300, Figure 5D), IL-2 (F=3.683; *p*=0.0191, Figure 5E), and VEGF (F=7.095; *p*=0.0014, Figure 5K). Post-hoc multiple comparisons testing comparing CSF protein levels after a single concussion in males vs females at specific timepoints showed no significant

Concussions Only	Two-way Mixed Effects Analysis			Post-hoc Holm-Sidak Multiple Comparisons Concussion (Male vs Female)			
Protein	Time	Sex	Time x Sex	1dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	**	ns	ns	ns	ns	ns
CXCL2	ns	ns	ns	ns	ns	ns	ns
GM-CSF	ns	**	ns	ns	ns	ns	ns
ICAM-1	ns	*	ns	ns	ns	ns	ns
IFNg	**	ns	ns	ns	ns	ns	ns
IL-1a	ns	ns	ns	ns	ns	ns	ns
IL-1b	ns	*	*	ns	ns	ns	ns
IL-2	ns	ns	*	ns	ns	ns	ns
IL-4	ns	ns	ns	ns	ns	ns	ns
IL-6	ns	ns	ns	ns	ns	* (M>F)	ns
IL-10	ns	*	ns	ns	ns	ns	ns
IL-13	ns	ns	ns	ns	ns	ns	ns
IL-18	*	*	ns	ns	ns	* (M>F)	ns
L-selectin	*	*	ns	ns	ns	ns	ns
TIMP-1	ns	ns	ns	ns	ns	ns	ns
TNFa	ns	*	ns	ns	* (M<F)	ns	ns
VEGF	ns	*	**	ns	ns	* (M>F)	ns

Table 4: 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. *$p<0.05$; **$p<0.01$

differences at 1 dpi and 14 dpi. However, at 3 dpi females had significantly higher TNFα levels than males (p=0.0275, Figure 5J). At 7 dpi, males showed significantly greater levels of three proteins compared to females: IL-6 (p=0.0200, Figure 5F), IL-18 (p=0.0410, Figure 5H), and VEGF (p=0.0113, Figure 5K).

3.6. Male and female sham animals exhibit baseline differences in CSF protein detection

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. Post-hoc multiple comparisons testing (Table 5) showed significant differences when comparing sham male and sham female to groups of the opposite sex. At 1 and 14 dpi, there were no significant differences in the levels of any of the 17 proteins when comparing either sham male or sham female with the opposite sex. However, at 3 dpi, sham male was shown to have significantly greater levels of CXCL3 (p=0.0030, Figure 6A) and VEGF (p=0.0145, Figure 6G) than sham female. At 7dpi, sham male was shown

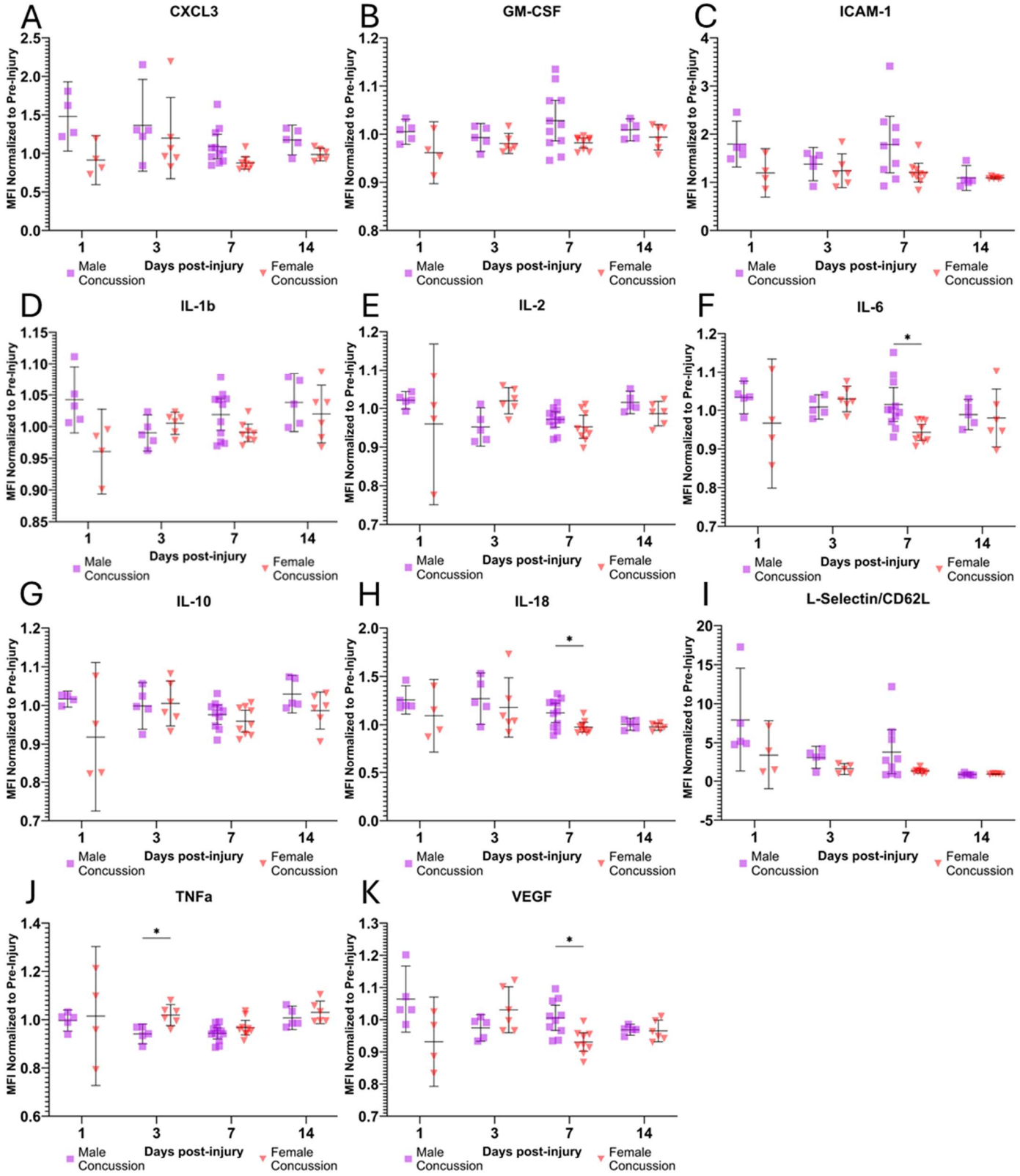


Figure 5: Males show a delayed inflammatory response to concussion compared to females. The MFI for each sample at each timepoint normalized to pre-injury is reported for CXCL3 (A), GM-CSF (B), ICAM-1 (C), IL-1b (D), IL-2 (E), IL-6 (F), IL-10 (G), IL-18 (H), L-selectin/CD62L (I), TNFα (J), and VEGF (K). These proteins were selected based on results from statistical testing reported in Table 4 showing sex as a significant factor affecting protein expression. Mean with 95% CI is plotted with individual points representing individual rats. *$p<0.05$.

All 4 groups	Post-hoc Holm-Sidak Multiple Comparisons Concussion (Male vs Female)			
Protein	1dpi	3 dpi	7 dpi	14 dpi
CXCL3	ns	** (MS>FS)	* (MS>FC)	ns
CXCL2	ns	ns	* (MS>FC)	ns
GM-CSF	ns	ns	ns	ns
ICAM-1	ns	ns	ns	ns
IFNg	ns	ns	* (MS<FS); * (MS<FC)	ns
IL-1a	ns	ns	ns	ns
IL-1b	ns	ns	ns	ns
IL-2	ns	ns	ns	** (MS<MC)
IL-4	ns	ns	ns	ns
IL-6	ns	ns	* (MC>FC)	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	* (MC<FC)	ns	ns
VEGF	ns	* (MS>FS)	* (MC>FC)	ns

Table 5: Statistical testing comparing all four experimental groups. Two-way mixed effects analysis with post-hoc Holm-Sidak multiple comparisons test comparing all 4 experimental groups. Analyte mean fluorescent intensities (MFI) at each time point were normalized to pre-injury measurements and compared between groups. MS = Male Sham, MC = Male Concussion, FS = Female Sham, FC = Female Concussion. *p<0.05; **p<0.01.

to have significantly greater levels of CXCL3 (p=0.0339, Figure 6A) and CXCL2 (p=0.0404, Figure 6B), as well as significantly lower levels of IFNγ (Figure 6C) compared to concussed female (p=0.0181) and sham female (p=0.0489). Additionally, using this statistical approach, which includes the most comparisons of all statistical tests, and thus the least statistical power in this study, 4 of the 5 significant pairwise comparisons reported in the previous tests were confirmed (Table 5) at this lower power. At 3 dpi, IFNγ was significantly greater in concussed females compared to concussed males (p=0.0409, Figure 6C). At 7 dpi, concussed males had significantly greater levels of IL-6 (p=0.0298, Figure 6E) and VEGF (p=0.0169, Figure 6G) compared to concussed females. Additionally, at 7 dpi, we previously reported the concussed males had significantly greater levels of IL-18 (Figure 5H), but in this lower powered analysis, this comparison is no longer significant (p=0.0608). At 14 dpi, concussed males had significantly greater levels of IL-2 (p=0.0076, Figure 6D) compared to uninjured males.

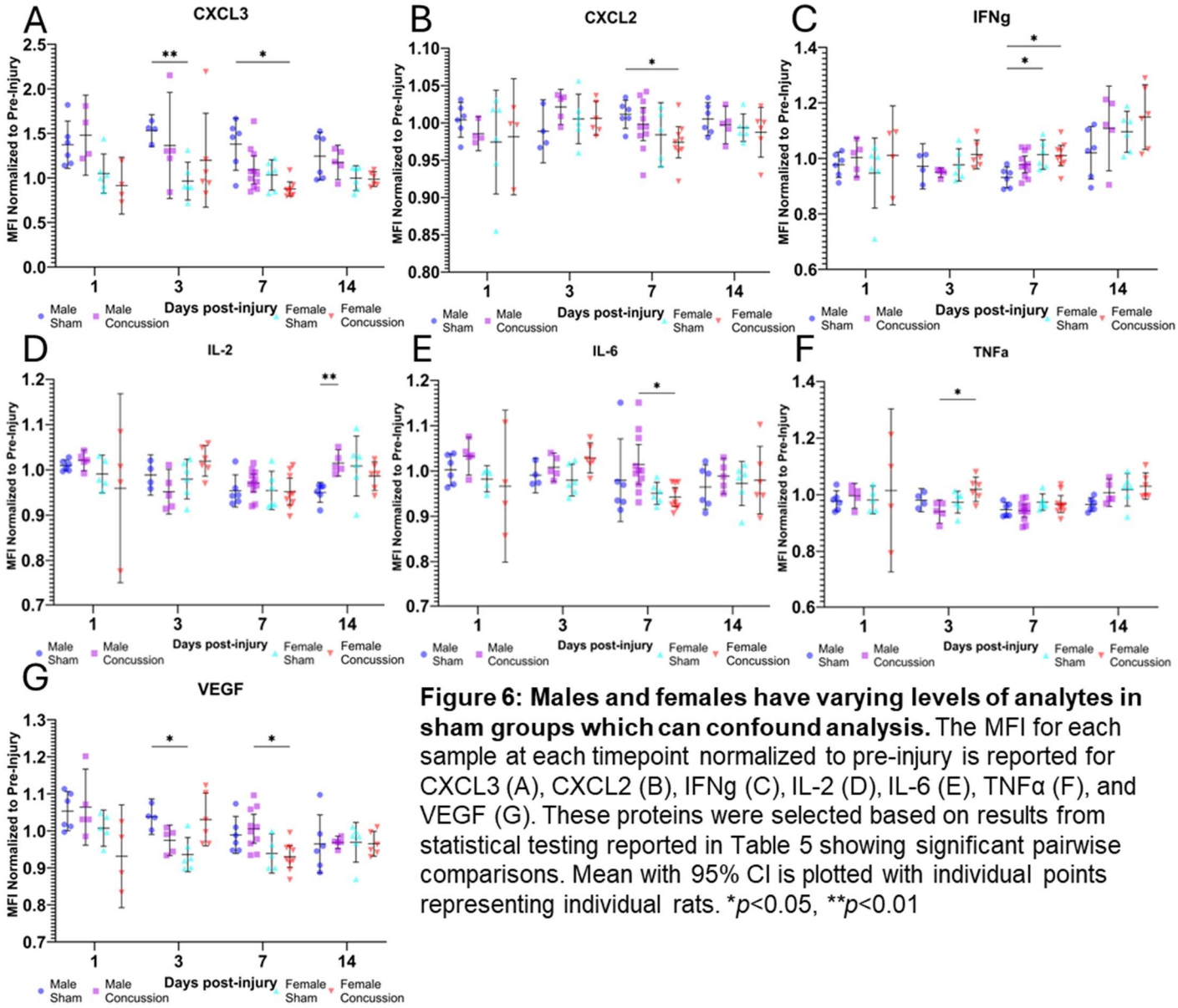
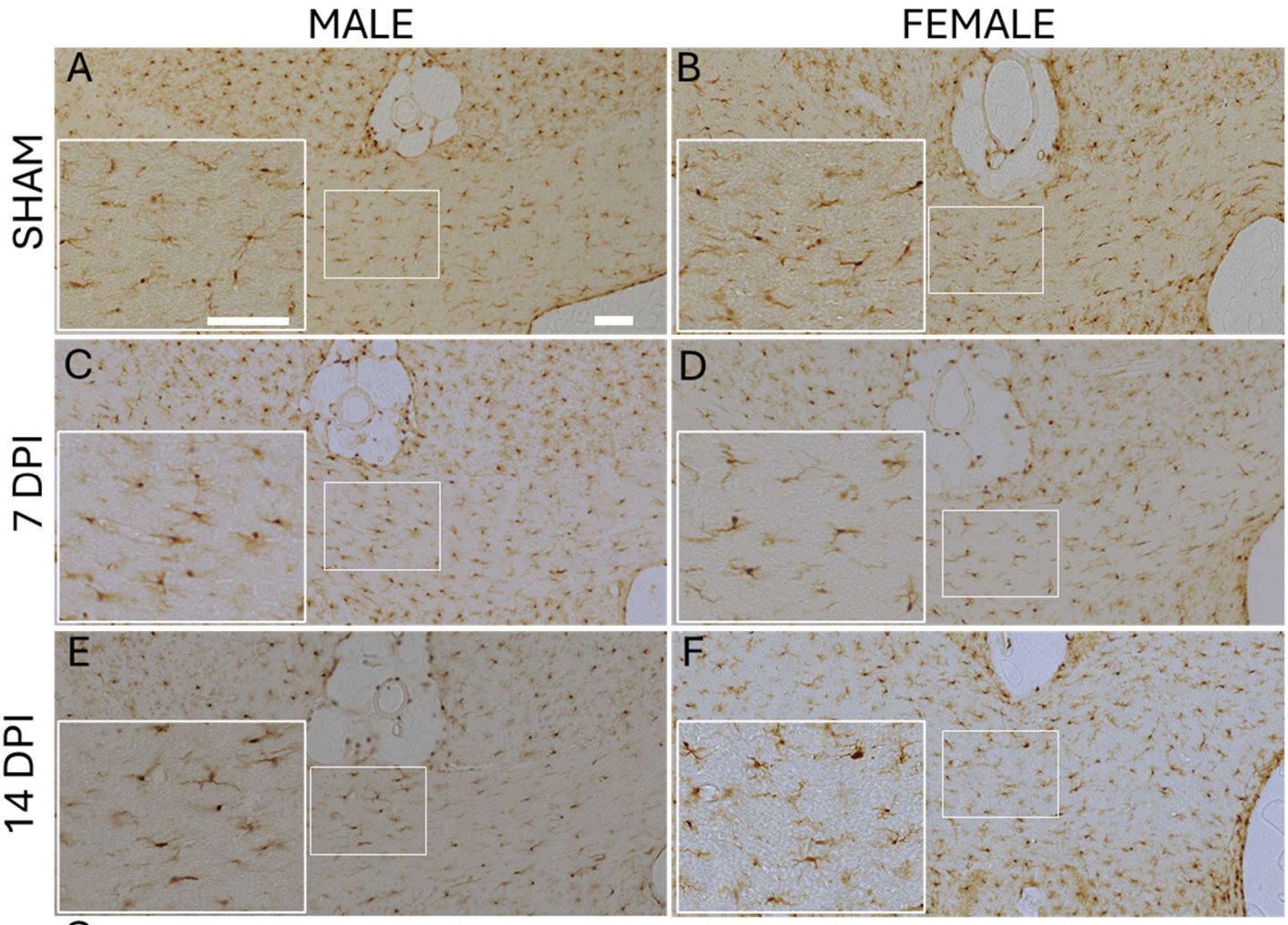


Figure 6: Males and females have varying levels of analytes in sham groups which can confound analysis. The MFI for each sample at each timepoint normalized to pre-injury is reported for CXCL3 (A), CXCL2 (B), IFNg (C), IL-2 (D), IL-6 (E), TNFα (F), and VEGF (G). These proteins were selected based on results from statistical testing reported in Table 5 showing significant pairwise comparisons. Mean with 95% CI is plotted with individual points representing individual rats. *p<0.05, **p<0.01

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+, Figure S1) and microglia (Iba1+, Figure 7) using stereological counting and performed a two-way ANOVA and post-hoc Holm-Šídák'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



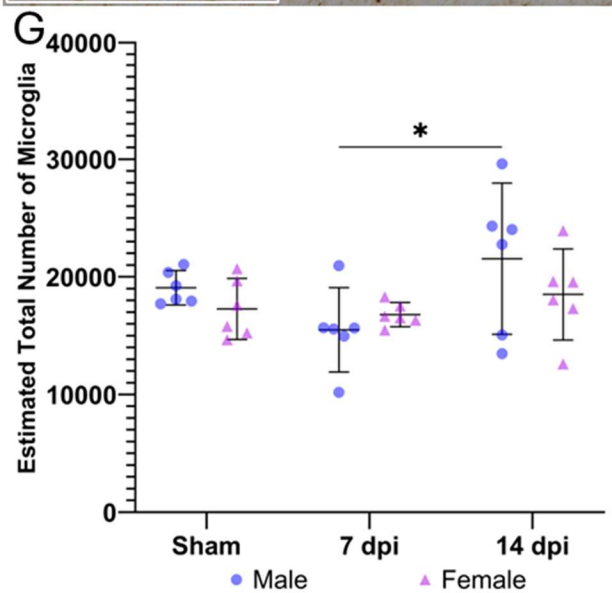
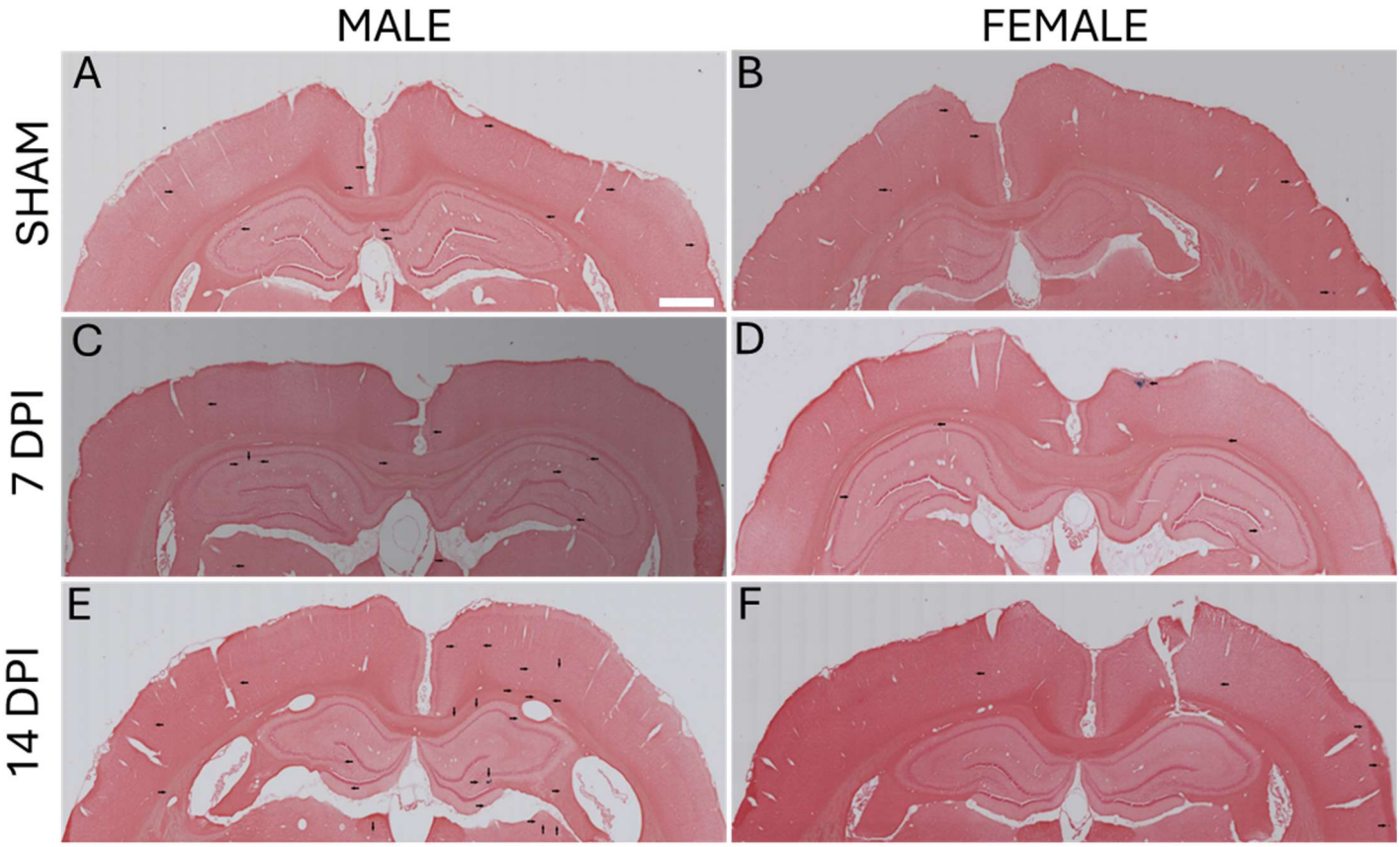


Figure 7: Males experience a significant increase in the estimated total number of microglia, but not females, 14 days after a concussion. Stereological quantification of Iba1+ cells was performed on serial sections of brain tissue. Representative images are shown (A-F). Quantification was performed 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 µm.

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 (Figure



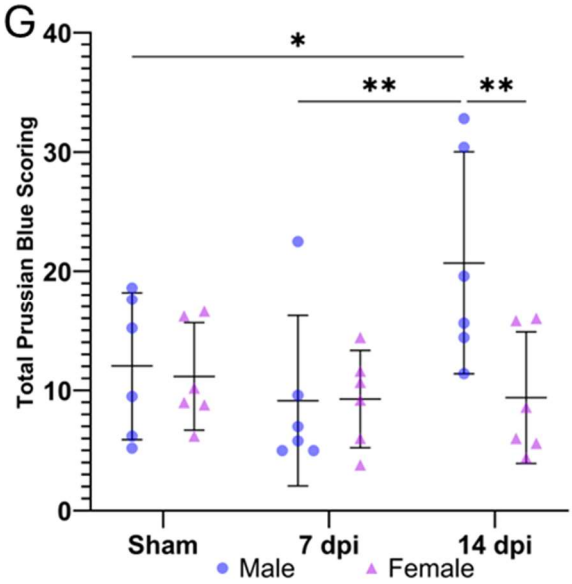


Figure 8: Males, but not females, experience a significant increase in the presence of microbleeds 14 days after a concussion. A semi-quantitative scoring system for microbleed presence in the cortex, corpus callosum, and hippocampus was used. Representative images are shown (A-F). Black arrows, added 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 µm.

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 (Figure 7G).

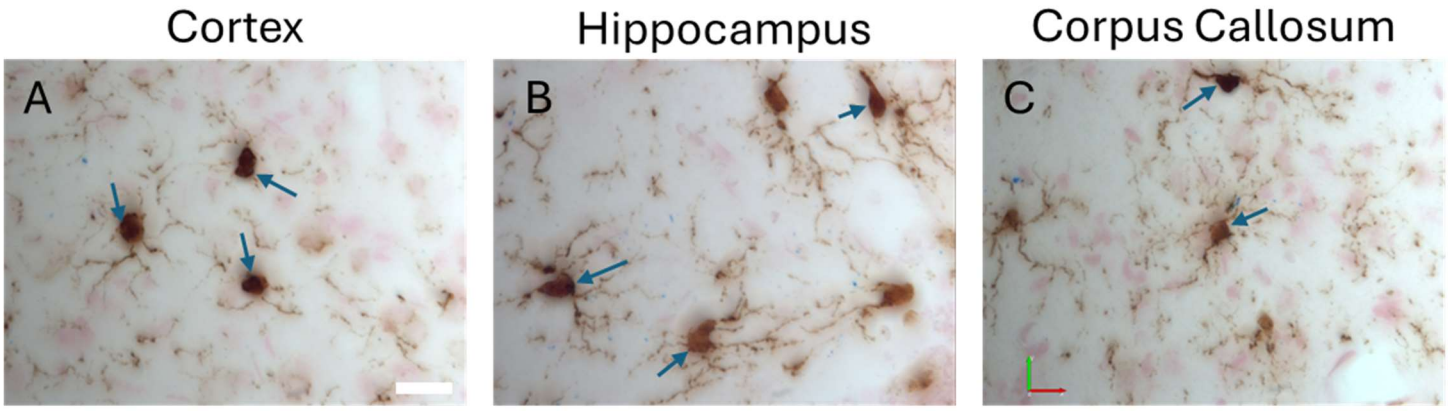


Figure 9: Microglia phagocytose microbleeds. Representative images show colocalized positive staining for Iba1 and microbleeds confirming microglia are responding to BBB permeability in the cortex (A), hippocampus (B), and corpus callosum (C). Scale bar = 20 µm.

The trends in this inflammatory cell data correspond well with trends seen in scoring of Prussian Blue staining for microbleeds (Figure 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-Šídák's multiple comparisons testing shows there are significant differences between groups (Figure 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 (Figure 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 neuroinflammatory response to mTBI as currently established methods (i.e. stand-alone CSF collection, brain tissue lysates, histology)

299 require large numbers of animals for studies. Third, the majority of mTBI literature fails to
300 investigate sex as a biological variable. This study addressed these limitations.

301 *4.1. Serial collection of CSF does not significantly alter CSF inflammation*

302 We first described a method for minimally invasive serial collection of CSF. CSF proteomics are
303 not widely studied in pre-clinical models due to the difficult nature of CSF collection. Previously
304 described methods of CSF collection do not allow for repeated collections, require specialized
305 tools and equipment, and can cause further brain injury or death[26,27,37]. The serial collection
306 method used herein did not cause the rats to experience unusually significant weight changes,
307 exhibit evidence of pain/distress (shallow or labored breathing, poor grooming, hunching, or
308 porphyria), show signs of infection or swelling, or die.

309 Further, since serial collection of CSF requires multiple punctures with a needle over time, we
310 compared a 7 dpi fresh CSF sample from rats who underwent serial collection at pre-injury, 1 dpi,
311 3 dpi, 7 dpi, and 14 dpi to a CSF sample from rats who only underwent a single CSF collection at
312 7 dpi (Figure 1). A two-way ANOVA revealed that collection frequency was not a significant factor
313 affecting the proteome results, at least for the 17 analytes we assessed. Interestingly, however, a
314 post-hoc Holm-Šídák's multiple comparisons test revealed that none of the analytes in our panel
315 showed significant differences between serial collection and single collection except TIMP-1 in
316 both males and females (Figure 2). TIMP-1 is an inhibitor of metalloproteinases that plays a role
317 in alleviating inflammatory pain associated with wound healing[33]. Given this role for TIMP-1, the
318 elevation in TIMP-1 resulting from repeated collection of CSF makes sense as repeated punctures
319 would cause increased inflammation and prolonged wound healing. This one elevated protein
320 provides confidence in our statistical testing as it shows that the statistical approach has enough
321 power to detect a significant result and supports the assumption that our negative statistical
322 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 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 IFNγ 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[9,16,34–36]. 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 1), with the exception of IFNγ. IFNγ (Figure 3) was shown to be significantly affected by injury.

IFNγ 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, IFNγ has been shown to be significantly increased acutely after mTBI in blood samples from children[14] and adults[36]. 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, IFNγ was shown to be significantly affected by injury in a two-way mixed effects analysis[38]. While this result from blood testing directly

348 reflects our finding in this study using CSF, it is important to note that Chaban *et al*. did not report
349 time as a significant factor influencing IFNγ levels. Interestingly, adult patients showed no
350 significant differences in IFNγ levels compared to controls in CSF samples taken approximately
351 17 months after concussion[9]. It should be noted that the athletes in this sample had a median of
352 5 concussions prior to CSF collection.

353 While IFNγ was the only protein in our panel that showed significance in our sex blended statistical
354 testing, TNFα, IL-2, IL-6, IL-10, and VEGF are also interesting, as these five proteins have shown
355 significant changes in sex blended human studies. Chaban *et al.* reported that TNFα expression
356 in adult mTBI patient blood samples was significantly affected by both time and injury[38]. The 207
357 patients in this study sustained a single mild TBI, but the injury was severe enough to result in
358 hospitalization. Vedantam *et al.* showed that IL-2 and IL-6 are both significantly increased
359 compared to controls at 1 dpi and IL-6 stayed significantly increased up to 6 months post-injury
360 in blood samples from adult mTBI patients, and these changes were associated with more severe
361 symptoms, depression, and/or PTSD. Again, these patients were recruited from injuries serious
362 enough to warrant a trip to the emergency room[36]. Ryan *et al*. reported that TNFα and IL-10 were
363 significantly decreased, while IL-6 was significantly increased in blood sampled from pediatric
364 mTBI patients within 2 weeks of injury[14]. Gard *et al*. reported that IL-2, TNFα, and VEGF were all
365 significantly increased in the CSF of adult mTBI patients 17 months post-injury[9]. While our results
366 do not fully reflect what has been published in sex blended clinical studies, it is important to note
367 that while these studies are sex blended, they are still predominantly male patient populations
368 with almost a 2:1 male:female ratio[9,14,36,38]. To fully understand these published results, as well as
369 our results in this study, it is vital to separate the sexes and compare the injury response in each
370 sex.

371 *4.3. A single concussion significantly affects pro-resolving proteins in females and pro-*
372 *inflammatory proteins in males*

373 The second step we took in our stepwise approach to analyzing this dataset was separating the
374 sexes and comparing the injury response in each sex. This approach gave us two sets of two
375 groups: Concussion vs sham in females (Table 2) and concussion vs sham in males (Table 3).
376 The results from this approach were quite intriguing. In females, injury was a significant factor
377 affecting the levels of soluble L-selectin (Figure 4B) and the interaction of time and injury was a
378 significant factor affecting the levels of IL-13 (Figure 4A) and VEGF (Figure 4C). In males, the
379 interaction of time and injury significantly affected the levels of IL-2 (Figure 4D) and TNFα (Figure
380 4E). IL-13 and VEGF are typically secreted by pro-resolving, anti-inflammatory immune cells,
381 while IL-2 and TNFα are pro-inflammatory proteins[39–41]. The trends shown in the data paint an
382 interesting picture. Females experience an acute upregulation of pro-resolving proteins, which
383 have also been shown to be neuroprotective[41,42], while males initially experience a decrease in
384 pro-inflammatory proteins that eventually increases at later timepoints, with IL-2 levels being
385 significantly greater in concussed males compared to sham males at 14 dpi, which reflects
386 previously published male-biased sex blended studies[9,36]. One possible explanation of this
387 paradigm is the differential presence of estrogen and progesterone, which have been shown to
388 be neuroprotective after TBI, reducing mortality and improving neurological outcomes, by
389 reducing neuronal apoptosis, inhibiting microglial and astrocytic activation, and decreasing brain
390 edema[17,20–22]. To further dissect the sex differences in the inflammatory response after
391 concussion, it is imperative to compare CSF protein levels after concussion across both sexes
392 directly.

393 *4.4. Males experience a biphasic and more prolonged inflammatory response compared to*
394 *females.*

395 The third phase of our stepwise approach to analyzing CSF protein concentration after
396 concussion was comparing the protein levels in both concussion groups, giving us two groups for
397 comparison: male vs female. In this comparison, many sex-based differences were revealed. Sex

398 was a factor significantly affecting the levels of 9 of 17 proteins and the interaction of sex and time
399 was a factor significantly affecting the levels of 3 of these proteins (Table 4). There is a clear trend
400 present in the time course representations of each of these proteins (Figure 5). While there are
401 only four significant pairwise comparisons in post-hoc multiple comparisons testing, in general,
402 males experience a more rapid inflammatory response, with all proteins being greater in males
403 than females, except TNFα, at 1 dpi. At 3 dpi, females have significantly greater TNFα than males,
404 as well as greater levels of IL-1β, IL-2, IL-6, and VEGF, suggesting that females undergo a
405 delayed inflammatory response. At 7 dpi, males experience a second peak in inflammation with
406 significantly greater levels of IL-6, IL-18, and VEGF, as well as greater levels of CXCL3, GM-CSF,
407 ICAM-1, IL-1b, IL-2, and L-selectin, than females. This persists at 14 dpi, with males exhibiting
408 greater levels of CXCL3, GM-CSF, IL-1b, IL-2, and IL-10, though none of these are significant,
409 than females.

410 Our results are supported by a previous study evaluating serum and brain cytokine concentrations
411 in mice 4 hours after the last injury in a repetitive blast-related mTBI model[28]. Baskin *et al.* reported
412 that female mice experience greater anti-inflammatory signaling acutely after mTBI, with
413 significantly higher concentrations of IL-10 and G-CSF and significantly lower concentrations of
414 IL-9, IL-12, and IP-10 in brain tissue homogenates, compared to male mice[28]. While few studies,
415 especially longitudinal ones, have compared protein levels in biofluids sampled from mTBI
416 patients across sexes, it is critical to analyze these sex differences more holistically, as biomarker
417 bioactivity may vary between sexes. For example, Di Battista *et al.* reported no significant
418 differences in plasma concentrations of IFNγ or TNFα between male and female mTBI patients[43].
419 However, in males, they found that higher IFNγ concentrations in blood were significantly
420 correlated with more severe concussion symptoms, whereas in females, higher concentrations of
421 IFNγ and TNFα were significantly correlated with less severe symptoms[43]. This data suggests
422 there are underlying differences in the mechanisms regulating how males and females respond

423 to inflammation. Studies comparing male and female injury responses to mTBI are valuable for
424 bridging the sex-based knowledge gap, and more studies investigating these sex-based
425 differences are clearly needed. With that said, directly comparing the injuries between sexes is
426 not the complete picture. It is imperative to understand uninjured baseline differences between
427 sexes as well, especially in longitudinal biomarker studies such as the present one.

428 *4.5. Uninjured males and females have varying cytokine levels in the CSF*

429 The last phase in our stepwise approach to analyzing this dataset incorporates the sham groups
430 into the comparisons. This gives us four groups for comparison: sham male, concussed male,
431 sham female, and concussed female. A major point to note with this level of statistical testing is
432 that by increasing the number of groups, the number of comparisons also increases, which
433 decreases the statistical power of post-hoc multiple comparisons testing[44]. This is critical to keep
434 in mind, as it can result in a comparison being reported as negative, even though with fewer
435 comparisons, it may have been positive. This phenomenon can be seen in this study. When
436 comparing concussed males to concussed females (Table 4), IL-18 (Figure 5H) was significantly
437 greater in males than females. However, by increasing the number of comparisons with added
438 groups, the difference in IL-18 between concussed males and concussed females is no longer
439 significant. All other significant pairwise comparisons remained significant, even with less power.
440 This suggests that IL-18 may still be an important biomarker to investigate in mTBI, but is not as
441 strongly affected by sex or injury as other proteins in this panel.

442 Incorporating all four experimental groups into our statistical testing revealed some interesting
443 baseline differences that may prove to be confounding to our previous results (Table 5). First, the
444 sham male group was shown to have significantly higher levels of CXCL3 (Figure 6A) and VEGF
445 (Figure 6G) at 3 dpi compared to sham female and significantly higher levels of CXCL3 (Figure
446 6A) and CXCL2 (Figure 6B) at 7 dpi than concussed female. Also the shame male group was
447 shown to have significantly lower levels of IFNγ (Figure 6C) at 7 dpi than sham female and

448 concussed female. These results likely do not affect interpretation of directly comparing
449 concussed males and concussed females (Table 4), but instead affect interpretation of the injury
450 responses separated by sex (Table 2 & 3). For example, sham males exhibited significantly lower
451 levels of IFNγ (Figure 6C) compared to both sham female and concussed female at 7 dpi, but
452 there was no statistical significance between concussed male and sham female, concussed
453 female, or sham male. It's possible that the lack of significance in this comparison is a
454 consequence of the limited study size, and a larger study would be able to confirm whether or not
455 there is a significant difference in IFNγ.

456 *4.6. Presence of microbleeds corresponds with increase in Iba1+ microglia number*

457 Despite a concussion being a mild traumatic brain injury, there are still noticeable differences in
458 brain pathology between sexes after a concussion. We performed histological characterization
459 using a quantitative and semi-quantitative methodology to assess the presence of Iba1+ microglia
460 (Figure 7) and GFAP+ astrocytes (Figure S1), as well as microbleeds (Figure 8), respectively.
461 Stereological quantification of Iba1+ microglia, in the corpus callosum shows a significant
462 increase in the estimated total number of microglia in the corpus callosum of males at 14 dpi
463 compared to 7 dpi (Figure 7G). Stereological quantification of GFAP+ astrocytes in the corpus
464 callosum showed no significant differences between any groups. The overall trend of this data
465 corresponds well with the semi-quantitative scoring of microbleed presence, where each instance
466 of a microbleed is graded on a scale of 0-3 (0 = none, 1 = a few granules, 2 = several granules,
467 3 = a lot of granules). This scoring reveals that males experience a significantly greater presence
468 of microbleeds at 14 dpi compared to male sham, males at 7 dpi, and females at 14 dpi (Figure
469 8G). This correlation makes sense, as microglia are known to phagocytose blood products after
470 hemorrhage[45,46]. To confirm this response, co-staining of Iba1 and Prussian Blue was performed
471 and showed multiple Iba1+ microglia phagocytosed microbleed byproducts (Figure 9). This data
472 suggests the possibility that the pro-inflammatory CSF protein expression seen in males after a

473 concussion could be driven by an increase in pro-inflammatory microglia polarization due to a
474 increase in BBB permeability. Future studies should investigate variability in microglial
475 phagocytosis between sexes as a possible mechanism underlying the sex-dependent differences
476 in neuroinflammation after concussion.

477 *4.7. Limitations*

478 This study was designed with three goals. First, to establish a method of minimally invasive serial
479 CSF collection that could be used by any laboratory without specialized tools to enable future
480 longitudinal studies utilizing clinically relevant outputs. Second, to determine if the serial collection
481 of CSF altered the CSF proteome in the absence of an experimental condition. Third, to establish
482 a framework for interrogating sex differences in levels of biomarkers for mTBI in rats. While this
483 study accomplishes all three goals, it is not without its limitations. First, the sample size in the
484 present study is small. Each group at each timepoint has n=4-6 rats. While group numbers are
485 sufficient for statistical analysis, groups of 10-12 rats would be ideal and would allow for testing
486 of behavioral outcomes in future interventional studies. Second, it is extremely likely that there
487 are other proteins that play a significant role in mTBI inflammation that are not addressed in this
488 study. This is mainly due to the lack of analytes commercially available in multiplex immunoassay
489 kits for rats. As more and more kits designed for rats come to market, studies like this will be able
490 to be expanded. Last, a limitation of this study is the absence of behavioral data. Future studies
491 should aim to correlate clinically relevant biomarkers over time with functional outputs like
492 behavior to maximize the amount of data that can be generated with the least number of animals.

493 **5. Conclusion**

494 This study was designed to accomplish three goals. First, to describe a method of minimally
495 invasive serial CSF collection. The method described in this study can easily be adapted by any
496 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.

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Funding Declaration

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Competing Interests

The authors declare that they have no competing interests.

Ethics Declaration

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

521 **Data Availability Statement**

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

524

525 1 Guan B, Anderson DB, Chen L, Feng S, Zhou H. Global, regional and national burden of
526 traumatic brain injury and spinal cord injury, 1990-2019: a systematic analysis for the
527 Global Burden of Disease Study 2019. *BMJ Open* 2023;**13**:.
528 https://doi.org/10.1136/BMJOPEN-2023-075049.

529 2 Capizzi A, Woo J, Verduzco-Gutierrez M. Traumatic Brain Injury An Overview of
530 Epidemiology, Pathophysiology, and Medical Management. *Med Clin N Am*
531 2020;**104**:213–38. https://doi.org/10.1016/j.mcna.2019.11.001.

532 3 Levy AM, Saling MM, Anderson JFI. Frequency and extent of cognitive complaint
533 following adult civilian mild traumatic brain injury: A systematic review and meta-analysis.
534 *Brain Impair* 2023:309–32. https://doi.org/10.1017/BrImp.2022.19.

535 4 Grovola MR, Von Reyn C, Loane DJ, Cullen DK. Understanding microglial responses in
536 large animal models of traumatic brain injury: an underutilized resource for preclinical and
537 translational research. *J Neuroinflammation* 2023;**20**:67. https://doi.org/10.1186/s12974-
538 023-02730-z.

539 5 Neale KJ, Reid HMO, Sousa B, McDonagh E, Morrison J, Shultz S, *et al.* Repeated mild
540 traumatic brain injury causes sex-specific increases in cell proliferation and inflammation
541 in juvenile rats. *J Neuroinflammation* 2023;**20**:. https://doi.org/10.1186/S12974-023-
542 02916-5.

543 6 Fraunberger EA, Dejesus P, Zanier ER, Shutt TE, Esser MJ. Acute and Persistent
544 Alterations of Cerebellar Inflammatory Networks and Glial Activation in a Rat Model of
545 Pediatric Mild Traumatic Brain Injury. *J Neurotrauma* 2020;**37**:1315–30.
546 https://doi.org/10.1089/neu.2019.6714.

547 7 Feigin VL, Nichols E, Alam T, Bannick MS, Beghi E, Blake N, *et al.* Global, regional, and
548 national burden of neurological disorders, 1990–2016: a systematic analysis for the
549 Global Burden of Disease Study 2016. *Lancet Neurol* 2019;**18**:459–80.

550 8 Gold EM, Vasilevko V, Hasselmann J, Tiefenthaler C, Hoa D, Ranawaka K, *et al.*
551 Repeated Mild Closed Head Injuries Induce Long-Term White Matter Pathology and
552 Neuronal Loss That Are Correlated With Behavioral Deficits. *ASN Neuro* 2018;**10**:.
553 https://doi.org/10.1177/1759091418781921/ASSET/IMAGES/LARGE/10.1177_17590914
554 18781921-FIG2.JPEG.

555 9 Gard A, Vedung F, Piehl F, Khademi M, Wernersson MP, Rorsman I, *et al.* Cerebrospinal
556 fluid levels of neuroinflammatory biomarkers are increased in athletes with persistent
557 post-concussive symptoms following sports-related concussion. *J Neuroinflammation*
558 2023;**20**:1–10. https://doi.org/10.1186/S12974-023-02864-0/FIGURES/2.

559 10 Visser K, de Koning ME, Ciubotariu D, Kok MGJ, Sibeijn-Kuiper AJ, Bourgonje AR, *et al.*
560 An exploratory study on the association between blood-based biomarkers and subacute
561 neurometabolic changes following mild traumatic brain injury. *J Neurol* 2023.
562 https://doi.org/10.1007/s00415-023-12146-7.

563 11 Giza CC, Hovda DA. The new neurometabolic cascade of concussion. *Neurosurgery*
564 2014;**75**:S24–33. https://doi.org/10.1227/NEU.0000000000000505.

565 12 Howell DR, Southard J. The Molecular Pathophysiology of Concussion. *Clin Sports Med*
566 2021;**40**:39–51. https://doi.org/10.1016/J.CSM.2020.08.001.

567 13 Shultz SR, Mcdonald SJ, Haar CV, Meconi A, Vink R, Van Donkelaar P, *et al.* The

potential for animal models to provide insight into mild traumatic brain injury: Translational challenges and strategies. *Neurosci Biobehav Rev* 2017;**76**:396–414. https://doi.org/10.1016/j.neubiorev.2016.09.014.

14 Ryan E, Kelly L, Stacey C, Huggard D, Duff E, McCollum D, *et al.* Mild-to-severe traumatic brain injury in children: altered cytokines reflect severity. *J Neuroinflammation* 2022;**19**:36. https://doi.org/10.1186/s12974-022-02390-5.

15 Villapol S, Loane DJ, Burns MP. Sexual dimorphism in the inflammatory response to traumatic brain injury. *Glia* 2017;**65**:1423–38. https://doi.org/10.1002/glia.23171.

16 Daisy CC, Varinos S, Howell DR, Kaplan K, Mannix R, Meehan WP, *et al.* Proteomic Discovery of Noninvasive Biomarkers Associated With Sport-Related Concussions. *Neurology* 2022;**98**:E186–98. https://doi.org/10.1212/WNL.0000000000013001.

17 Brotfain E, Gruenbaum SE, Boyko M, Kutz R, Zlotnik A, Klein M. Neuroprotection by Estrogen and Progesterone in Traumatic Brain Injury and Spinal Cord Injury. *Curr Neuropharmacol* 2016;**14**:641. https://doi.org/10.2174/1570159X14666160309123554.

18 Liu S (Steve), Pickens S, Barta Z, Rice M, Dagher M, Lebens R, *et al.* Neuroinflammation drives sex-dependent effects on pain and negative affect in a murine model of repeated mild traumatic brain injury. *Pain* 2023. https://doi.org/10.1097/j.pain.0000000000003084.

19 Beckmann L, Obst S, Labusek N, Abberger H, Köster C, Klein-Hitpass L, *et al.* Regulatory T Cells Contribute to Sexual Dimorphism in Neonatal Hypoxic-Ischemic Brain Injury. *Stroke* 2022;**53**:381–90. https://doi.org/10.1161/STROKEAHA.121.037537/FORMAT/EPUB.

20 Chakrabarti M, Das A, Samantaray S, Smith JA, Banik NL, Haque A, *et al.* Molecular mechanisms of estrogen for neuroprotection in spinal cord injury and traumatic brain injury. *Rev Neurosci* 2016;**27**:271–81. https://doi.org/10.1515/revneuro-2015-0032.

21 Wang J, Hou Y, Zhang L, Liu M, Zhao J, Zhang Z, *et al.* Estrogen Attenuates Traumatic Brain Injury by Inhibiting the Activation of Microglia and Astrocyte-Mediated Neuroinflammatory Responses. *Mol Neurobiol* 2021;**58**:1052–61. https://doi.org/10.1007/s12035-020-02171-2.

22 Zlotnik A, Leibowitz A, Gurevich B, Ohayon S, Boyko M, Klein M, *et al.* Effect of estrogens on blood glutamate levels in relation to neurological outcome after TBI in male rats. *Intensive Care Med* 2012;**38**:137–44. https://doi.org/10.1007/S00134-011-2401-3.

23 Wang KK, Yang Z, Zhu T, Shi Y, Rubenstein R, Tyndall JA, *et al.* An update on diagnostic and prognostic biomarkers for traumatic brain injury. *Expert Rev Mol Diagn* 2018:165–80. https://doi.org/10.1080/14737159.2018.1428089.

24 Liu MC, Akinyi L, Scharf D, Mo J, Larner SF, Muller U, *et al.* Ubiquitin C-terminal hydrolase-L1 as a biomarker for ischemic and traumatic brain injury in rats. *Eur J Neurosci* 2010;**31**:722–32. https://doi.org/10.1111/j.1460-9568.2010.07097.x.

25 Yao C, Williams AJ, Ottens AK, Lu XCM, Liu MC, Hayes RL, *et al.* P43/pro-EMAPII: A potential biomarker for discriminating traumatic versus ischemic brain injury. *J Neurotrauma* 2009;**26**:1295–305. https://doi.org/10.1089/neu.2008.0811.

26 Zoltewicz JS, Mondello S, Yang B, Newsom KJ, Kobeissy F, Yao C, *et al.* Biomarkers track damage after graded injury severity in a rat model of penetrating brain injury. *J Neurotrauma* 2013;**30**:1161–9.

https://doi.org/10.1089/NEU.2012.2762/ASSET/IMAGES/LARGE/FIGURE5.JPEG.

27 Han JR, Yang Y, Wu TW, Shi TT, Li W, Zou Y. A Minimally-Invasive Method for Serial Cerebrospinal Fluid Collection and Injection in Rodents with High Survival Rates. *Biomedicines* 2023;**11**:. https://doi.org/10.3390/biomedicines11061609.

28 Baskin BM, Logsdon AF, Janet Lee S, Foresi BD, Peskind E, Banks WA, *et al.* Timing matters: Sex differences in inflammatory and behavioral outcomes following repetitive blast mild traumatic brain injury. *Brain Behav Immun* 2023;**110**:222–36. https://doi.org/10.1016/j.bbi.2023.03.003.

29 Heyburn L, Batuure A, Wilder D, Long J, Sajja VS. Neuroinflammation Profiling of Brain Cytokines Following Repeated Blast Exposure. *Int J Mol Sci 2023, Vol 24, Page 12564* 2023;**24**:12564. https://doi.org/10.3390/IJMS241612564.

30 Breen EJ, Tan W, Khan A. The Statistical Value of Raw Fluorescence Signal in Luminex xMAP Based Multiplex Immunoassays. *Sci Rep* 2016;**6**:. https://doi.org/10.1038/SREP26996.

31 Breen EJ, Polaskova V, Khan A. 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* 2015;**71**:188–98. https://doi.org/10.1016/J.CYTO.2014.10.030.

32 Batllori M, Casado M, Sierra C, Salgado M del C, Marti-Sanchez L, Maynou J, *et al.* Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins. *Fluids Barriers CNS* 2019;**16**:1–9. https://doi.org/10.1186/S12987-019-0154-5/TABLES/2.

33 Knight BE, Kozlowski N, Havelin J, King T, Crocker SJ, Young EE, *et al.* TIMP-1 Attenuates the Development of Inflammatory Pain Through MMP-Dependent and Receptor-Mediated Cell Signaling Mechanisms. *Front Mol Neurosci* 2019;**12**:. https://doi.org/10.3389/FNMOL.2019.00220/FULL.

34 Giza CC, McCrea M, Huber D, Cameron KL, Houston MN, Jackson JC, *et al.* 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* 2021;**4**:E2037731. https://doi.org/10.1001/JAMANETWORKOPEN.2020.37731.

35 Hicks SD, Onks C, Kim RY, Zhen KJ, Loeffert J, Loeffert AC, *et al.* Refinement of saliva microRNA biomarkers for sports-related concussion. *J Sport Heal Sci* 2023;**12**:369–78. https://doi.org/10.1016/j.jshs.2021.08.003.

36 Vedantam A, Brennan J, Levin HS, McCarthy JJ, Dash PK, Redell JB, *et al.* Early versus Late Profiles of Inflammatory Cytokines after Mild Traumatic Brain Injury and Their Association with Neuropsychological Outcomes. *J Neurotrauma* 2021;**38**:53–62. https://doi.org/10.1089/neu.2019.6979.

37 Amen EM, Brecheisen M, Sach-Peltason L, Bergadano A. Refinement of a model of repeated cerebrospinal fluid collection in conscious rats. *Lab Anim* 2017;**51**:44–53. https://doi.org/10.1177/0023677216646069/ASSET/IMAGES/LARGE/10.1177_0023677216646069-FIG2.JPEG.

38 Chaban V, Clarke GJB, Skandsen T, Islam R, Einarsen CE, Vik A, *et al.* Systemic

654 Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the
655 Prospective Trondheim Mild Traumatic Brain Injury Study. *J Neurotrauma* 2020;**37**:2120–
656 30. https://doi.org/10.1089/neu.2019.6963.

657 39 Barreto GE, Thelin EP, Zhang Y, Madathil SK, Wilfred BS, Urankar SE, *et al.* Early
658 Microglial Activation Following Closed-Head Concussive Injury Is Dominated by Pro-
659 Inflammatory M-1 Type. *Front Neurol* 2018;**9**:414936.
660 https://doi.org/10.3389/FNEUR.2018.00964.

661 40 Simon DW, McGeachy MJ, Baylr H, Clark RSB, Loane DJ, Kochanek PM. The far-
662 reaching scope of neuroinflammation after traumatic brain injury. *Nat Rev Neurol*
663 2017:171–91. https://doi.org/10.1038/nrneurol.2017.13.

664 41 Miao W, Zhao Y, Huang Y, Chen D, Luo C, Su W, *et al.* IL-13 Ameliorates
665 Neuroinflammation and Promotes Functional Recovery after Traumatic Brain Injury. *J*
666 *Immunol* 2020;**204**:1486–98. https://doi.org/10.4049/jimmunol.1900909.

667 42 Lladó J, Tolosa L, Olmos G. Cellular and molecular mechanisms involved in the
668 neuroprotective effects of VEGF on motoneurons. *Front Cell Neurosci* 2013;**7**:64456.
669 https://doi.org/10.3389/FNCEL.2013.00181/BIBTEX.

670 43 Di Battista AP, Churchill N, Rhind SG, Richards D, Hutchison MG. The relationship
671 between symptom burden and systemic inflammation differs between male and female
672 athletes following concussion. *BMC Immunol* 2020;**21**:. https://doi.org/10.1186/s12865-
673 020-0339-3.

674 44 Lee S, Lee DK. What is the proper way to apply the multiple comparison test? *Korean J*
675 *Anesthesiol* 2018;**71**:353–60. https://doi.org/10.4097/kja.d.18.00242.

676 45 Yu F, Wang Y, Stetler AR, Leak RK, Hu X, Chen J. Phagocytic microglia and
677 macrophages in brain injury and repair. *CNS Neurosci Ther* 2022;**28**:1279–93.
678 https://doi.org/10.1111/cns.13899.

679 46 Chen W, Zhang Y, Zhai X, Xie L, Guo Y, Chen C, *et al.* Microglial phagocytosis and
680 regulatory mechanisms after stroke. *J Cereb Blood Flow Metab* 2022;**42**:1579–96.
681 https://doi.org/10.1177/0271678X221098841.

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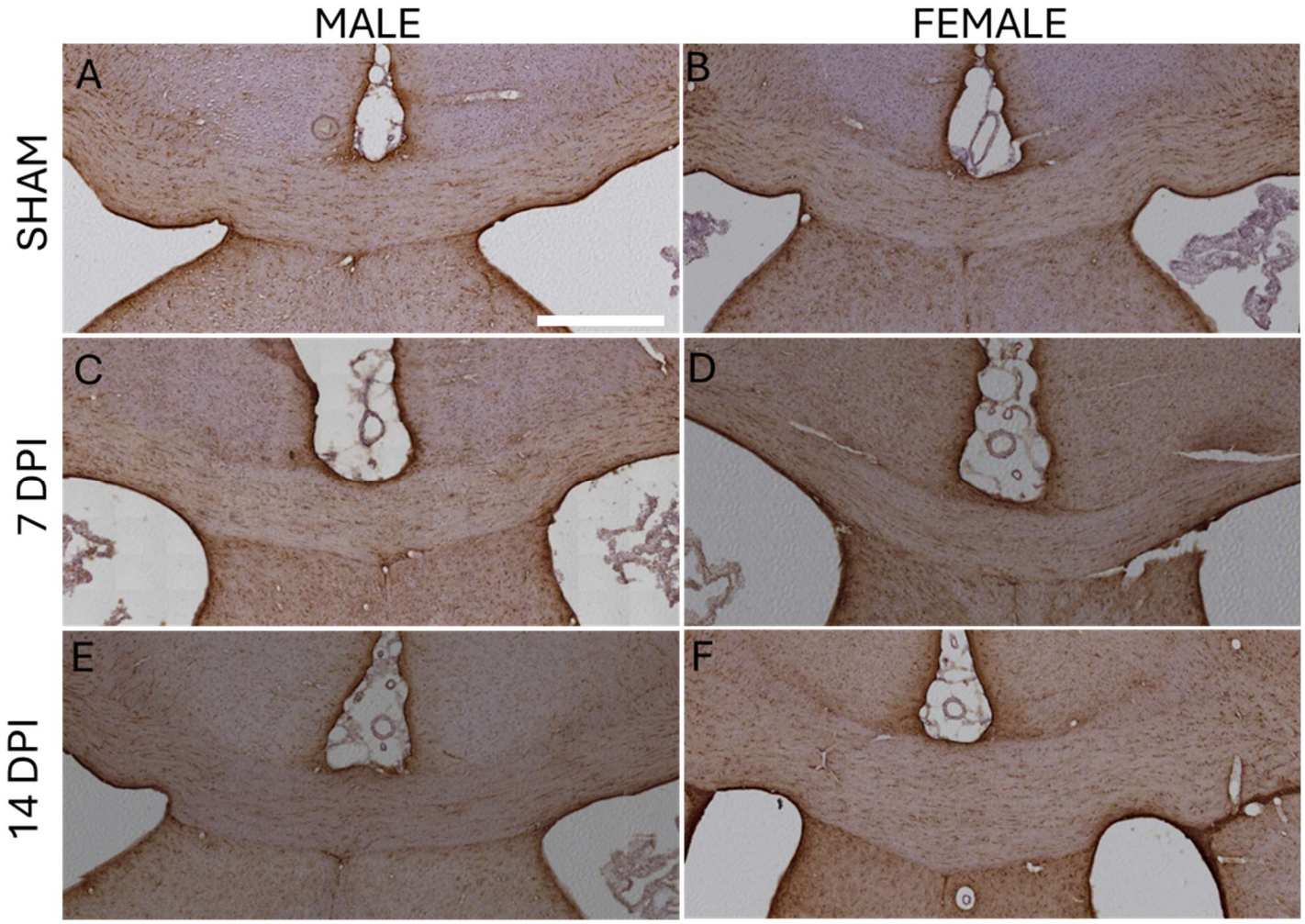

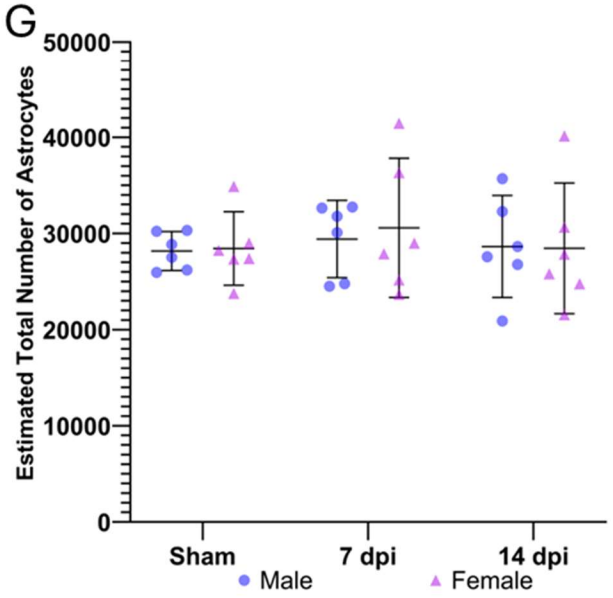

Figure S1: There are no differences in the number of astrocytes across groups. Stereological quantification of GFAP+ (brown) cells counterstained with hematoxylin (blue) was performed on serial sections of brain tissue. Representative images are shown (A-F). Quantification was performed only in the corpus callosum and is reported as the estimated total number of astrocytes (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. Scale bar = 100 µm.

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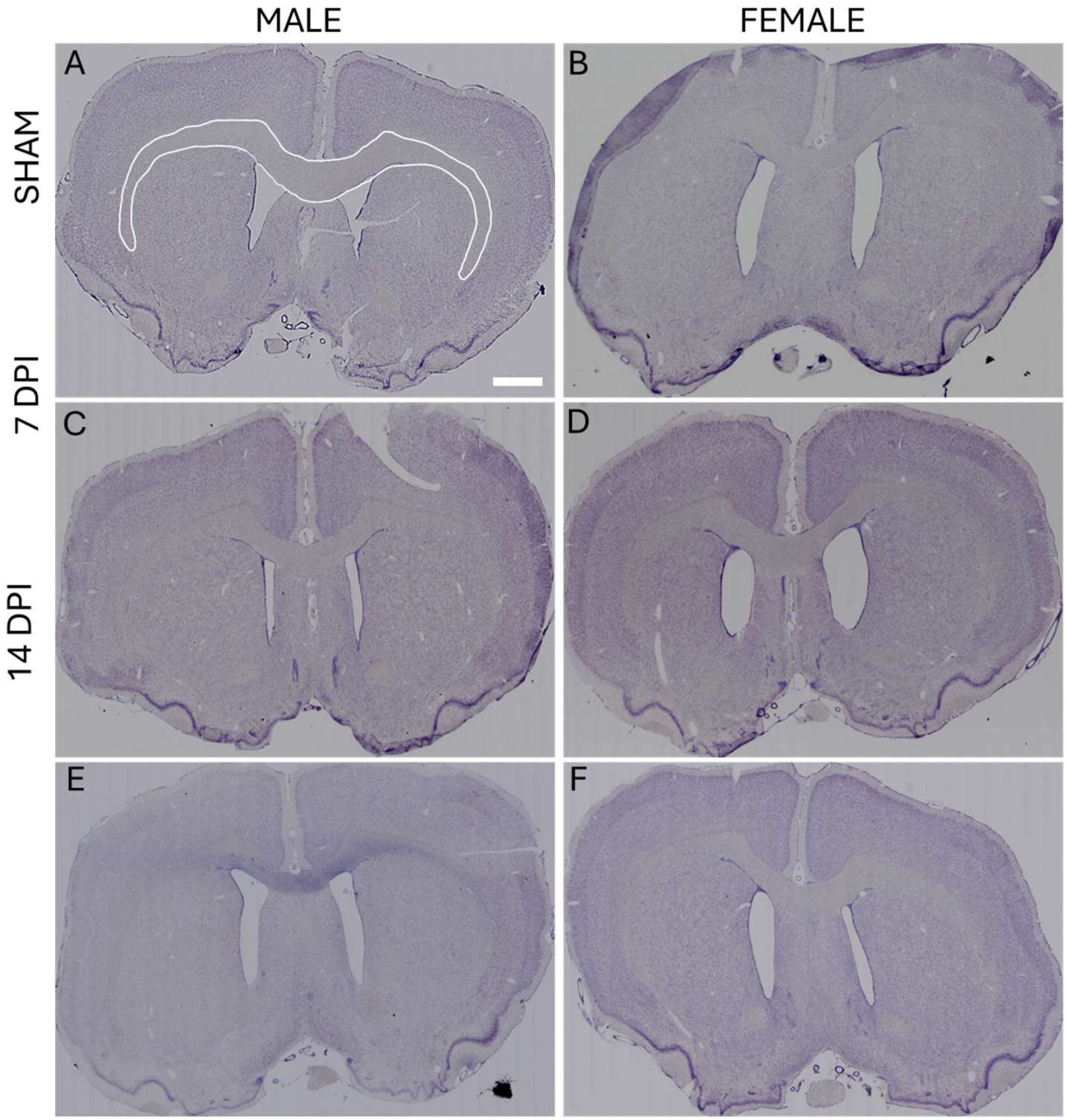

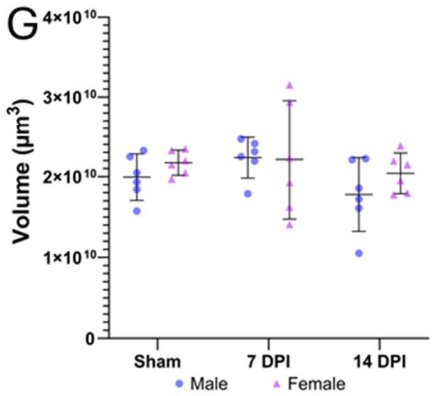

Figure S2: There are no differences in corpus callosum volume across groups. Cavalieri estimation was performed on serial sections of brain tissue to estimate volume of corpus callosum. Representative images are shown (A-F). An example ROI for corpus callosum volume estimation can be seen in A, depicted with a white outline. Volume estimations are presented as mean with 95% CI (G). Statistical analysis was performed using a two-way ANOVA and post-hoc Holm-Sidak multiple comparisons test. Scale bar = 1000 μm.

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