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Temporal Sex Differences in Blood Brain Barrier and Vasculature Recovery After Traumatic Brain Injury

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by

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ABSTRACT OF THE THESIS

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by

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Master of Science, Graduate Program in Biomedical Sciences
University of California, Riverside, June 2026
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Traumatic brain injury (TBI) in males has been extensively studied but less so in females. There is a general understanding that females and males' recover differently after TBI but this has not been extensively documented. A significant gap is how the vasculature and blood-brain barrier (BBB) is altered in females after TBI. Adult C57/BL6 male and female mice underwent a moderate cortical TBI. At selected time points BBB leakage and lesion volumes were assessed by magnetic resonance imaging (MRI) at baseline, 3-, 7-, 14-, 30-, and 60-days post injury (DPI). We also obtained classical vessel characteristics and complexity using our vessel painting technique at 60 DPI. We observed that females had a significant increase in vascular complexity at 60 DPI compared to the male mice. In contrast, males exhibited an increased BBB leakage and a more elevated lesion volumes over the 60 DPI time course. Our novel findings confirm that the response to a penetrating TBI in female mice is radically different than in male mice. These data provide additional evidence that treatment of TBI should be different between males and female subjects, particularly long-term treatment strategies.

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Introduction

Traumatic Brain Injuries (TBI) are a common physical affliction, with worldwide occurrences affecting approximately 70 million each year (Dewan et al., 2019). They are considered to be a leading cause of death and disability globally (Blaya, 2021). The resulting severity of a TBI can vary greatly from mild (GCS 14 – 15), moderate (9 – 13), and severe (3 – 8) as determined by the Glasgow Coma Scale (GCS) (Teasdale et al., 2014). There are many incidences in which TBI may occur, some of the most common including falls and motor vehicle accidents (Maas et al., 2022). TBI's are prevalent among many groups, including civilians, professional sports personnel, and military service members (McKee & Robinson, 2014, Marklund et al., 2019 ,Bailey et al., 2013). Long term consequences that result from TBI are problematic, as there can be both recovery and deficits that persist even 20 years out from a TBI (Wilson, 2017). This can result in functional impairments as well as debilitating diseases, such as cognitive deficits, Alzheimer's disease, post traumatic epilepsy, stroke, and in the most extreme cases death (Wilson, 2017). There is a need for greater post care management of a TBI due to these persisting complications. It is also of great concern that there is currently no established treatment to help with repairing both chronic and acute forms of TBI, and no consensus on rehabilitation (Marklund, 2019). This drives the need for further research to help elucidate how the brain functionally behaves after a TBI has occurred.

Current analysis has shown that men are 40% more likely to receive a TBI in comparison to females under the age of 75 (Gupte et al., 2019), and furthermore males are three times as likely to suffer from a TBI related death (Peterson et al., 2025). This can be partially explained with how societally men are put into more vulnerable workforce positions that can increase their chance of receiving a TBI (Biegon, 2021). However, this does not negate the fact that there is a greater need for studies that analyze how the sexes may differ in disease and recovery. It should also be stated that there is a likelihood that the amount of TBI's that women experience may be underreported, especially in the cases of intimate partner violence (IPV) (Monahan, 2017). There has also generally been an underrepresentation of females within clinical and preclinical TBI studies, with women representing ~30% or less of subjects in clinical studies (Biegon, 2021). This is unfortunately evident even though there are clear dimorphism differences that can be translated to different mechanistic responses within the brain (Currier Thomas et al., 2022). It is understood that women will undergo massive hormonal changes throughout their lifetime, and these gonadal hormones can exert different effects to the brain structure and function. For example, there has been research suggesting that both estrogen and progesterone are able to provide neuroprotective benefits to the brain after TBI (Roof et al., 1994). Receiving a TBI from occurrences such as fall is sex-independent (Späni et al., 2018), it is therefore imperative that both men and women be included in studies that involve TBI research. As it is predicted that 50% of people will suffer severe consequences resulting from a TBI within their lifetime, it is important to recognize and further analyze the impact that TBI's can have over time across the sexes

(Blaya et al., 2022). This will help us gain a deeper understanding how the brain repairs itself after physical damage and bring attention to where sex differences may lie. From this, we can create better individual recovery treatments that can be further explored in clinical trials.

Previously, as published by the Obenaus lab, observations were made that both behavioral and physiological outcomes were altered after a TBI in male mice. Results exhibited that male mice demonstrated acute vascular loss with a slow but progressive return to a similar vascular density at the site of injury (Singh et al., 2024). These studies all demonstrated vasculature loss with recovery over the 14-21 days post injury (DPI) period. A notable finding was that at 60 DPI, there was a reduction of vascular density which then led to a strong decline of cerebral blood flow in the cortex (Singh et al., 2024). However, all these analyses were performed only within male mice cohorts and it is unknown if female mice would respond in the same continuous manner. Due to time constraints, our lab had previously only been able to observe and derive from male data sets. Female data was also obtained to help address a limitation present in our Singh et al. 2024 study, so this data builds off those findings. For accuracy, the same mouse and TBI model were utilized in order to compare the differences of male and female TBI outcome. Our aim with this continuation of the study is to give a greater focus on how male and female mice may recover differently when it comes to cerebrovasculature and the continuation of blood-brain barrier leakage over time. Ultimately, we are interested in

observing more of the morphological and physiological changes that may be present between the sexes overtime.

For our analysis, our main interest was observing how BBB leakage and vessel characteristics would change over the course of 60 DPI. Our interest in looking at the BBB in particular lies in its significance for maintenance of brain homeostasis and determining the overall health of the brain. Because the brain parenchyma (neurons, glial cells) is very delicate, there is a need for protection from any harmful components circulating in the blood (bacteria, viruses, toxins…). One of these protective components is the BBB, which consists of tight junctions between cerebral microvascular endothelial cells (Alluri, 2014). These endothelial cells of the BBB metabolize many toxic and neuroactive substances, so it also functions as a metabolic barrier. TBIs can disrupt the BBB and cause a breakdown, which can lead to a cascade of complications. Because of the importance of the BBB, we explored how the extravasation progressed overtime. There is also an understanding that both angiogenesis and vasculogenesis have been shown to occur after a brain injury (Salehi et al., 2017), so there is some functional recovery that typically occurs after TBI (Zhang, 2014). It is important to understand whether the sexes would have different patterns of disturbances and recovery over time.

To gain a more comprehensive understanding of how male and female mice brains may differ in repair, we performed a longitudinal study to observe how BBB disruption and the vasculature surrounding the brain had changed over time. Within

this study, we focused on these parameters and effectively continued our previous observations from males. Looking at the structural changes between male and female brains will help elucidate how they may differ in response to TBI, as there is a great need to further investigate where the differences may lie. Given the current understanding of how preclinical male and female models recover after TBI, we hypothesized that the female TBI cohort would have a significantly better recovery in comparison to the male cohort (Rubin & Lipton, 2019). Overall, we found that males had a greater amount of BBB disruption when compared to the females and had an elongated period of recovery. The TBI female cohort exhibited physiological recovery after 60 DPI, and regained complexity of the vessels at the site of impact from both the axial and coronal perspective.

Materials and methods

Animals

The experiments were performed in accordance with the ARRIVE guidelines (Percie Du Sert et al., 2020) and were approved through University of California Irvine Animal Care and Use Committee. Adult male and female C57/B6 mice (JAX#000664, 2-3months old) were acquired from Jackson Laboratory and were group housed (5/cage) with 12h light/dark cycle in ventilated cages. Mice were also acclimated for a minimum of 7d after arrival. Male and female C57BL/6 mice were randomly designated to either receive a sham surgery (n = 11 for males, n = 8 for females) or a moderate TBI (n = 10 for males, n

= 9 for females) and underwent MRI across a 60 DPI time course. Animal replicates were derived from previous experiments and pilot experiments. Our published findings detail the behavioral outcomes for our male mice cohorts. Two male TBI mice died over the course of the experimental period and were excluded from our analysis.

Controlled cortical impact (CCI) model

The CCI model for traumatic brain injury (TBI) was performed as previously reported (Lin et al., 2022). Briefly, mice were anesthetized with isoflurane (1–3%), which were then maintained at 37ºC and then placed within a stereotaxic device. Under aseptic conditions, a scalp incision was performed, with the supporting connective tissue removed and a circular 5mm craniotomy (Bregma AP − 1.25 cm, ML + 1.25 cm) performed. Then, a 1.5mm rounded impactor tip was zeroed to the pial surface and retracted 1mm. An electromagnetic impactor was then applied (Leica, NeuroscienceTools, O'Fallon) with the following parameters: 1 mm depth, 200ms dwell-time, speed 5 m/s. Visible extravascular bleeding was removed. The skin was stitched closed without replacing the bone. Buprenorphine (100ng/g body weight, intramuscular) was injected, and mice were returned to a warmed chamber until ambulatory. Sham mice were exposed to identical anesthesia duration but did not experience the craniotomy procedure nor the impact.

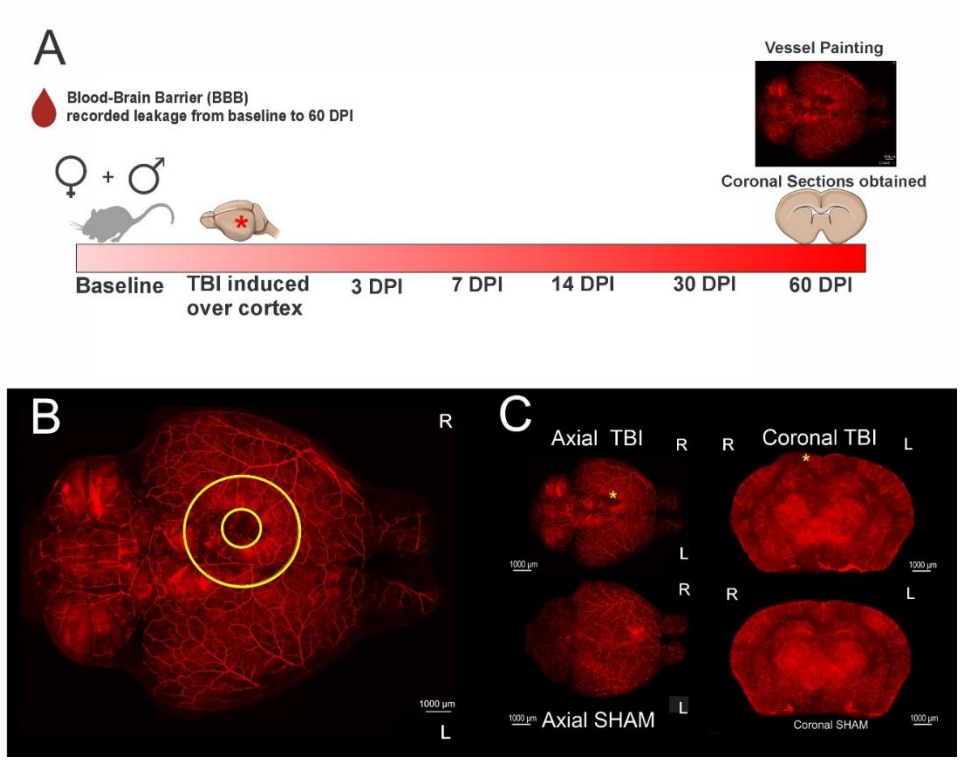

Figure 1 *– **(A)** A time course analysis included time points of baseline, 3, 7, 14, 30, and 60 days post injury (DPI) after cortical contusion injury (CCI) over the somatosensory cortices in male and female mice. T1-weighted MRI (pre/post) were taken at each time points for analysis of the blood brain barrier (BBB) leakage. T2-weighted MRI was collected for TBI lesion volumes. Vessel painting was performed for each animal at 60 DPI. (**B**) – Lesion and perilesional areas were analyzed for a further analysis and isolation of the site of impact on the axial surface. (**C**) – Representative axial and coronal vessel painted images. Vessels were stained with Dil to reveal structural changes after 60 DPI. Whole brain axial images were at 2X magnification for further quantification of vessel morphology at 60 DPI. The brain was transected at the level of the dorsal hippocampus at the site of the lesion. Vascular loss is present at the site of impact at 60 DPI.*

Magnetic Resonance Imaging (MRI) and blood-brain barrier (BBB) analyses

In vivo MRI was performed at baseline and after TBI induction (3, 7, 14, 30, and 60 DPI). MRI was carried out in the preclinical and translational neuroimaging center at UCI on a horizontal 30 cm bore, 9.4Tesla MR scanner (Bruker Avance) with a 72 mm diameter volume excitation RG coil. The mice were anesthetized with 2% isoflurane and tail veins were cannulated to facilitate contrast injection (0.1mmol/kg Gadoterate Meglumine diluted with sterile saline, Dotarem, Guerbet, Princeton, NJ). Sham and TBI

mice were then placed in the MRI machine and then T2-weighted, T1-weighted, PWI with Gd scans, and susceptibility-weighted MRI scans were executed.

The Jim9 software was used to determine the whole brain and lesion volume on the T2 scans. Jim9 and the Image Algebra function were utilized to examine the T1 scans before and after Gadolinium injection (called T1pre and T1post). Gadolinium is a contrast agent that does not cross the BBB in healthy conditions. If Gadolinium signal is detected in the brain parenchyma, it means the BBB is altered. Regions of interest (ROIs) were drawn to separate the ipsilateral and contralateral sides of the brain on the T1 scans. BBB leakage was then recorded through obtaining the signal intensity histogram values, and leakage sum was calculated through converting the pixel size to the proper area. To eliminate the noise signal of the BBB a threshold of 0.25 minimum contrast was used before extracting the values. Data obtained was then stored in MS Excel and analyzed with the GraphPad Prism software .

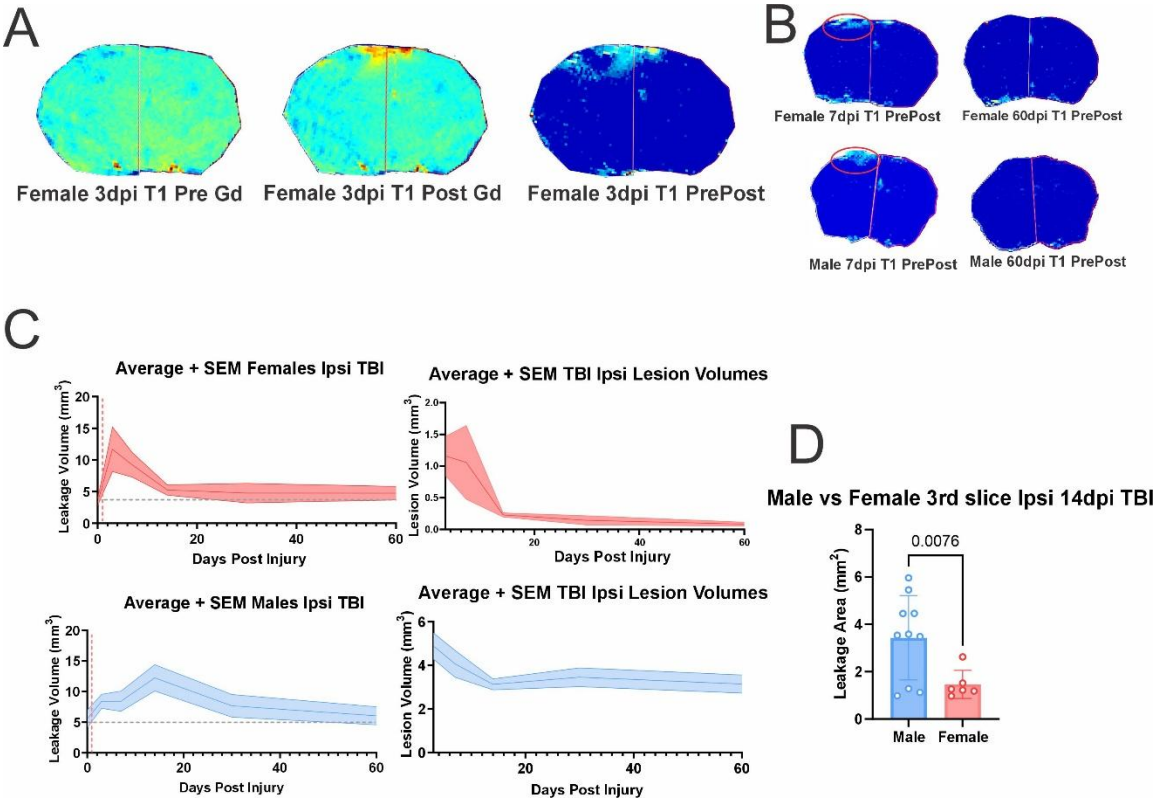

*Figure 2 – (**A**) – Representative T1-weighted (T1) Pre/post images at 3DPI. T1W1 were acquired prior and after Gadolinium (Gd) contrast agent injection to visualize BBB leakage. Subtraction of post- from the pre-image resulted in a net Gd extravasation into the tissues that was then quantified in each hemisphere. As can be seen there is considerable BBB leakage present at 3 DPI both on the ipsilateral and contralateral hemispheres. (**B**) – The T1 prepost scans from 7 and 60 DPI show the difference in blood brain barrier (BBB) leakage over time. At 7 DPI the injury for both the males and females is very prominent at the cortex, but the male has more leakage. At 60 DPI both males and females go back to around baseline levels of BBB leakage. (**C**) – BBB leakage is a dynamic process after TBI. Female mice exhibited a more pronounced early elevation after TBI peaking at around 7 DPI with a faster decline and return to sham levels by 21 DPI. In contrast, the BBB leakage peak in males was observed at 14 DPI, and their recovery processes was more gradual along with a prolonged lesion volume. T2-weighted lesion volumes in male and female TBI mice. The temporal evolution of lesion volumes was dramatically different in females with small lesion volumes which rapidly declined in size by 14 DPI. Male mice showed a pronounced lesion volume that remained elevated over the 60 DPI observation period. Again, there are overt differences in lesion volumes over time between sexes. (**D**) – The 3rd slice of the T1 scan was compared between the male and female mice. A t-test showed that there is a statistically significant difference between the leakage area in males and females at this timepoint.*

Vessel painting and analyses

To visualize cortical angioarchitecture we utilized our vessel painting protocol as previously reported (Singh, 2024). Briefly, 1,1'-Dioctadecyl 3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, D282, Invitrogen, Carlsbad) was

delivered by intracardiac injection prior to transcardial fixative perfusion at 60 DPI and brains were then extracted after 24h post fixation in 4% PFA. Brains were rinsed with PBS for 24h and stored in a PBS + 0.02% sodium azide solution until microscopic acquisition. There were 2 brains that were unusable due to poor vessel staining. Vascular image acquisition was performed through the use of Keyence fluorescent microscope, with proper calibration to visualize the vessels. Images were acquired at 2X magnification. Axial surface images were taken to look at the hemisphere as well as the lesion and perilesion sites. For coronal visualization, sectioning was performed at the site of impact. Anterior and posterior sections were imaged. Classical vascular analysis for vessel density, junctional branch points, total end points, and average and total vessel lengths were obtained using the Angiotool software (Zudaire et al., 2011). Fractal analyses for vascular complexity were performed using the Fiji plugin FracLac to obtain local fractal dimensions (LFD) (Karperien et al., 2013). A higher LFD value indicates more complexity within the region. Our regions of interest (ROIs) for the vessel analysis included ipsilateral and contralateral axial hemisphere and lesion and perilesional ROIs from the axial hemisphere. We also undertook cortical ROIs of the parenchymal vessels from coronal sectioning. Coronal sections were taken at the site of impact and separated by the anterior and posterior. Ipsilateral side cortex analysis was taken due to time limitations.

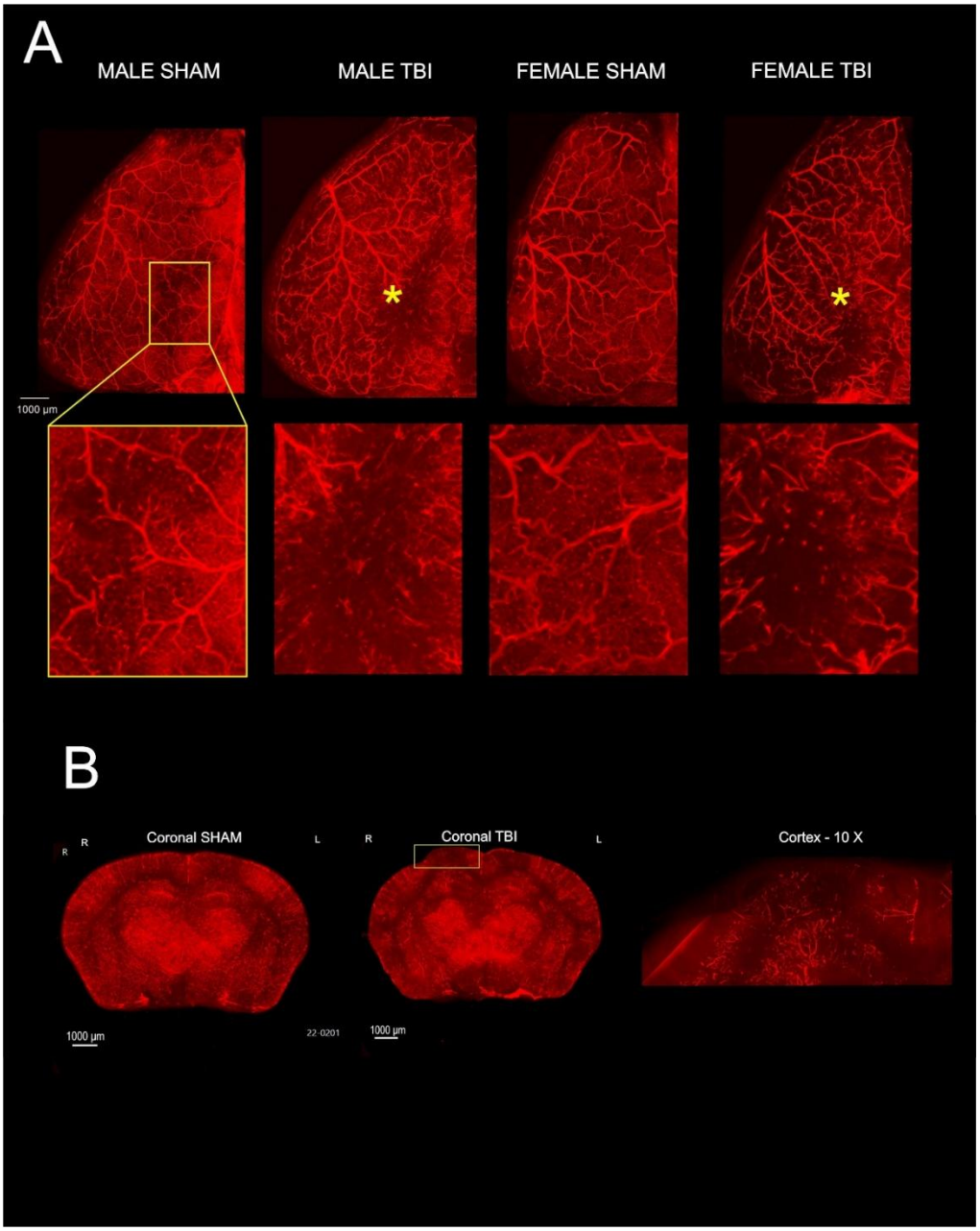

*Figure 3 – (**A**) – Comparison of vessel density in female and male vessels at 60 DPI. The highlighted box depicts the area of interest where the hit was taken. At the site of injury both male and female mice exhibit loss of vascularity compared to the shams. (**B**) – Loss of vessel density at the site of impact. Vessel density was reduced at the site of the injury and extended deep into the somatosensory cortex*

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Statistical Analysis

Utilizing FracLac and AngioTool, we obtained the data from axial and coronal angioarchitecture. From FracLac, a Local Fractal Dimension (LFD) graph was obtained from each animal. The output that is derived from the vessel painted brain are the pixels intensity from the image. That is to say, the software is able to derive and understand the complexity of the image through performance of a box counting fractal analysis inside of each of the windows. Raw values were obtained from AngioTool, which took measurements of the vessels. Values were then compiled and organized through excel files. For FracLac, the dimensions measured included skewness, kurtosis, max LFD, and peak frequency. For AngioTool, vessel density, lacunarity, total vessel length, average vessel length, and junction per mm^2 were analyzed. The software is able to derive and understand the complexity of the image this through a series of grids and by counting the number of pixels that occupy the space within the grid. The relationship between the number of pixels that occupy the space and the empty space determines the complexity. It is plotted on a log-log scale, and a higher fractal dimension indicates a more complex branching pattern of the vessels. The skewness tells you the symmetry of the LFD histogram distribution. If there has been a reduction skewness, that means that the vascular network has been simplified and the complexity is shifting away from the higher values, meaning that it has lost complexity. The kurtosis of the LFD histogram is the peakedness, with a reduction meaning a loss of overall complexity. The max LFD value corresponds to the highest fractal dimension that was found in the image. If there is a

lower peak LFD, that means that the most complex vascular regions were lost in the process. Finally, peak frequency corresponds to the amount of vessels that were found at the most common complexity value. If there is a decrease, that indicates that there was a reduction of the amount of vascular components to analyze.

For AngioTool, the values that were analyzed were vessel density, total vessel length, average vessel length, lacunarity, and the junctions of branching per mm^2. This was utilized to analyze angiogenesis at 60 DPI. The program was able to separate the vessels from the background, and a visual outline was created. A skeleton was then created, which was able to visualize all of the classical vessel dimensions.

Cortical Contusion Key Findings

Vessel Angioarchitecture in female and male mice after CCI

Our first analysis of the entire axial hemisphere that inspected the vessels of the cortical surface and included the lesion site found no significant ($p=0.978$) differences in vessel density between sham and CCI mice. Similarly, there were no significant differences for total vessel length, average vessel length nor junction densities. These negative findings were somewhat expected as we have demonstrated significant, albeit abnormal, revascularization at the injury site in previous studies (Lin et al 2022). All values below reported as mean ± standard error of the mean.

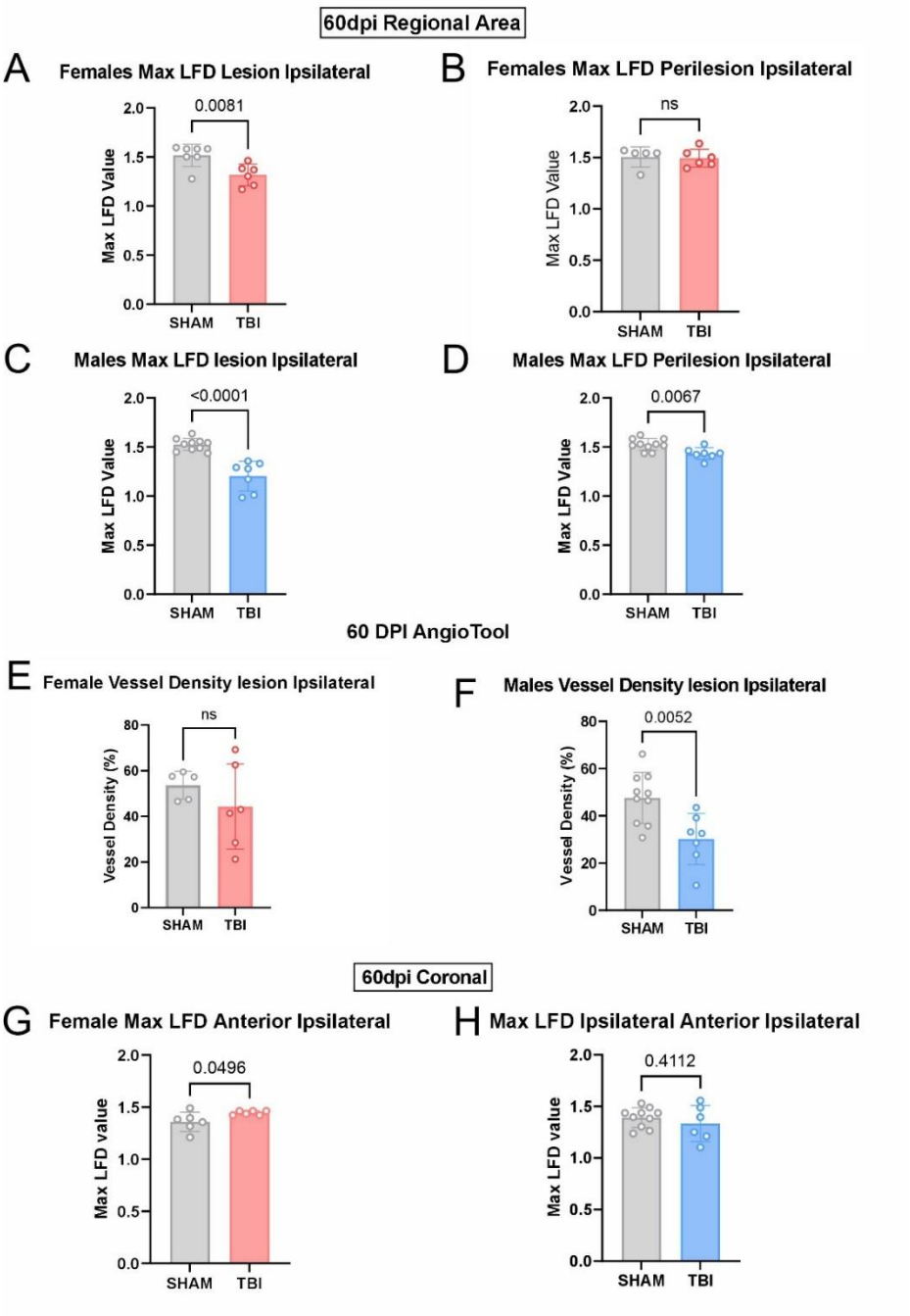

*Figure 4 – Vascular complexity at the lesion site. **(A) – (H)** - Both males and females exhibited significant decrements in vascular complexity at the lesion site. However, in the perilesion region, vascular complexity was only decreased in the males. This would suggest that females may have the ability for improved vessel recovery around the lesion. Vessel density is reduced in males at 60 DPI. In female TBI mice at 60 DPI there was a considerable increase in variability with some improved recovery while other mice had poor recovery of vessel density. This further confirms significant differences between male and female mice. Vascular complexity in female TBI is increased but not in male mice. There is a significant increase in complexity (Max LFD) in females, consistent with the formation of new vessels. For males, we saw that there was a decrease of vessel complexity in comparison to the SHAM.*

Our analyses revealed that there was a decrease in vessel density (sham 53.69 ± 2.76 vs. CCI 44.32 ± 7.64 % of area) although not significantly (p=0.3151) due to a 3-fold increase in the CCI variance.

We then investigated vascularity at the injury site and adjacent regions, termed region and perilesion respectively. Other vascular features, such as total vessel length and junction density were similarly reduced but not significantly different. In concurrence, lacunarity (space without vessels) was dramatically increased (sham 0.060 ± 0.005 vs. CCI 0.160 ± 0.042, p=0.0621) in line with reduced vessel density. We observed a significant (p=0.009) decrease in average vessel length within the lesion site (sham 4.32 ± 0.888 vs. CCI 0.628 ± 0.298 mm). Thus, vessel segments within the lesion site are reduced in length.

In the perilesion region, there were no differences between sham and CCI mice in vessel density, junctions or total vessel lengths. Again, average vessels were dramatically reduced (sham 6.65 ± 2.19) vs. CCI 2.43 ± 1.04 mm. p=0.155) although not significantly. The average vessel lengths were increased in perilesion area relative to the lesion. Thus, vascularity within the lesion and adjacent regions is increased in females. These changes in female CCI mice contrast with the findings in males where there is a significant difference in vessel density and junctions as we previously reported (Singh et al 2024). In males there was no difference between average vessel length in the lesion or perilesion sites.

We then undertook analysis of parenchymal vasculature within the dorsal cortex of female and male mice. The region of analysis encompassed retrospenial, motor and somatosensory cortices at the level of the dorsal hippocampus. There were subtle differences between males and females. For example, male CCI mice had reduced vessel density (12.7%) whereas females had increased vessel density (11.8%) albeit not significantly different. Broadly, these sex opposing effects were observed in most of the vascular metrics but were also not significantly different.

In summary, at 60 DPI there was re-vascularization present at the impact site, extending our previous findings which stopped at 30 DPI. There are subtle sex differences in vascular features suggesting a potential differential in the temporal evolution of vascular repair.

Vascular complexity in female and male mice after CCI

Fractal analyses of the vessel complexity were able to provide additional measurements to understand how the vessels repair. We used fractal analyses of the axial and coronal vasculature at 60 DPI in both male and female CCI mice compared to shams. The result of fractal analysis is a histogram of local fractal dimension (LFD, x axis) and frequency of occurrence (y-axis) from which we extract curve features: 1) skewness of the curve, 2) Kurtosis of the curve, 3) maximum of the LFD, and 4) peak frequency (reports number of vessels). These measures provide indices of vessel number (peak frequency) and vascular complexity (LFD).

The axial hemisphere fractal analysis observed no significant differences between male or female CCI mice compared to shams in any of the fractal features. As a reminder, the whole axial surface including the lesion and brain regions distant from the lesion were included in this global analysis. When we performed fractal analysis of the lesion area we observed a significant ($p<0.0001$) reduction LFD (complexity) in male CCI mice with no significant ($p=0.480$) change in peak frequency (number of vessels) confirming our angiographic analyses from above. In female CCI mice we also found a significant reduction in LFD ($p=0.008$) but not in peak frequency ($p=0.963$). Compared to shams, male CCI mice vascular complexity (LFD) in the lesion was reduced by 21.1% and in female CCI complexity was reduced 13.2%. Thus, while axial vessel density was not overtly altered, males exhibited a more dramatic decrease in vascular complexity than females.

The perilesional region exhibited the same general findings with male and female CCI mice having significantly reduced LFD values, $p=0.008$ and $p=0.007$, respectively. In male CCI mice there was a 6.1% decrease in LFD compared to sham mice and in female CCI mice there was a 0.08% LFD decrease. No other fractal parameters were significantly altered. In summary, both the lesion and perilesional regions on the axial hemisphere at the site and adjacent to the injury exhibited reduced vascular complexity.

In coronal analyses – no significant changes were observed in male CCI mice although LFD values were still decreased (6.1%). In contrast, within the ipsilateral cortex, female CCI mice reported a significant ($p=0.0496$) increase in LFD with a 6.3% increase within the cortex of vascular complexity. This increased parenchymal cortical vessel complexity is suggestive of a compensatory response to brain injury.

Temporal evolution of blood-brain barrier (BBB) disruption after CCI

As described previously, all male and female mice underwent a battery of neuroimaging, which included an assessment of BBB disruption. We performed DSC PWI which utilizes a bolus infusion of Gadolinium, a T1 relaxation shortening contrast agent for a proper assessment of BBB leakage. Acquisition of a pre-contrast and a post-contrast T1WI MRI allows for digital subtraction and brain regions with increased T1 signal are indicative of increased BBB leakage.

BBB leakage analysis at 3,7, 14, 30 and 60 DPI was performed on shams and CCI mice at each time point including males and females. In female mice the volume of BBB disruption was maximal at 3 DPI (11.68 ± 3.52 mm^3) and at 7 DPI was still elevated (10.03 ± 2.09 mm^3). At 14, 30 and 60 DPI BBB disruption in the female mice returned to baseline volumes (3.33 ± 0.58 mm^3).

Interestingly, in male CCI mice we observed a different recovery pattern. Baseline BBB leak volumes were elevated in male CCI mice (5.54 ± 1.41 mm^3)

compared to female CCI mice. Male CCI mice exhibited an increased BBB leak volume at nearly all time points which peaked at 14 DPI (12.25 ± 2.16 mm^3). The BBB leak volume at 30 DPI then declined by 37.38% to (12.25 ± 2.16 mm^3). At 60 DPI in male CCI mice there was a further 21.29% decrease in BBB leak volume relative to 30 DPI (6.03 ± 1.47 mm^3). In summary male mice exhibited a lengthened period of BBB leakage over the 60 DPI observation period that was not seen in females who had a sharp but transient BBB leakage peak with recovery to baseline by 14 DPI. These novel sex differences may foreshadow sex-dependent vascular recovery mechanisms.

Blood-Brain Barrier Leakage and Vasculature Dimensions Correlation

We were also interested in observing the potential relation between the amount of blood brain barrier leakage present and the vasculature features obtained at 60 DPI to see what the correlation may have looked like. What we found was in the male data sets had a stronger correlation in comparison to the females. It was interesting and important to note that suggesting that although there is a return of vessels and vessel complexity in the males, they may not be fully functional and could be leaky which could be the reason for the positive correlation between the two. In regards to the females,

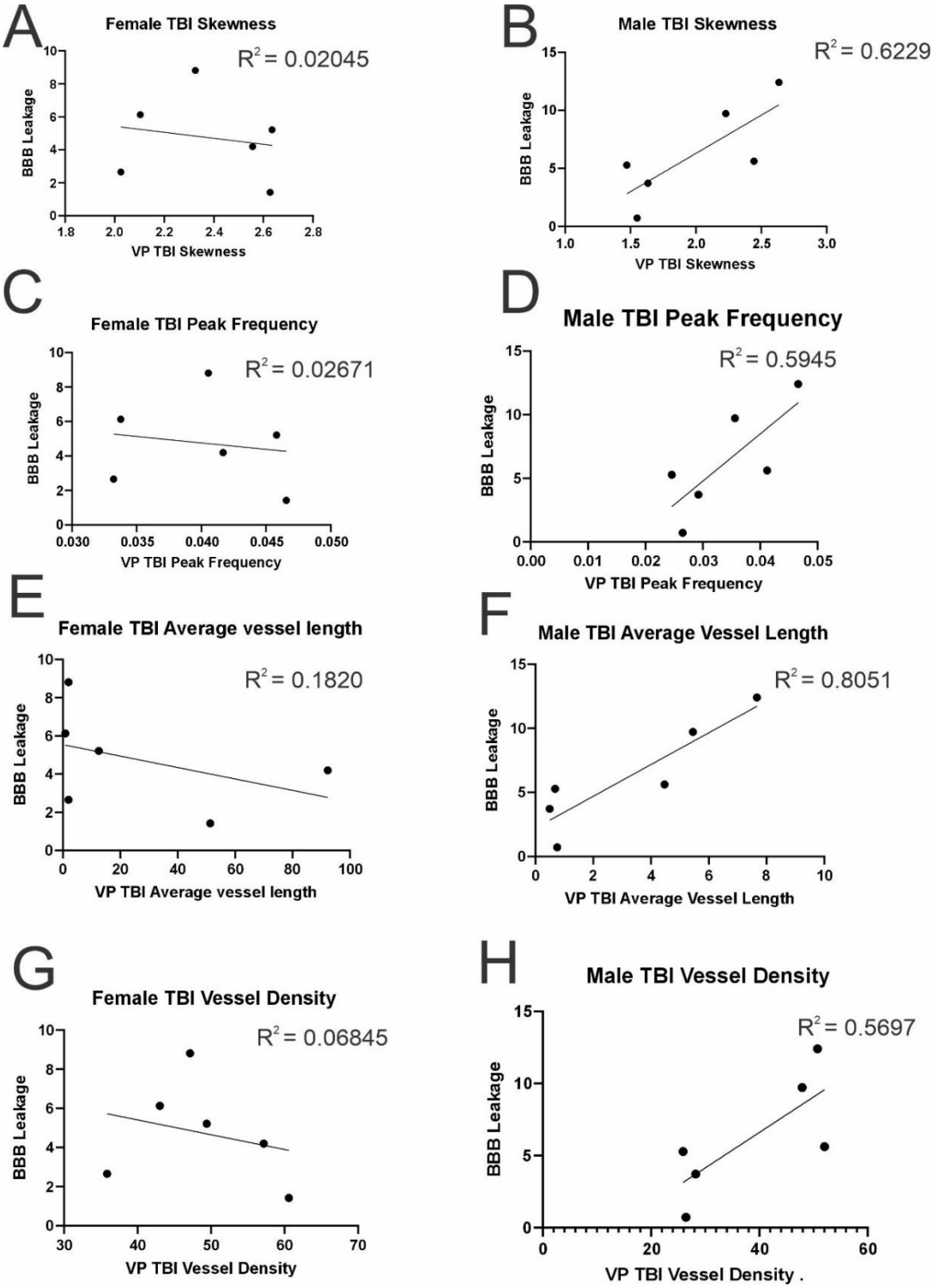

*Figure 5 – **(A)** – **(H)** – We observed a strong correlation in male skewness, peak frequency, average vessel length, and vessel density. For females, the correlations were weaker and less predictable.*

Discussion

Our findings demonstrate distinct differences between sexes and how they recover after TBI. We report that there are continued vascular perturbations that continue late (60 DPI) after the initial TBI event. These changes are seen in vessel length, vascular complexity, and BBB alteration. There are robust correlations between the BBB, and the vessel features that suggest a difference in vasculature to TBI between the sexes. In females there was a greater amount of vessel complexity within the site of injury in comparison to the male mice. This continues to support the data that there is clear sexual dimorphism in recovery from TBI.

Our findings align with those previously published. In preclinical TBI models, female cohorts typically recover better after TBI when compared to males in physical alterations as well as behavioral paradigms (Rubin & Lipton, 2019). The differences between male vs. female outcomes in studies appears to be based on hormonal factors, as both progesterone and estrogen exert neuroprotective effects that help better aid in recovery within preclinical models (Roof & Hall, 2000). These hormonal effects have been seen in reducing edema, preventing cell death, and improving behavior after a TBI (Roof et al., 1994, Rubin & Lipton, 2019,).

At long time points after the initial TBI, vascular features and characteristics were different and distinct between male and female mice. We observed that in males the vessels appeared to be shorter in length in comparison to females at 60

DPI. Our current understanding is that vessels are dynamic and constantly change in response to glial signaling even prior to any TBI (Attwell et al., 2010, Risser et al., 2007). Once TBI occurs, existing vessels are damaged and become leaky (Zhao et al., 2015), and within hours after injury there are new vessels that appear (Guo et al., 2016). We have reported that new vascular growth occurs after a TBI event and we show that these new vessels have the capability of showing functional perfusion starting at 7 DPI, with vessel density peaking around 14 – 21 DPI (Lin et al., 2022). However, vascular recovery that does occur does not necessarily mean that it is permanent. Our recently published study found that cerebral blood flow and volumes initially showed improvements from 7 to 30 DPI, but then declined precipitously at 60 DPI and overall showed a worse outcome than the initial injury time point (Singh et al., 2024). This pattern has also been shown within other studies, where Hayward et al., (2011) found that there was a rapid recovery shown in the cerebral blood flow from 24 hours to 3 DPI, that then decreased at 60 DPI. There is a significant gap within the literature about this initial recovery period within female preclinical TBI models which needs to be further explored to see if similar patterns would emerge as reported in our study.

Another finding from our study was the observed peak of BBB leakage that occurred earlier in females (7 DPI) when compared to males (14 DPI) who exhibited a slower decline in leakage. This has important implications regarding sex differences that may be present within the neurovascular unit. In endothelial cells, estrogen helps promote barrier integrity with the production of tight junction proteins such as claudin-5 (Cldn5)

and Occludin (Bake & Sohrabji, 2004, Pislaru & Haas, 2026, Robert, 2023,). There has also been *in vitro* evidence showing that astrocytes have sexual dimorphism differences in their response to TBI (Santos-Galindo et al., 2011), where males exhibited a stronger inflammatory reaction in comparison to females. Another aspect that we observed was a smaller TBI lesion site in our female TBI cohort compared to our male TBI cohort. This also aligns with what has been reported in various TBI models, as lesion volumes tend to be smaller in females in comparison to males when the same type of TBI is performed (Geddes et al., 2016, Igarashi et al., 2001, Missios et al., 2009, Robertson & Saraswati, 2015, Villapol et al., 2014) .

Our findings and those previously reported in the literature bring into question what the underlying mechanisms may be for these sex differences in our mouse TBI model. Further experiments are needed to gain a better understanding of what may be responsible for these differences, and how targeted treatments directed to improve structural support in response to vascular damage. To further isolate the potential sex changes we report, one could conduct experiments in ovariectomy (OVX) and orchiectomized (ORX) mouse models. This strategy would eliminate hormone production for these animals and thus potentially minimize hormonal effects (Chisholm & Sohrabji, 2016). We could also incorporate the use of estrogen receptor beta (Erβ) selective agonists, as they are able to facilitate the neuroprotective benefits of estrogen without the feminization factors. This has shown promising results in a TBI model (Soustiel et al.,

2005), and it has been shown that the Erβ pathway is able to provide neuroprotection without having feminization occur (Cordey & Pike, 2005).

For future experiments, we plan to incorporate additional sex comparisons to our TBI studies, including mild TBI models (concussion), as they are the most common type of TBI experienced and can lead to long term deficits over time. We will also investigate the vascular perturbations in the pediatric and elderly population after TBI, as these are the most vulnerable populations. Examining age-related differences in TBI recovery as well as sex differences would create a larger encompassing picture of how we could create targeted treatments based on sex.

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