Sex Differences in Stroke Outcome Are Associated With Constitutive Gut Dysbiosis and Stroke-induced Gut Permeability

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Research

Keywords: Ischemia, sex difference, inflammation, gut permeability, gut dysbiosis, dextrans

Posted Date: August 3rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-50069/v1

Sex differences in stroke outcome are associated with constitutive gut dysbiosis and stroke-induced gut permeability

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Abstract:

Background: Sex differences in experimental stroke are well documented, such that adult males show worse outcomes compared to adult females, including greater infarct volume, increased stroke-induced mortality and more severe sensory-motor impairment. Based on recent evidence that gut dysbiosis may be an early response to stroke, the present study tested the hypothesis that in the acute phase, stroke will result in greater permeability of the gut blood barrier and gut dysbiosis in males as compared to females.

Method: Male and female Sprague Dawley rats (5-7 months of age) were subject to endothelin (ET)-1-induced middle cerebral artery occlusion (MCAo). Fecal samples, blood draws and sensory motor tests were conducted pre and 2d after MCAo. Fecal samples were analyzed for 16s sequencing and short chain fatty acids (SCFAs). Gut permeability was assessed in serum samples using biomarkers of gut permeability as well as functional assays using size-graded dextrans.

Results: We confirmed stroke-induced sex differences, including greater mortality and sensory motor deficit in males as compared to age-matched female rats. Remarkably, fecal 16s sequencing showed greater bacterial diversity in females at baseline (prior to stroke) while 2 days after stroke, these measures were similar between the sexes. In contrast, fecal levels of short chain fatty acids (SCFAs) which are usually beneficial, were higher in males. MCAo-induced gut permeability was much worse in males as compared to females, as indicated by histological analysis, biochemical markers in serum, and serum measurement of fluorescent-labeled dextrans following oral gavage. Additionally, males had higher serum levels of proinflammatory cytokines IL-17A, MCP-1, IL-5 and EGF compared to females after stroke. Predictive modeling indicated that markers of gut permeability were associated with stroke-induced sensory-motor impairment.

Conclusions: Poor stroke outcomes in adult males is mirrored by increased gut permeability in this group. Additionally, these data suggest that constitutive sex differences in the diversity and

richness of gut microbial communities may predispose better functional outcomes after stroke in females, and support the idea that preventative modification of the gut microbiome may reduce the risk for stroke in vulnerable populations such as the elderly or those with co-morbid conditions.

Keywords: Ischemia, sex difference, inflammation, gut permeability, gut dysbiosis, dextrans

Introduction:

Ischemic stroke is the 5th leading cause of mortality and a major cause of long-term disability in the United States (Mozaffarian et al., 2015). Sex differences in stroke have been well documented in preclinical studies with young adult males displaying worse outcomes after stroke, including greater mortality, a larger infarct volume and more severe sensory motor impairment. In fact, females have been reported to have as low as one third the infarct volume of age-matched males (Toung et al., 1998). This has been attributed to the neuroprotective properties of the endogenous ovarian steroid hormones. Accordingly, ovariectomy increases infarct volume in young females (Fukuda et al., 2000, Selvamani and Sohrabji, 2010d, Rusa et al., 1999, Dubal et al., 1998), and estrogen treatment to either males or females reduces infarct volume (Toung et al., 1998, Suzuki et al., 2007, Selvamani and Sohrabji, 2010d, Selvamani and Sohrabji, 2010b, Simpkins et al., 1997). It is generally believed that ovarian hormones act directly on the brain or the immune system to alter stroke outcomes, however, recent evidence indicates that the gut microbiome constitutes a target for estrogen's actions.

The intestinal microbiome has been shown to influence brain specific activities that are related to stroke such as blood brain barrier integrity (Braniste et al., 2014), microglial activity (Erny et al., 2017) and peripheral inflammation (Benakis et al., 2016). Estrogen has been shown to prevent the loss of beneficial bacteria and to promote their growth and proliferation (reviewed in (Chen and Madak-Erdogan, 2016), as well as reducing lipopolysaccharide (LPS)-induced inflammation and pathogenic populations of bacteria (Baker et al., 2017, Blasco-Baque et al., 2012). In addition, estrogen also modulates the gut barrier/integrity (Baker et al., 2017), through regulation of tight junction proteins (Braniste et al., 2009). Collectively this represents another way in which sex differences may occur in stroke outcomes.

While several recent studies have shown that gut dysbiosis is associated with common risk factors for stroke, such as age (Spychala et al., 2018), obesity (Ley et al., 2006) and metabolic disease (Sato et al., 2014), only one previous study has reported on sex specific gut

disruption in association with stroke injury. While aged males and females typically have worse stroke outcomes than young animals, this study showed that aged males fare worse than aged females after stroke, and display persistent changes in gut dysbiosis as well as more prominent T-cell responses in the brain (Ahnstedt et al., 2020).

Despite the fact that sex differences in stroke outcomes are dramatically different in young males and females, gut dysbiosis and gut permeability have not been reported for this age group. The present study tested the hypothesis that stroke-induced gut dysbiosis and gut permeability would be severe in adult males as compared to adult females. Surprisingly, our data show that males and females show significant differences in gut microbial communities prior to stroke, but few differences after stroke. Additionally, there are constitutive differences in fecal levels of SCFAs with males having higher levels than females. In contrast, disruption of gut morphology and gut permeability is more severe in males after stroke, as compared to females, which is consistent with greater stroke-induced mortality and sensory-motor impairment in this group. The data suggest that constitutive differences in gut communities may predispose males to be more vulnerable to stroke-induced mortality and impairment.

Materials and Methods

Animals: Sprague-Dawley female and male rats were purchased from Envigo Laboratories (IN) as adults (5–7 months, 230–420 g). Animals were maintained in a 12:12 light/dark cycle in AAALAC-accredited vivarium facilities. Food and water were available ad libitum. A week after arrival, females were subjected to daily vaginal smears for 14–21 days to determine estrous status (Jezierski and Sohrabji, 2001). Adult females with a normal estrous cycle of 4–6 days were included in the study. Within each sex, animals were assigned randomly to stroke or sham groups. All animals were fed pelleted food (Harlan #8604 Teklad diet) for at least 4 weeks prior to their assignment to the study. All procedures were reviewed and approved by the Texas A&M

University Institutional Animal Care and Use Committee in accordance with OLAW guidelines for the humane treatment of animals in research.

Middle cerebral artery occlusion (MCAo): MCAo was induced by intracerebral injection of endothelin-1 (ET-1) to the MCA as previously described (Selvamani and Sohrabji, 2010d, Selvamani et al., 2014, Selvamani and Sohrabji, 2017b, Selvamani et al., 2012, Park and Sohrabji, 2016b). Animals were anesthetized (200 mg/ml/kg ketamine and 10 mg/ml/kg xylazine) and placed in a stereotaxic apparatus. ET-1 (3 microliters of 0.5 µg/µl, 600 pmol; American Peptide Co, CA) was injected at a rate of 1µl/min to the left middle cerebral artery (AP: +0.9, ML: −3.4, relative to bregma, DV: −8.5, relative to dura). Animals were observed every 6 hours after stroke until termination at 48h. Mortality was recorded at 12, 24, 36 h after stroke.

Infarct volume: Infarct volume was determined using our previous procedures (Selvamani and Sohrabji, 2010d, Selvamani and Sohrabji, 2010a). Briefly, animals were given an anesthetic overdose, and the brain was rapidly removed from the cranium and sliced into 2 mm coronal sections using a brain matrix (Roboz, US). Brain slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, MO) at 37°C for 20 min and stained slices were photographed using an Olympus digital camera attached to a surgical microscope. Images were coded and infarct volume was measured using image analysis software, Image J (NIH, MD) by an experimenter who was blind to the codes. Total brain infarct was calculated from 3–4 slices (per animal) and expressed as the ratio of infarct volume in the ischemic hemisphere to the total volume of the non-ischemic hemisphere.

Behavioral analysis: Motor impairment following MCAo was assessed using the vibrissae-evoked forelimb placement task (VIB) and the adhesive-tape removal test (ART) as described previously (Balden et al., 2012, Selvamani et al., 2014, Selvamani and Sohrabji, 2017b).

The vibrissae-elicited forelimb placement test was performed prior and 2 days after the MCAo surgery. Animals were subject to same-side placing trials and cross-midline placing trials elicited by brushing the ipsi and contra-lesional vibrissae against the edge of a table. During the same-side forelimb placing trials, the animal's ipsilesional vibrissae were stimulated against the edge of a table and forelimb placing response on that side was scored by an investigator, who was blinded to experimental conditions. In the cross-midline placing trials, the animal was held gently by the upper body such that the ipsi lesional vibrissae lie perpendicular to the table top and the forelimb on that side is gently restrained as the vibrissae was brushed on the top of the table to evoke a response from the contra lesional limb and vice versa. Between each trial the animal was allowed to rest all four limbs briefly on the table top to help relax its muscles. Ten trials were performed during each test.

For the adhesive tape removal test, a piece of adhesive backed foam tape (Scotch Permanent Mounting Squares, 12.7 × 12.7 mm) was used as tactile stimuli attached to the palmar surface of the paw of each forelimb. For each forelimb, the time it took to remove the stimulus (tape) from the forelimbs was recorded during three trials per day for each forepaw. Animals were allowed to rest for 1 min between sessions, and each test session had a maximum time limit of 120 s.

ELISA Assays: Blood samples were obtained by saphenous draw at baseline (0 day) and 2 days after stroke and centrifuged to obtain serum. ELISA assays were used to determine LPS, LPS binding protein (LBP), mucins 2, intestinal fatty acid binding protein (iFABP) and cytokines. Serum LPS (endotoxin) levels were measured using a commercial kit, Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, MA) as per manufacturer's instruction and our

published protocol (Park et al., 2019). Serum MUC1 and 2, iFABP and LPS binding protein (LBP) levels were assayed by a solid-phase sandwich ELISA method (Mybiosources, USA) using a colorimetric assay. Plates were read on a microplate reader (TECAN, VT) at a wavelength of 450 nm. The concentration of the samples was obtained by interpolation from the standard curve. Levels of a panel of inflammatory cytokine/ chemokine in serum were quantified using a rat cytokine/chemokine panel which detects 27 analytes (Millipore, MA). The procedure was performed according to the manufacturer's directions and our published procedures (Bake et al., 2017, Park and Sohrabji, 2016b)

Fecal metagenomics analyses:

Fecal samples were collected at baseline (2d prior to stroke) and 2d after stroke. The samples were frozen immediately after collection and an aliquot was used for DNA extraction using a MoBio Power soil DNA isolation kit (MoBio Laboratories, CA) following the manufacturer's instructions. Illumina sequencing of the bacterial 16S rRNA genes were performed using primers 515F (5'-GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) to 806RB (5' GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015), at the MR DNA laboratory (Shallowater, TX). Sequences were processed and analyzed using a Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Callahan et al., 2016) v 2018.6 pipeline. Briefly, the sequences were demultiplexed and the amplicon sequence variant (ASV) table was created using DADA2 (Douglas et al., 2019). Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance ASVs, containing less than 0.01% of the total reads in the dataset were removed. All samples were rarefied to even sequencing depth, based on the lowest read depth of samples.

Alpha diversity was measured with the Chao1 (richness), Shannon diversity, and observed ASVs metrics (only observed ASV metrics are reported here for brevity). Beta diversity was evaluated with the phylogeny-based unweighted UniFrac (Park et al., 2019a) distance metric

and visualized using Principal Coordinate Analysis (PCoA) plots. The F:B ratio was calculated as the ratio of the dominant phyla Firmicutes and Bacteroidetes.

Gut permeability analysis: Size-graded dextran labeled with either Fluorescein isothiocynate (FITCD,10kDa) or Rhodamine (RhoD, 70kDa) was administered by oral gavage (60mg/100g of body weight) to animals 30 min prior to MCAo or sham surgery. Blood was collected from the tail tip at 30, 60 and 90 mins after the MCAo or sham procedures and stored in the dark at 4 °C for 4 h. Blood samples were then centrifuged for 2 mins at 1200 rpm and the supernatant was diluted into 1:10,000 fold with 1XPBS and added in a 96-well microplate to determine the concentration of fluorescently-labeled dextran in serum by spectrophotometer (Tecan, USA) with an excitation frequency of 490nm and emission of 520nm for FITCD-10kDa, and excitation frequency of 540 nm and an emission of 625 nm for RhoD-70kDa using a standard serially diluted FITC-dextran and Rho-dextran (100, 50, 25, 10, 5 and 1 ng/ml). Serum from a naïve rat (not administered with labeled dextran) was used to determine the background.

Gut Histology: Cryosections of the ileum were stained for Hematoxylin and Eosin (H&E) as previously described (Kumar et al., 2017). Sections were visualized and photographed with the FSX100 Cell Imaging System (Olympus) at 10X magnification. The height of the villus and crypt layers was measured from these images and reported as a ratio of villus height/crypt height.

Immunohistochemistry: Immunofluorescence for Zonula Occludens (ZO-1) was performed as previously described (Kumar et al., 2017). Briefly, a portion of the ileum was embedded in Cryo-OCT compound (Leica Microsystems, Buffalo Grove, IL, USA) and frozen in liquid nitrogen. Cryosections (10 microns) were collected on glass slides and incubated in blocking buffer (5% bovine serum albumin, 0.1% Triton X-100 in PBS, pH 7.4) for 20 min at room temperature. Sections were then incubated overnight at room temperature with primary antibodies to either

ZO-1 (Custom antibody services; Thermo Fisher Scientific), at 1:400 dilution. Secondary antibodies, (Alexa Fluor 568 nm for ZO-1) (Thermo Fisher Scientific) were used at 1:1000 dilution for 1 h at room temperature. Sections were then washed three times in PBS, and cover slipped with mounting media containing the nuclear dye DAPI (Fluoroshield, Abcam). Sections were visualized and photographed on the FV12-IX83 confocal microscope.

Short-chain fatty acid Analysis: The following SCFA were analyzed in fecal samples: butyric acid, isobutyric acid, valeric acid, isovaleric acid, propionic acid levels. Fecal samples were weighed and lyophilized overnight, and extracted with a methanol:chloroform:water based extraction method. Samples were spiked with 0.1mM d7 Butyric Acid as an internal standard. SCFAs were detected and quantified on a gas chromatography triple quadrupole mass spectrometer (TSQ EVO 8000, Thermo Scientific, Waltham, MA) at the Texas A&M University Integrated Metabolomics Analysis Core.

Predictive modeling of stroke outcomes:

In view of the sex difference in mortality after stroke and the ART, logistic and/or linear regression models were applied to determine whether these outcomes could be predicted by gut metabolites or gut permeability markers.

Mortality as Response Variable: A logistic regression was used to model binary mortality response. The explanatory variables were gender, treatment, butyric acid, isovaleric acid, propionic acid, and valeric acid. The form of the logistic regression model was as follows:

$$log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 Male + \beta_2 Sham + \beta_3 Butyric\ acid + \beta_4 Isovaleric\ acid + \cdots \beta_6 Valeric\ acid$$

Here, β_0 is the model intercept, and each of the other β terms are log odds ratios. For example, β_1 is the log odds ratio comparing males to females, holding all other variables constant. Similarly, β_3 is the log odds ratio comparing two animals who differ by one unit on butyric acid,

holding all other variables constant. All models were fit using iteratively reweighted least squares, according to the standard logistic regression assumptions. Statistical confidence was again set to 95%.

ART as Response Variable: A linear regression was used to formally model the ART response variable and conduct statistical inference. The explanatory variables considered were the binary variables gender and treatment (ET1 or Sham) and the numeric metabolite variables butyric acid, isovaleric acid, propionic acid, valeric acid, Muc2 as well as gut permeability markers LPS, LBP, iFABP. The form of the linear regression models was as follows:

Average ART = $\beta_0 + \beta_1 Male + \beta_2 Sham + \beta_3 Butyric\ acid + \beta_4 Isovaleric\ acid + \cdots + \beta_{10} Muc2$

Here, β_0 is again the model intercept, β_1 is the mean difference in ART comparing males to females while holding all other variables constant, β_2 is the mean difference in ART comparing the sham group to the ET1 group while holding all other variables constant, and the $\beta_3, \beta_4, \cdots, \beta_{10}$ coefficients are mean differences in ART associated with one-unit increases in the metabolite variables while holding all other explanatory variables constant. Separate models were fit using pre- and post-treatment explanatory variables, respectively. All models were fit using least squares. Model estimates, confidence intervals, and p-values were computed under the standard least-squares regression assumptions (Sheather, 2009). Statistical confidence was set at 95%.

Statistical analysis: ANOSIM (Analysis of Similarity) test within PRIMER 7 software package (PRIMER-E Ltd., Luton, UK) was used as multivariate statistics on beta-diversity results. GraphPad prism software was used for statistical analysis (GraphPad Software, San Diego, CA). Initial sample size was 26 males (10 Sham, 16 MCAo) and 22 females (9 Sham, 13 MCAo). Following MCAo, sample size ranged from 5-13, depending on the assay. Survival plot

was calculated the using Kaplan Meier test. For behavioral tests, a paired Student's t test was used, comparing the values obtained pre- and post-stroke. For all other comparisons a two-way ANOVA was used with planned comparisons. Group differences between bacterial families as analyzed by ttest corrected for False Discovery Rate. FDR corrected bacterial abundance were converted to a z-score and plotted as a heat map. Group differences were considered significant at $p<0.05$ in each case. All data are expressed as mean ± S.E.M. Specific animal numbers used for an assay is described in each figure legend.

Results:

Stroke outcomes are worse in male rats as compared to age-matched female rats

Stroke outcome was assessed quantitatively by survival, infarct volume and sensory motor deficit. Males had significantly higher mortality rate due to stroke as compared to females (Fig. 1A; $p=0.0014$). By 12 hours post stroke, mortality in male rats was 60%, while mortality in the female rats was 8.33%. In the 24 to 36-hour time frame post stroke, the overall female rat mortality rose to 20.83%, while male mortality rose to 63.33%.

Infarct volume of the surviving rats was quantified from TTC-stained images (Fig 1B) and normalized to the contralateral hemisphere. There was no significant difference in the infarct volume of the surviving males and females at 2d post stroke (Fig. 1C, $p=0.209$). Our previous studies indicate that infarct volume is significantly larger in males at 5d post stroke compared to females (Selvamani and Sohrabji, 2017b).

Sensory motor deficit was evaluated using ART and VIB test. There was no difference between the pre and post-stroke performance of any group on the ipsilesional side for either test (data not shown). On the contralesional side, latency to remove the adhesive tape was significantly impaired in all male rats, such that none were able to remove the tape within the maximum allotted time of 120 seconds (Fig. 1D). Female rats also showed an increased latency to tape

removal after stroke compared to their pre-stroke performance, however, the latency in this group was significantly lower than that of males (Fig. 1D; p=.0003).

On the ‘‘same-side’’ VIB placement task, where the animal is expected to reach out with the paw located on the same side as the stimulated vibrissae, both males and females were equally adept at the task prior to MCAo. After MCAo, both males and females were unimpaired on the limb ipsilesional to the stroke. On the contralesional limb, male rats were severely impaired and completely unable to perform the task after MCAo. In contrast, female rats had a deficit after MCAo indicated by a significantly lower score, however, their score was significantly better than that of the male rats (Fig. 1E; p<.0001). Collectively, these results clearly show that the male rats had a worse stroke outcome as confirmed by their lower survival and greater sensory motor deficit as compared to age-matched female rats.

Sex differences in bacterial diversity

Gut dysbiosis was evaluated by three measures, the richness of bacterial families (alpha diversity), clustering of bacterial communities (unweighted UniFrac; beta diversity) and the ratio of the major phyla Firmicutes and Bacteroidetes. The ratio of Firmicutes to Bacteroidetes (F:B) is an estimate of health and an elevated F:B ratio is seen in aging and disease states such as metabolic syndrome (Natividad et al., 2018) and stroke (Park et al., 2019a, Spychala et al., 2018).

Bacterial sequencing revealed a significant sex difference in the composition of bacterial communities. Alpha diversity as indicated by observed ASVs (Fig 2A; p=0.0163) Chao1 and Shannon (not shown) was significantly different in males and females, with females displaying greater richness of bacterial families. Unweighted UniFrac analysis showed that there was virtually no overlap between bacterial communities between males and females indicating a significantly different beta diversity pre-stroke (Fig 2B, p=0.001, R=0.411). In contrast, there were no sex differences in the F:B ratio at baseline (pre-stroke; Fig 2C; p=0.7056).

Remarkably, sex differences in bacterial communities were absent after stroke (Fig 2D, E). Further, there was no differences in the F:B ratio between males and females or compared to their pre-stroke ratio (Fig. 2F). This is consistent with our previous study that the F:B ratio is not changed after stroke in young females (Park et al., 2019a) and the current data suggests that this may be a feature of young animals.

Analysis of bacterial abundance prior to stroke indicated significant sex differences in bacterial families within the order Clostridiales, all within the phyla Firmicutes (Fig 2G). Some of these bacteria are implicated in synthesis of short chain fatty acids, which have been shown to improve stroke outcomes, however some families (eg., Clostridiaceae) were elevated in males while others (Lachnospiraceae) were elevated in females.

<u>Males and females displayed significant constitutive differences in fecal SCFA levels.</u>

Since many of the sex regulated constitutive differences in gut microbiota were associated with SCFA synthesis, we next analyzed fecal levels of SCFAs. SCFAs, specifically butyric acid, are shown to be neuroprotective for stroke (Park and Sohrabji 2016) (Patnala et al. 2017). Fecal samples, collected before (0 day) MCAo or sham surgery, were analyzed for 5 SCFAs including butyric acid, isobutyric acid, valeric acid, isovaleric acid and propanoic acid. Surprisingly, in all cases males had higher levels of SCFAs (Fig. 3A-E).

<u>MCAo has a more severe effect on gut histology and intestinal tight junction proteins in male rats than female rats</u>.

Despite the lack of sex differences in gut microbial communities or gut dysbiosis after stroke, analysis of gut morphology revealed significant stroke-associated sex differences. Typically, an 'injured' gut has shorter, blunted villi and crypt hyperplasia. In males there was significant perturbation of gut after stroke (Fig. 4A), as evidenced by short, wider villi as compared to the sham male or the stroke-injured female in H&E stained sections. Moreover, crypt width was also

increased resulting in a shorter villus to crypt ratio (Fig. 4B; p=0.046) in males subjected to MCAo as compared to sham males or females subjected to MCAo (Fig. 4B; p=.0364). There were no sex differences in the villus/crypt ratio in sham groups.

Gut barrier properties were further assessed qualitatively by immunohistochemistry for the tight junction protein, ZO-1. Continuous expression of ZO-1 is noted at the brush border of the villi in sham males and females (Fig. 4Ci, Cii; white arrows). The pattern is also well-maintained in females that were subject to stroke (Fig. 4Ciii), however in males, the villus structure was distorted and the brush border was indistinguishable after stroke (Fig. 4Civ).

<u>Male rats exhibit higher levels of gut permeability markers and inflammation associated proteins than age matched females after stroke.</u>

Gut barrier integrity was assessed by measuring serum levels of iFABP, and LPS-binding protein (LBP) respectively, which are commonly used surrogate markers of gut permeability (Troseid et al., 2013; Volynets et al., 2016; Sikkora e al., 2019). Levels of iFABP, a 15 kD protein, were elevated in both female and male rats after stroke as compared to the sham animals indicating that gut becomes more permeable post stroke to this relatively small protein in both sexes (Fig. 5A; main effect of stroke $F_{(1,29)}$: 4.417, p=0.044). In the case of serum levels of LBP (60kD), there was a main effect of sex (Fig. 5B, $F_{(1,29)}$: 12.24; p=0.0016), however, planned comparisons indicated that LBP levels were similar in sham and stroke females (p=0.3452), while LBP was significantly elevated in stroke males as compared to shams (p=0.025). In contrast to iFABP, LBP (which is ~4-fold larger) is only elevated in males with stroke, suggesting that gut permeability is likely more severe in males. Two other markers of gut permeability, LPS and Muc-2, were elevated in males irrespective of stroke. Thus there was a 13% elevation of endotoxin LPS (Fig 5C; $F_{(1,29)}$: 8.43, p=0.007) and a 3.5 fold elevation of Muc-2 (Fig 5D; $F_{(1,29)}$: 25.97, p<0.0001) in males as compared to females, irrespective of stroke, indicating a low-grade gut leakiness in males may predispose systemic inflammation and

worsen stroke outcomes. Overall, these data are consistent with the gut dysmorphology seen in males after stroke.

Gut permeability is more severe in males in the hyperacute phase of stroke.

Functional analysis of gut permeability was assessed by measuring serum levels of dextrans after oral gavage (Fernandez- Carrera et al. 2017) (Woting & Blaut 2018). Serum levels of either dextrans (10kDa, 70 kDa) was undetectable in sham animals. In the case of animals with MCAo, there was a significant sex difference in the amount of dextran detected in serum. (Fig. 6A and 6B). In the case of FITCD (10kDa), there was a significant interaction effect of time and sex ($F_{(6,36)}$: 2.489, p=0.0407), such that FITCD was detected in both males and females. However, in males FITCD was detected as early as 30 mins (first time point measured) and persisted till 90 mins (last time point tested), while in females, FITCD was detected only at the 90 min time point, indicating that gut permeability occurred rapidly in males (interaction effect $F_{(2,28)}$: 3.969; p=0.0305). In the case of RhoD (70kDa), overall lower amounts of this dextran were detected in serum as compared to FITCD, likely due to its larger size. Similar to FITCD, males had higher serum levels of RhoD across all time points as compared to females (main effect of sex, F(1,13): 5.312; p=0.0383). These data indicate that gut permeability is a response to stroke (since dextran was not detected in shams) and is an early response to stroke that is more severe in males.

Male rats exhibit higher serum levels of inflammation associated cytokines as compared to age matched females after stroke

Recent studies have shown that gut permeability results in trafficking of gut resident T-cells, including γδ-T cells (Arya and Hu, 2018, Benakis et al., 2016). While flow cytometry analysis is beyond the scope of the present study, we assessed, instead, serum levels of secretions of γδ-T

cells. These include TH1 (IL-17A) and TH2 (IL-5) cytokines and chemokines (MCP-1, EGF) using a multiplex ELISA assay. In all cases, we observed a significant interaction between stroke and sex. Levels of IL-17A (Fig 7A) were influenced by sex (main effect of sex; $F_{(1,30)}$: 31.64; p=0.0001) and by MCAo (main effect of stroke, $F_{(1,30)}$: 5.982; p=0.02), which was mainly restricted to males (interaction effect, $F_{(1,30)}$: 4.17; p=0.05). There was a similar interaction of stroke and sex for IL-5 ($F_{(1,30)}$: 16.00, p=0.0004), EGF ($F_{(1,30)}$:25.21, p=0.0004) and MCP-1 ($F_{(1,30)}$: 23.27, p<0.0001), with males showing a significant elevation of these chemokines after stroke (Fig 7B, 7C and 7D).

To evaluate if the sex-by-stroke interaction was restricted to γδ-T secretions, we also assessed two other cytokines/chemokines that are not typically considered γδ-T cell secretions. In the case of LIX (CXCL5), this chemokine is not different in males and females and not regulated by stroke at 2d (Fig. 7E). RANTES is reduced after stroke but not differently regulated in males and females (Fig. 7F). Overall, putative γδ-T cells secretions appear to show a sex-specific effect after stroke.

Predictive modeling: Since males and females differed significantly in terms of mortality after stroke and performance on ART, predictive modeling was used to determine whether gut metabolites or markers of gut permeability could serve as predictors of stroke outcomes.

Mortality as Response Variable: Model estimates, 95% confidence intervals, and p-values for the logistic regression models for mortality response are shown in Table 1. Gender was highly statistically significant (p=0.008), with a 95% confidence interval that estimates that males have between 0.1 and 0.8 units higher average log odds of death than females, all other variables held constant. Neither the treatment variable nor any of the metabolites had model coefficients that were statistically significantly different from zero.

ART as Response Variable: Model estimates, 95% confidence intervals, and p-values for the linear regression models for ART response are shown in Table 2. Among the pre-treatment predictor variables, no coefficients are statistically significantly different from zero with 95% confidence. Post-treatment levels of variables: as expected, the treatment (MCAo/Sham) coefficient was highly significant (p=0.002), with a 95% confidence interval that estimates that Sham animals had between 117.8 and 34.4 units lower than the ET1 animals, with all other variables held constant. Among the metabolites, LBP was statistically significantly associated with average ART response, with an estimated coefficient that indicates a negative relationship between LBP and average ART. Both iFABP and Muc2 show a trend toward statistical significance (p=0.051 and p=0.057 respectively), with a negative relationship with average ART for iFABP and positive relationship with average ART for Muc2. There is some evidence of an interaction between iFABP and gender (results not shown), with a negative relationship between iFABP and average ART among females but a positive relationship among males.

DISCUSSION

This study confirms previous reports (Liu et al., 2011, Manwani et al., 2015, McCullough et al., 2016) including our own work (Selvamani and Sohrabji, 2017a) that young male rats have a worse stroke outcome, demonstrated by their higher mortality and worse performance in sensory motor tasks as compared to age-matched female rats. The present data also shows that sex differences in stroke outcomes are associated with (a) constitutive differences in gut microbial communities and (b) subsequent stroke-induced changes in gut permeability.

Sex differences in stroke outcomes are related to gonadal hormones in young animals (Manwani et al., 2015) although sex chromosomes may also affect stroke sensitivity in aged animals (McCullough et al., 2016). Moreover, sex differences in stroke outcomes are also noted in aged males and females, where, presumably, estrogens are unlikely to be a critical factor.

Young females have a smaller infarct and better cerebral blood flow than age-matched males both in normoglycemic (Alkayed et al., 1998) and diabetic (Toung et al., 2000b) animals. Bilateral ovariectomy, which decreases circulating levels of estrogens, increases infarct volume in females (Toung et al., 2000a), while estrogen therapy to young females improves stroke outcome (Rusa et al., 1999, Rau et al., 2003, Carpenter et al., 2016, Selvamani and Sohrabji, 2010c). Interestingly, estrogen also reduces infarct volume in males, while the precursor steroid, testosterone, increases infarct volume in this group (Hawk et al., 1998). Estrogen has been shown to improve stroke-related outcomes by influencing inflammation (Villa et al., 2016), neurogenesis (Shao et al., 2012) and oxidative stress (Behl and Moosmann, 2002) and new evidence suggests that estrogen is an important modulator of gut barrier/integrity, which is especially critical in metabolic health (Baker et al., 2017). Correspondingly, the gut microbiome is actually one of the principal regulators of circulating estrogen (Yurkovetskiy et al., 2013). The estrobiome is the collective term for gut bacterial genes whose products metabolize estrogens (Plottel and Blaser, 2011, Kwa et al., 2016) and modulate the enterohepatic circulation of estrogens (estrone [E_1], estradiol [E_2], and estriol [E_3]).

The current study shows that there are significant constitutive sex differences in bacterial communities. Unweighted UniFrac analysis revealed that there was virtually no overlap between the bacterial communities, while female rats displayed greater diversity of bacterial species. Microbiota diversity is usually associated with health, and microbial diversity is decreased with age (Odamaki et al. 2016), metabolic syndrome (Tomas et al. 2016) and inflammatory bowel disease (Tamboli et al. 2004). A recent large human study showed that baseline dysbiosis was corelated with an increased risk of stroke (Zeng et al., 2019). Similarly, recent preclinical studies have also shown that age-related differences in stroke outcomes are linked to a constitutive difference in microbial communities. For example, young mice have better stroke outcomes than aged mice, and Spychala and colleagues reported constitutive age difference in the F:B ratio and unweighted UniFrac (Spychala et al., 2018). Moreover, while stroke affected the F:B ratio

and beta diversity at both ages, gut dysbiosis was much worse in the aged male group. Similarly, young female rats have better stroke outcomes than middle-aged acyclic females and our recent work showed a constitutive difference in bacterial diversity and the F:B ratio at baseline in these two groups. After stroke, however, young females appeared more resilient and less susceptible to changes in the biome, while middle-aged female rat showed elevated F:B ratio and a significant reduction in bacterial diversity (Park et al., 2019b). Collectively, the present data indicate that females may be in overall better health at baseline, allowing them to emerge with a less severe outcome after stroke.

A more granular assessment of bacterial families that were differentially abundant in males and females shows that several of them are implicated in the synthesis of SCFA. SCFA, such as butyric acid, are reported to have beneficial, anti-inflammatory effects on stroke (Park and Sohrabji, 2016a, Kim and Chuang, 2014, Kim et al., 2007, Patnala et al., 2017). Butyric acid has been shown to reduce intestinal permeability (Peng et al. 2009) and propionic acid has been shown to inhibit gut dysbiosis and induce expression of ZO-1 (Zhao et al. 2019). Surprisingly, males, whose stroke outcomes are worse than females, had significantly higher levels of all 5 SCFA tested at baseline. SCFAs are believed to activate signaling cascades that control immune functions signal via cell surface G-protein coupled receptors (GPCRs) (Parada Venegas et al., 2019). Although beyond the scope of this study, it would be important to know if expression of these GPCRs (such as GPR41, GPR43, and GPR109A) are different in males and females.

An important way in which the microbiome could affect stroke outcomes is its role in maintaining the integrity of the gut blood barrier. Through the mucosal system and the interepithelial tight junctions, the GI tract continuously regulates trafficking of molecules between the host and the luminal environment. The gut blood barrier is an important component of gut function as it keeps potentially pathogenic bacteria and intestine-specific proteins from leaking out into circulating blood (Obrenovich, 2018). Emerging evidence suggests that gut permeability

may precede many of the inflammatory events associated with disease (Arrieta et al., 2006). For example, in the IL-10 deficient mouse, intestinal permeability is shown to precede mucosal inflammation (Madsen et al., 1999). Gut dysbiosis can disrupt the mucosal layer (Kho and Lal, 2018) as do various morbidities such as obesity, metabolic disorders and autoimmune diseases (Wen and Wong, 2017).

Our data also show that the gut is an early responder to stroke. Within minutes of MCAo, gut permeability is altered as measured by extravasation of oral-gavage dextrans, and a new report indicates that a low grade permeability (4kD) can be detected 3d post MCAo (Ahnstedt et al., 2020). Disruption of the epithelial barrier likely results in part from stroke-induced activation of the vagus nerve as well as by inflammatory signals from the brain which act on the gut epithelium to increase gut permeability and gut motility (reviewed in (Arya and Hu, 2018). As a consequence, gut-resident immune cells, gut metabolites and microbes can translocate from the luminal compartment into host circulation (reviewed in (Bischoff et al., 2014)). At the same time, gut permeability can also increase availability of oxygen in the large bowel, which is deleterious for many keystone bacteria and can selectively drive luminal expansion of other species, thus altering homeostatic gut microbial populations (Kelly and Colgan, 2016). In this study, we observed a small but significant elevation of LPS as well as a 2-fold elevation of muc-2 in males as compared to females, in sham animals (as well as stroke), suggesting that there might be a pre-existing substrate for poor stroke outcomes.

Stroke-induced disruption of the epithelial barrier was much worse in male rats, as evidenced by the decreased villus length to crypt length ratio in the gut, irregular expression of ZO-1 at the villus border, elevated serum levels of iFABP and LBP. In addition, gut permeability is often associated with higher levels of inflammatory cytokines (Fukui, 2016). Male rats had higher levels of proinflammatory cytokines such as IL-17, which are associated with $\gamma\delta$T cells, although the current study did not assess T cells *per se*. Young male mice also exhibit increased expression of inflammatory genes associated with T-cell function and inflammatory

cytokines in the ischemic brain after stroke compared with females (Dotson et al., 2015). These measures of gut permeability partially predict the extent of sensory motor deficits. Thus, both sexes show some impairment in these behavioral tasks, which is reflected in elevated levels of iFABP. However, the deficit is more severe in males where LBP (a larger protein) is also elevated in serum but not in females. While the current focus is on acute stroke disability, measures of gut permeability may predispose long-term disability including stroke-induced depression or cognitive impairment. Translationally, markers of gut permeability may therefore provide a useful way to identify high risk individuals or populations.

In conclusion, the current study shows that in male rats, stroke is associated with deterioration of normal gut architecture, greater gut permeability and higher levels of inflammatory cytokines than age-matched female rats. More importantly, males have significantly less diverse intestinal microbial communities even at baseline and perhaps this pre-existing condition creates an ongoing vulnerability to stroke. These data suggest that preventative long-term modification of diet or dietary metabolites may be necessary to improve stroke outcomes.

TABLES

Table 1: Estimates, confidence intervals, and p-values for logistic regression model for mortality response

Coef.	Explanatory Variable	Estimate	95% Conf. Interval	P-value
β_0	Intercept	0.423	[-0.037, 0.882]	0.071
β_1	Gender (M vs. F)	0.439	[0.122, 0.755]	0.008
β_2	TX (Sham vs. ET1)	-0.220	[-0.511, 0.071]	0.135
β_3	Butyric acid	0.001	[-0.057, 0.057]	0.987
β_4	Isovaleric acid	0.755	[-0.849, 2.359]	0.347
β_5	Propionic acid	-0.129	[-0.657, 0.400]	0.625

Table 2: Estimates, confidence intervals, and p-values for linear regression models for ART response.

		Pre-treatment predictors			Post-treatment predictors		
Coef.	Explanatory Variable	Estimate	95% Conf. Interval	P-value	Estimate	95% Conf. Interval	P-value
β_0	Intercept	-36.981	[-299.248, 225.285]	0.767	150.305	[-0.520, 301.130]	0.051
β_1	Gender (M vs. F)	7.309	[-61.280, 75.898]	0.823	2.765	[-45.252, 50.782]	0.900
β_2	TX (Sham vs. ET1)	-47.924	[-101.351, 5.503]	0.075	-76.062	[-117.769, -34.354]	0.002
β_3	Butyric acid	-1.714	[-14.932, 11.504]	0.785	2.602	[-7.211, 12.415]	0.568
β_4	Isovaleric acid	29.160	[-250.990, 309.310]	0.827	-0.865	[-173.703, 171.972]	0.991
β_5	Propionic acid	77.531	[-68.629, 223.692]	0.274	-4.052	[-46.608, 38.505]	0.836
β_6	Valeric acid	-48.220	[-301.846, 205.406]	0.690	-48.224	[-200.572, 104.125]	0.497
β_7	LPS	1.299	[-3.408, 6.006]	0.563	0.486	[-2.967, 3.939]	0.760
β_8	LBP	-2.859	[-8.315, 2.596]	0.280	-4.667	[-8.996, -0.338]	0.037
β_9	iFABP	-1.234	[-25.262, 22.793]	0.914	-14.466	[-29.024, 0.093]	0.051
β_{10}	Muc2	119.763	[-75.352, 314.878]	0.209	121.323	[-4.593, 247.240]	0.057

List of Abbreviations

Term	Full name
AP	Anterior-Posterior
ASV	Amplicon sequence variant
DAPI	4′,6-diamidino-2-phenylindole
DV	Dorso-Ventral
EGF	Epidermal Growth Factor
ET-1	Endothelin-1
FITC	Fluorescein isothiocyanate
iFABP	Intestinal Fatty Acid Binding Protein
IL-17A	Interleukin-17A
LBP	LPS-binding protein
LIX	Lipopolysaccharide-induced CXC *chemokine*
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattract protein-1
ML	Medio-lateral
Muc-1	Mucin-1
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
Rho	Rhodamine
SCFA	Short chain fatty acid
ZO-1	Zonnula occludins-1

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: Please contact author for data requests

Competing interests: The authors declare they have no competing interests

Funding: Supported by NS074895 and AG042189 to FS

Authors' contributions: YEH and KKM performed the experiments including animal surgeries, histology and behavior and drafted the manuscript, AE assisted with behavior and surgeries, RP performed the analysis of fecal 16s sequencing data, AD performed the predictive modeling analysis, FS conceived the study and participated in the design of the study, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements: We thank Ms. Taylor E. Branyan for assistance with confocal microscopy images

LEGENDS:

Figure 1: Effect of MCAo on mortality, infarct volume and sensory motor ability in males and females: A) Kaplan Meier survival plot shows significantly greater mortality after MCAo in males as compared to females ($p=0.0014$). B) Representative images of TTC-stained brain sections from female and male rats 2d after MCAo. C) Histogram depicting mean (+/- SEM) of infarct volume (ns: not significant) D) Sensory motor impairment assessed by (E) Adhesive Removal test (ART) and (F) the Vibrissae Evoked Forelimb Placement task (VIB). A: main effect of stroke, b:main effect of sex, c: interaction effect; *: $p<0.05$.

Figure 2: Fecal metagenomic analysis pre- and post-stroke in male and female rats: Alpha diversity was determined by Observed ASVs at (A) baseline and (D) 2d after MCAo. Beta

diversity measured by unweighted UniFrac (B) baseline and (E) 2d after MCAo. Histogram depicting the mean (+/- SEM) of the ratio of Firmicutes to Bacteroidetes at (C) baseline and (F) 2d after MCAo. Pre-stroke n= 13-22, Post stroke: n= 8-13; $p<0.05$. (G) Heat map depicting bacterial groups that are differentially expressed in males and females (FDR corrected; $p<0.01$).

Figure 3: Baseline levels of SCFA in fecal samples from males and females: A) butyric acid B) isobutyric acid, C) isovaleric acid D) proprionic acid, E) valeric acid. In all cases, males had higher levels of SCFA. N=20-29; *: $p<0.05$

Figure 4: Histological analysis of the gut in sham and stroke animals. A) H&E stained sections of the ileum from sham and MCAo males and females. B) Histogram depicting the mean (+/- SEM) ratio of villus:crypt height for each group. N=7-9 per group, *: $p<0.05$. C) Immunohistochemistry for the tight junction protein ZO-1 in thin sections of the ileum from male and female sham and MCAo groups. White arrows indicate the location of the epithelial barrier. Inter-epithelial expression of ZO-1 was virtually absent in male rats 2d after MCAo compared to the other groups.

Figure 5: Serum levels of gut proteins/metabolites: Gut proteins/metabolites levels were assayed in serum as a surrogate measure of gut permability. A) Serum iFABP (15kDa) was elevated after stroke in both males and females. B) Serum LBP (60kDa) show a sex difference which was mainly due to increased levels of the protein in male rats after MCAo compared to females. Levels of serum C) LPS and D) Mucin-2 was significantly elevated in males irrespective of stroke/sham group. Key: ^{a}: main effect of stroke, ^{b}: main effect of sex, *: $p<0.05$

Figure 6: Functional analysis of gut permeability in the hyperacute phase of stroke: Sham and stroke males and females received an oral gavage of fluorescently labeled dextran prior to

surgery. Serum levels of oral-gavage (A) FITC-labeled dextran (FITCD, 10kD) and (B) Rhodamine-labeled dextran (RhoD, 70kD) were sampled 30, 60 and 90 min after MCAo (or Sham). n=3 for shams, n=8 ET-1 groups. [a]: main effect of sex, [c]: interaction effect (sex and time).

Figure 7: Serum levels of inflammatory cytokines pre and post MCAo in males and females. Levels of (A) IL-17A, (B) IL-5, (C) EGF, D) MCP-1 were significantly elevated after MCAo only in males. Levels of (E) LIX were not regulated by stroke, while expression of (F) RANTES was decreased in both males and females after stroke. Histograms represent mean +/- SEM, n=3-6 in sham groups, n=5-11 in MCAo groups. Key: [a]: main effect of stroke, [c]: interaction effect (stroke and sex), *: $p<0.05$.

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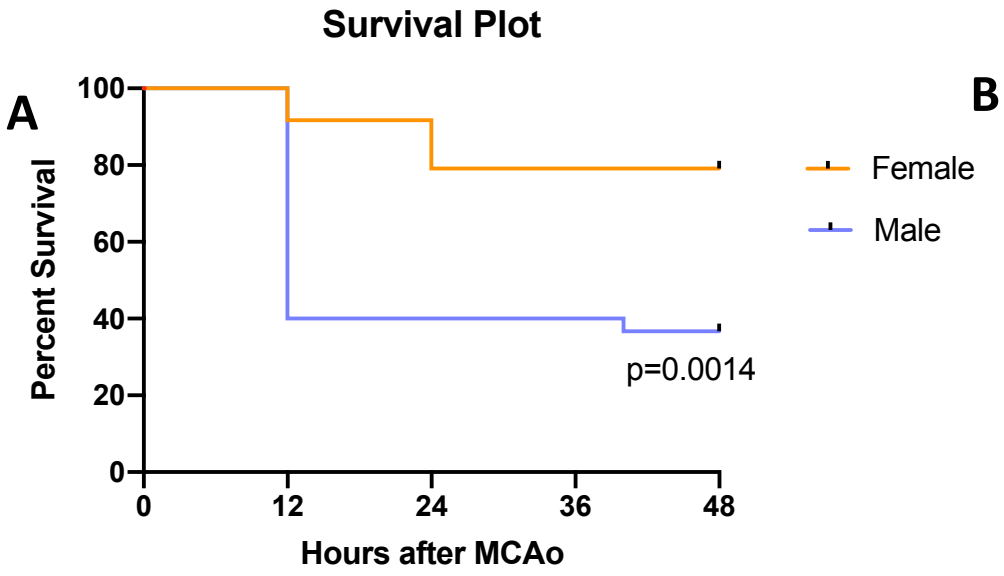
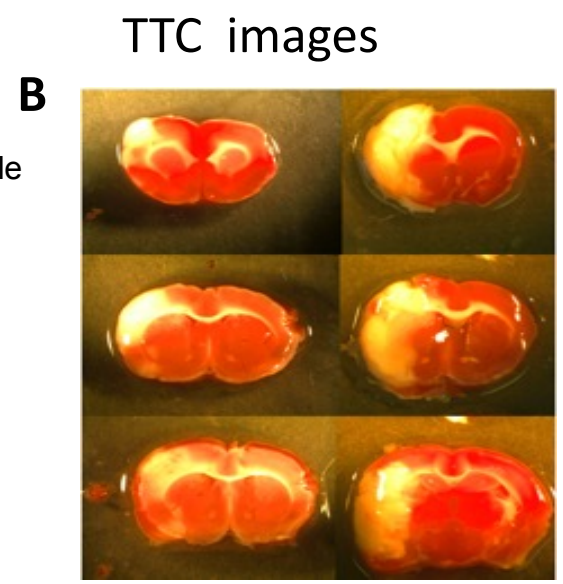
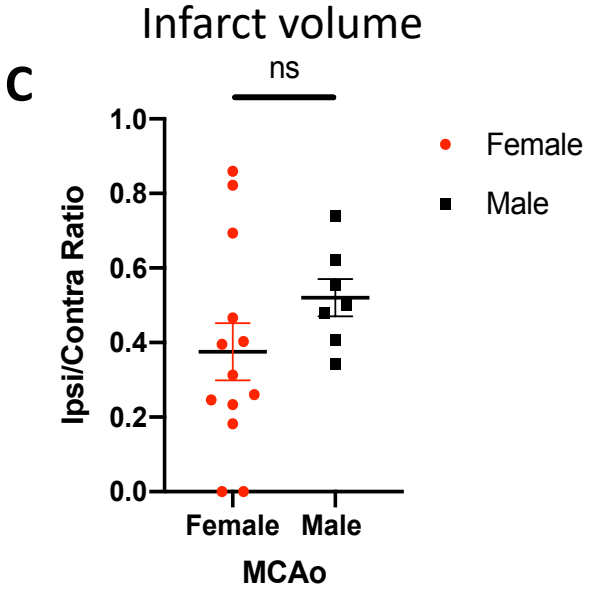
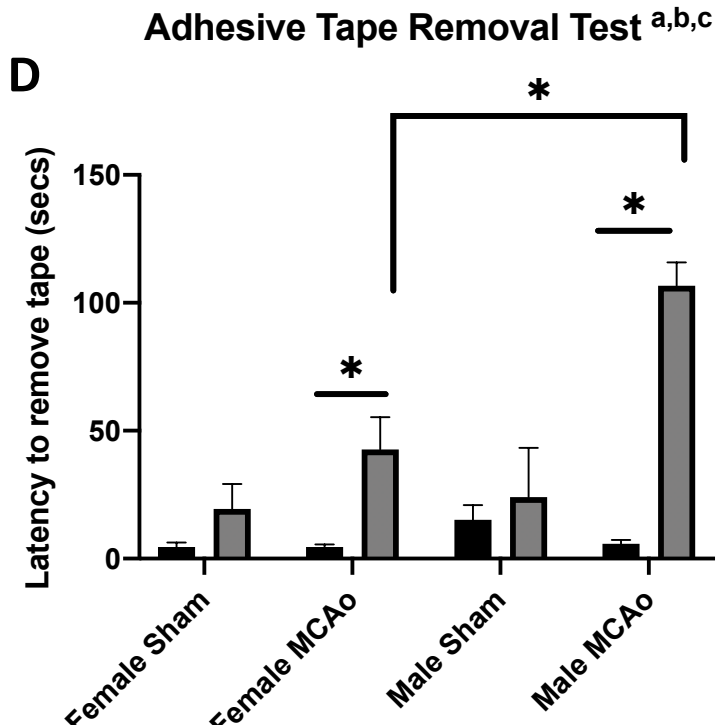
Figure 1


Survival Plot
A
Percent Survival
100
80
60
40
20
0
0
12
24
36
48
Hours after MCAo
Female
Male
p=0.0014


TTC images
B


Infarct volume
C
ns
Ipsi/Contra Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Female
Male
MCAo


Adhesive Tape Removal Test a,b,c
D
Latency to remove tape (secs)
150
100
50
0
*
*
*
Female Sham
Female MCAo
Male Sham
Male MCAo

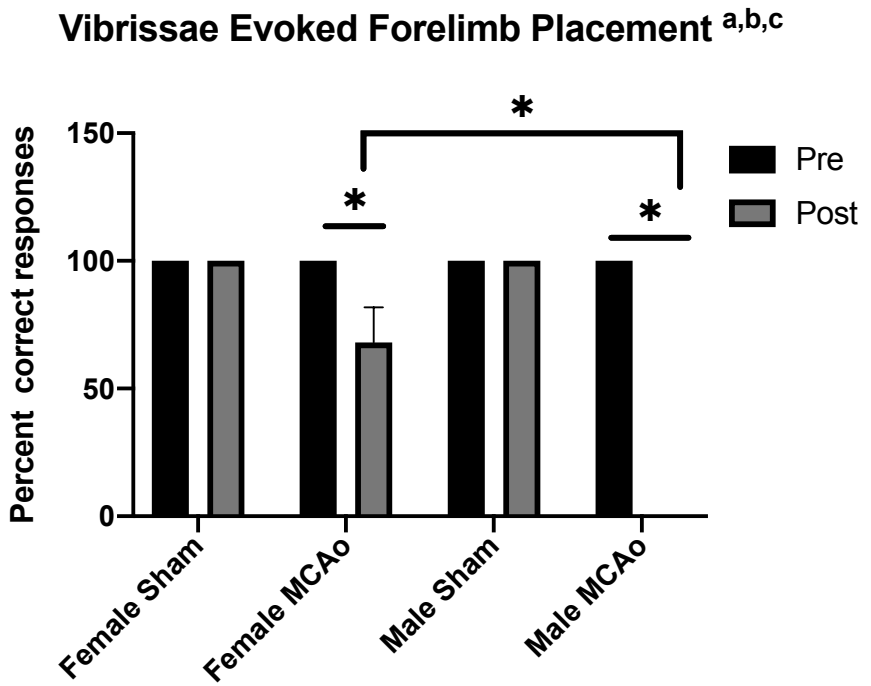

Vibrissae Evoked Forelimb Placement a,b,c
E
Percent correct responses
150
100
50
0
*
*
*
Pre
Post
Female Sham
Female MCAo
Male Sham
Male MCAo

Figure 2: Constitutive sex differences in fecal metagenomics

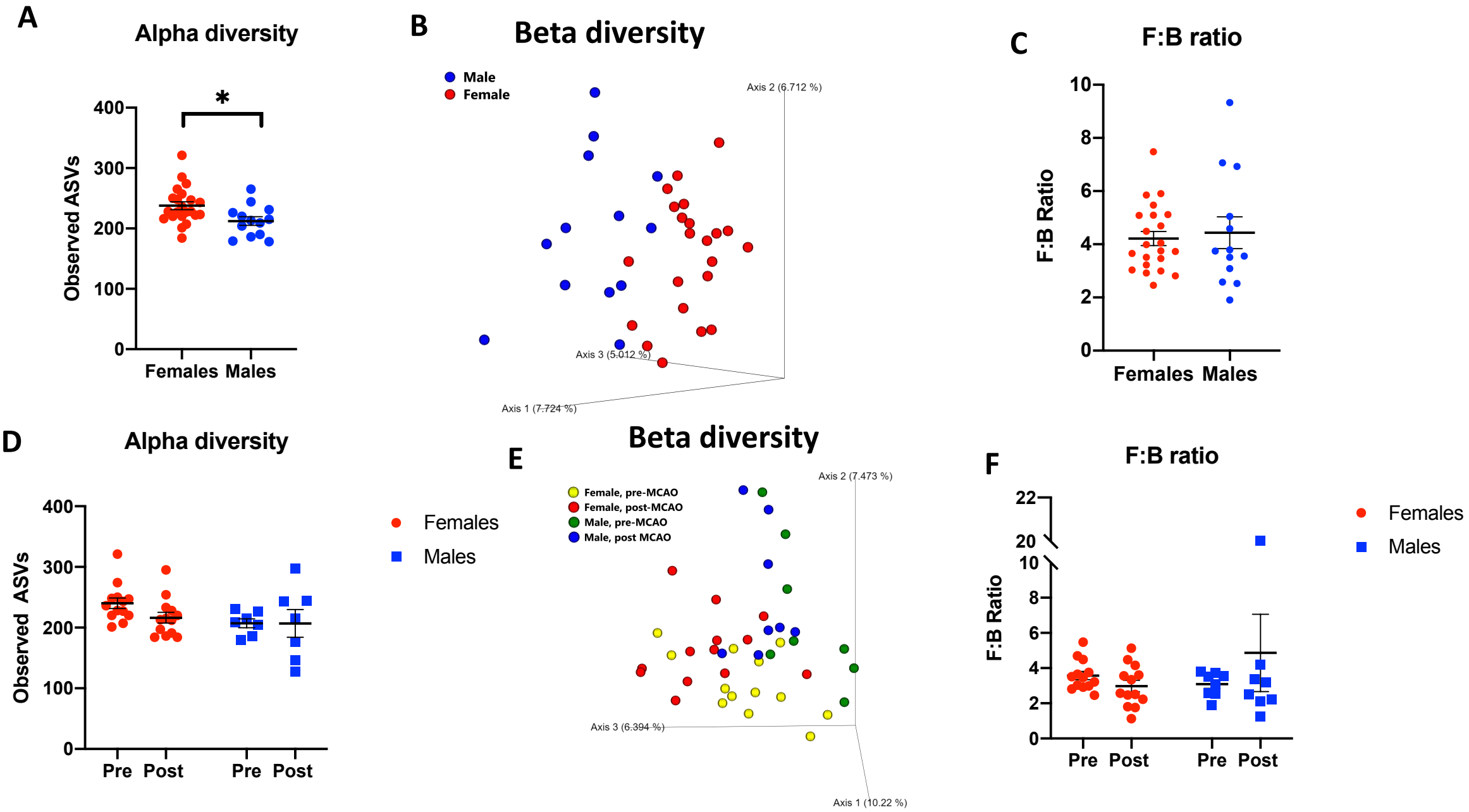


Figure 2G

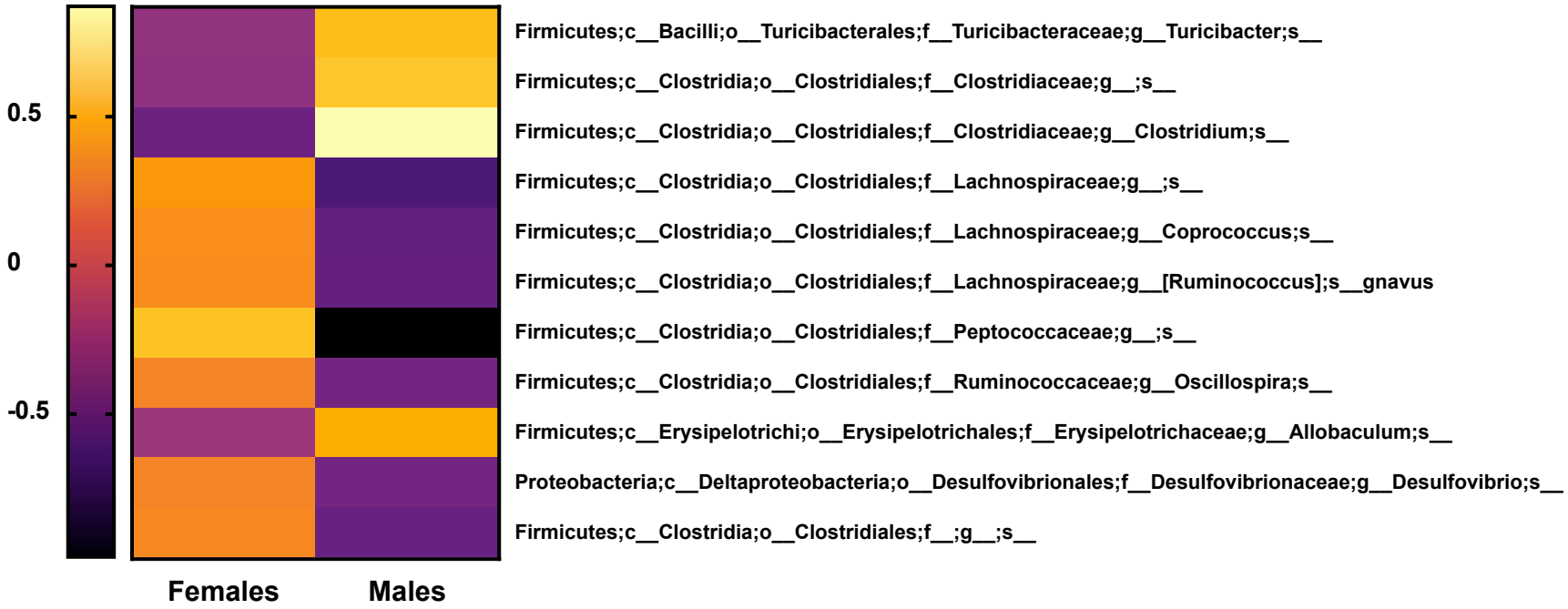

0.5
0
-0.5
Females
Males
Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus];s__gnavus
Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__
Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Allobaculum;s__
Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio;s__
Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;s__

Figure 3

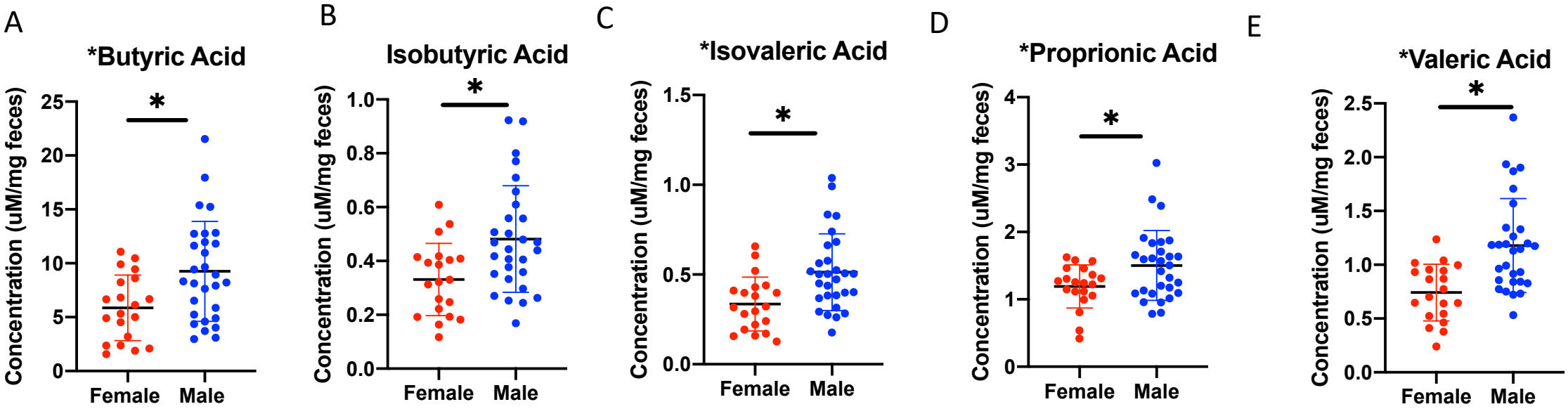

A
*Butyric Acid
Concentration (uM/mg feces)
25
20
15
10
5
0
*
Female
Male
B
Isobutyric Acid
Concentration (uM/mg feces)
1.0
0.8
0.6
0.4
0.2
0.0
*
Female
Male
C
*Isovaleric Acid
Concentration (uM/mg feces)
1.5
1.0
0.5
0.0
*
Female
Male
D
*Proprionic Acid
Concentration (uM/mg feces)
4
3
2
1
0
*
Female
Male
E
*Valeric Acid
Concentration (uM/mg feces)
2.5
2.0
1.5
1.0
0.5
0.0
*
Female
Male

Figure 4

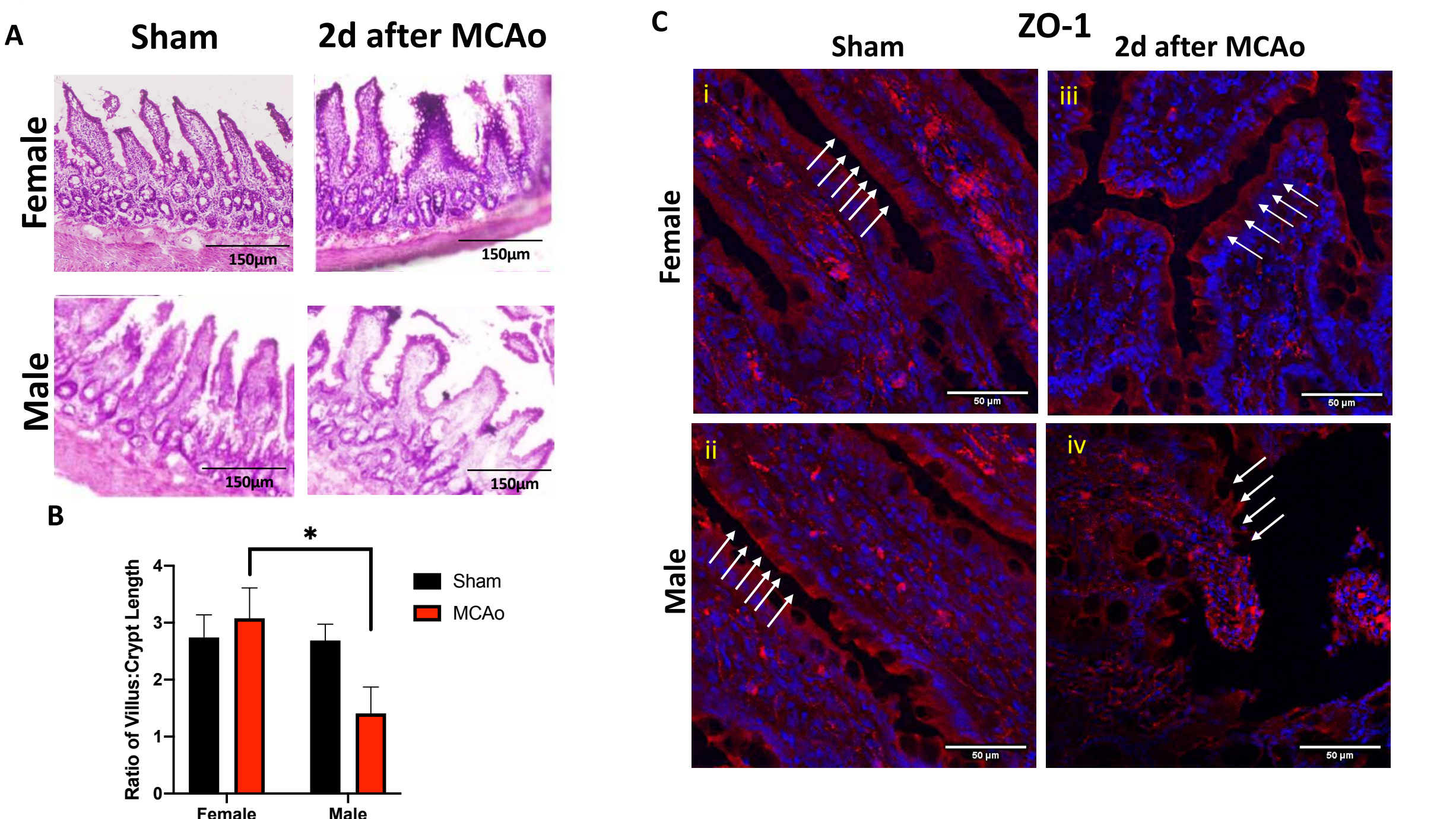

A
Sham
2d after MCAo
Female
Male
150μm
150μm
150μm
150μm
B
*
Ratio of Villus:Crypt Length
4
3
2
1
0
Female
Male
Sham
MCAo
C
ZO-1
Sham
2d after MCAo
Female
Male
i
iii
ii
iv
50 μm
50 μm
50 μm
50 μm

Figure 5:

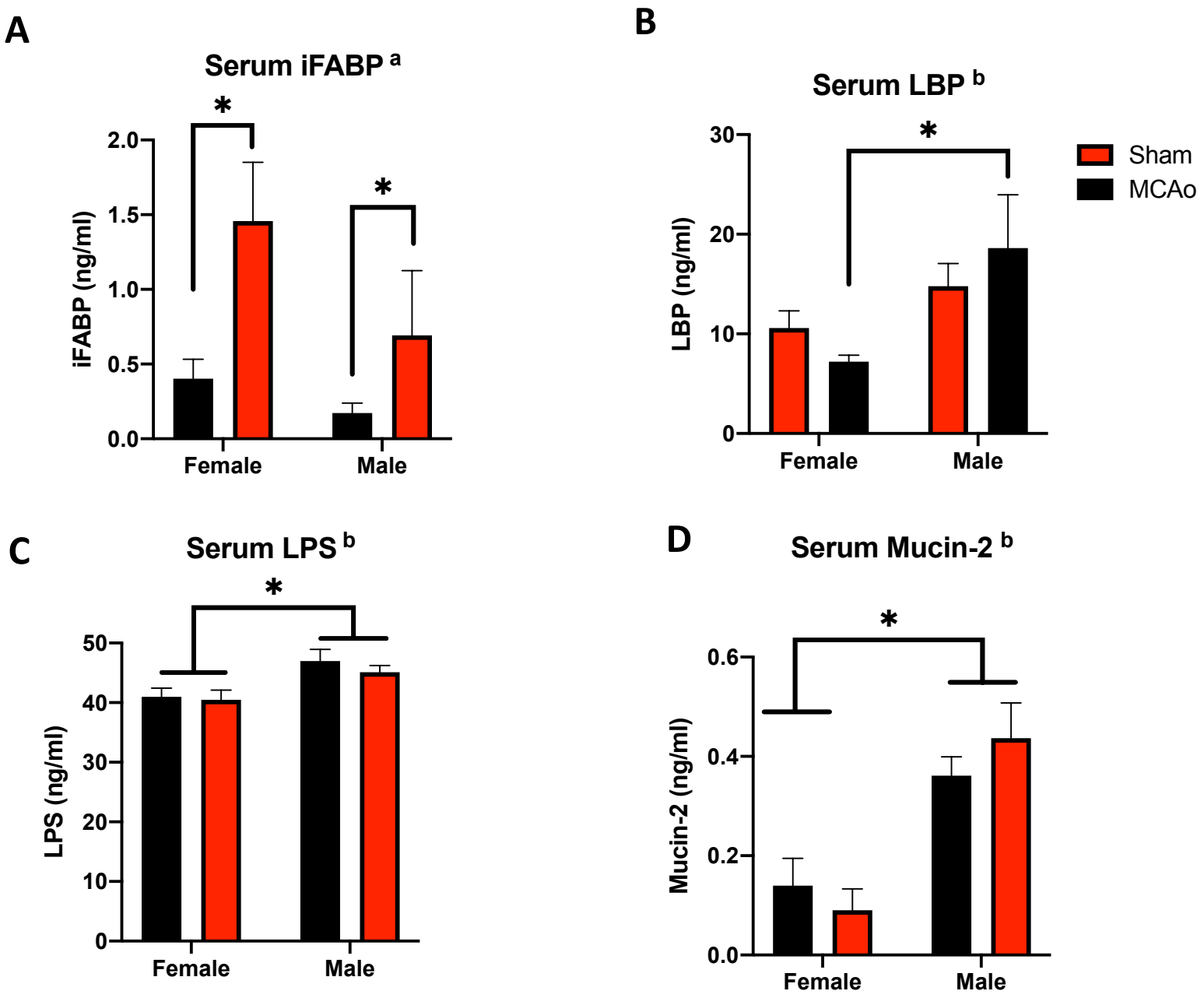

A
Serum iFABP a
iFABP (ng/ml)
2.0
1.5
1.0
0.5
0.0
Female
Male
B
Serum LBP b
LBP (ng/ml)
30
20
10
0
Female
Male
Sham
MCAo
C
Serum LPS b
LPS (ng/ml)
50
40
30
20
10
0
Female
Male
D
Serum Mucin-2 b
Mucin-2 (ng/ml)
0.6
0.4
0.2
0.0
Female
Male

Figure 6

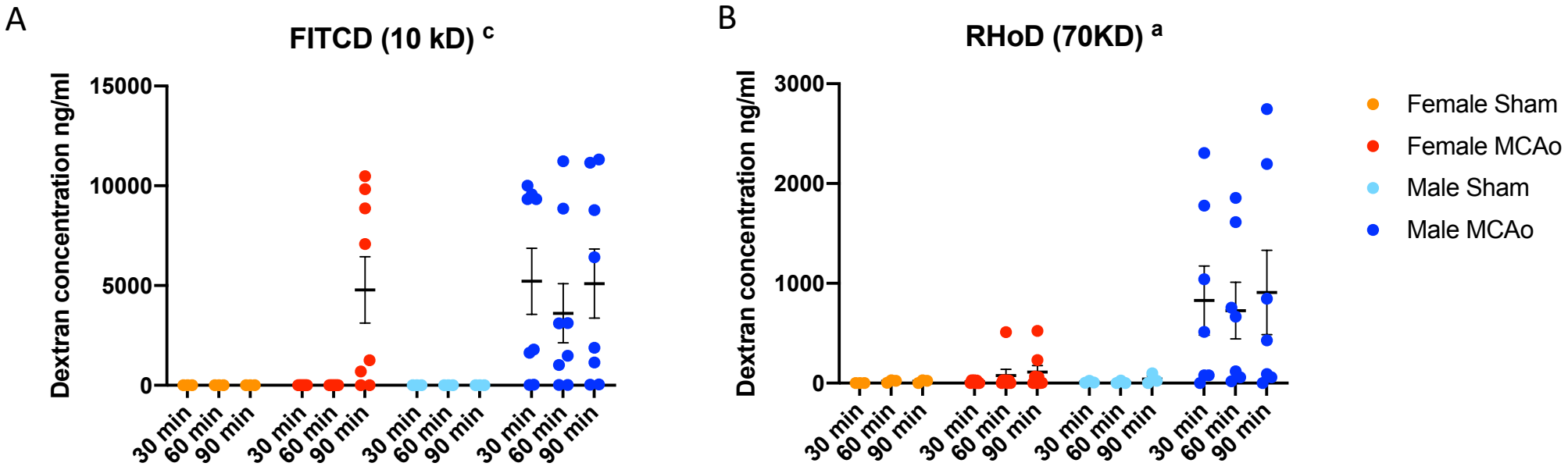
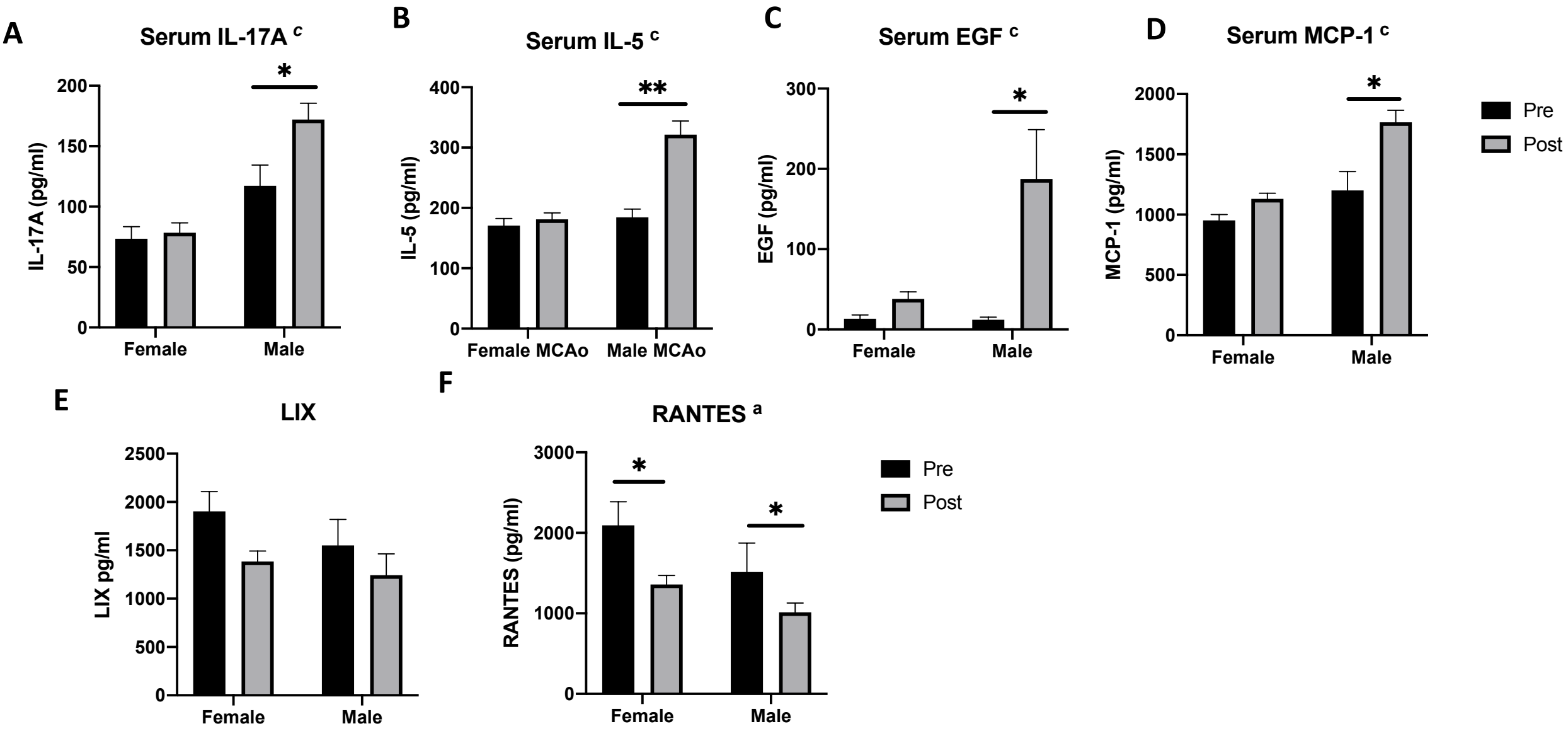

A
FITCD (10 kD) c
Dextran concentration ng/ml
15000
10000
5000
0
30 min
60 min
90 min
B
RHoD (70KD) a
3000
2000
1000
Female Sham
Female MCAo
Male Sham
Male MCAo

Figure 7


A
Serum IL-17A c
IL-17A (pg/ml)
200
150
100
50
0
*
Female
Male
B
Serum IL-5 c
IL-5 (pg/ml)
400
300
200
100
0
**
Female MCAo
Male MCAo
C
Serum EGF c
EGF (pg/ml)
300
200
100
0
*
Female
Male
D
Serum MCP-1 c
MCP-1 (pg/ml)
2000
1500
1000
500
0
*
Female
Male
Pre
Post
E
LIX
LIX pg/ml
2500
2000
1500
1000
500
0
Female
Male
F
RANTES a
RANTES (pg/ml)
3000
2000
1000
0
*
*
Female
Male
Pre
Post

Figures

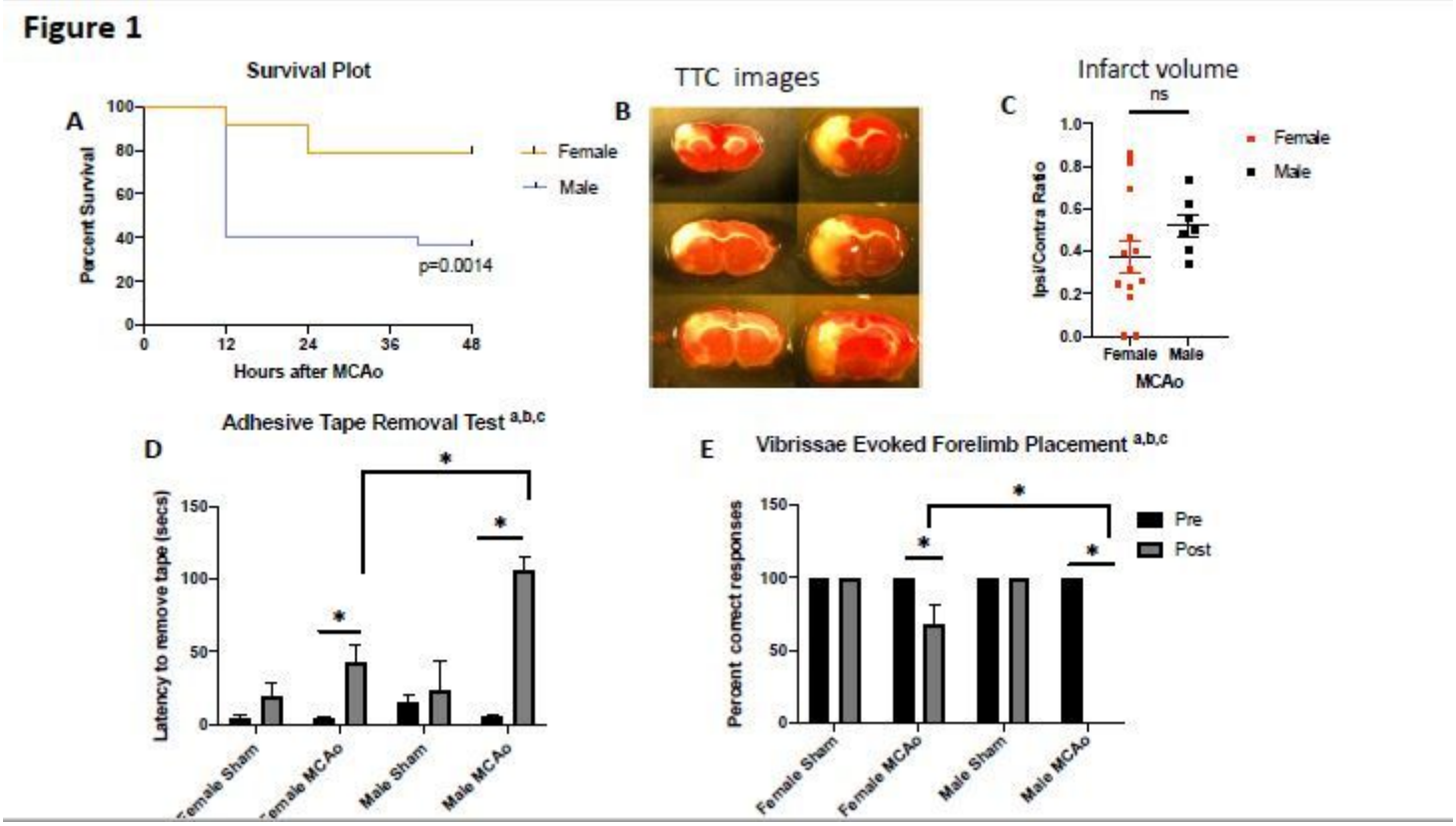


Figure 1

Effect of MCAo on mortality, infarct volume and sensory motor ability in males and females: A) Kaplan Meier survival plot shows significantly greater mortality after MCAo in males as compared to females (p=0.0014). B) Representative images of TTC-stained brain sections from female and male rats 2d after MCAo. C) Histogram depicting mean (+/- SEM) of infarct volume (ns: not significant) D) Sensory motor impairment assessed by (E) Adhesive Removal test (ART) and (F) the Vibrissae Evoked Forelimb Placement task (VIB). A: main effect of stroke, b:main effect of sex, c: interaction effect; *: $p<0.05$.

Figure 2: Constitutive sex differences in fecal metagenomics

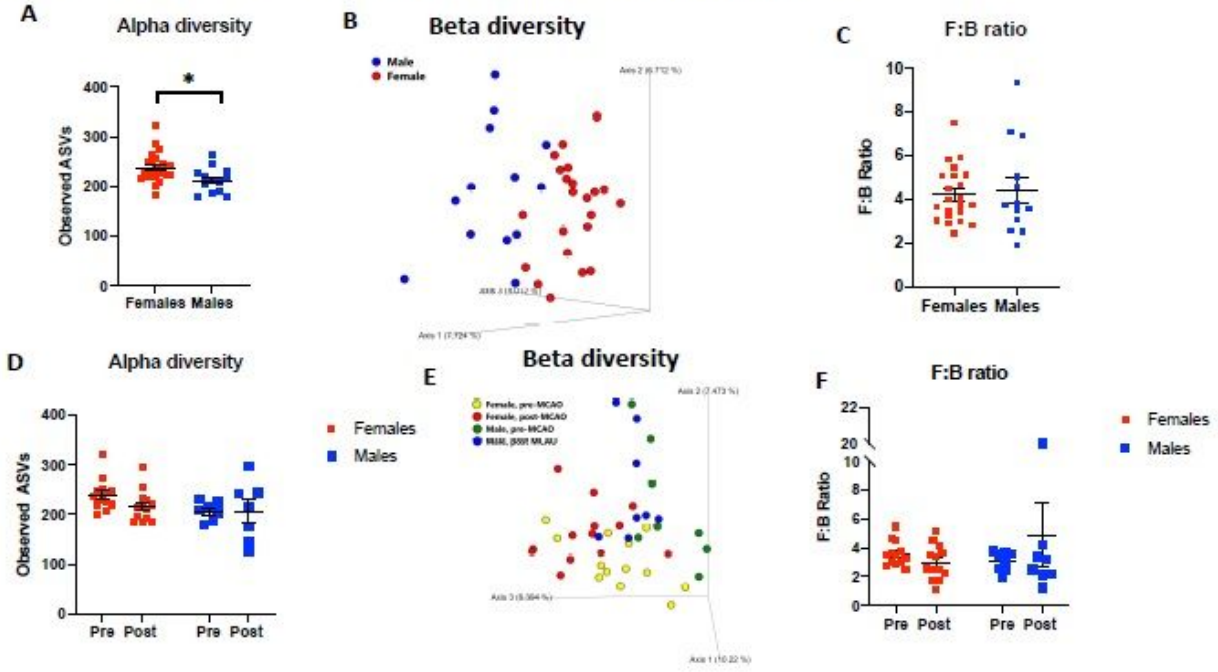


Figure 2G

Figure 2

Fecal metagenomic analysis pre- and post-stroke in male and female rats: Alpha diversity was determined by Observed ASVs at (A) baseline and (D) 2d after MCAo. Beta diversity measured by unweighted UniFrac (B) baseline and (1 E) 2d after MCAo. Histogram depicting the mean (+/- SEM) of the ratio of Firmicutes to Bacteroidetes at (C) baseline and (F) 2d after MCAo. Pre-stroke n= 13-22, Post

stroke: n= 8-13; p<0.05. (G) Heat map depicting bacterial groups that are differentially expressed in males and females (FDR corrected; p<0.01).

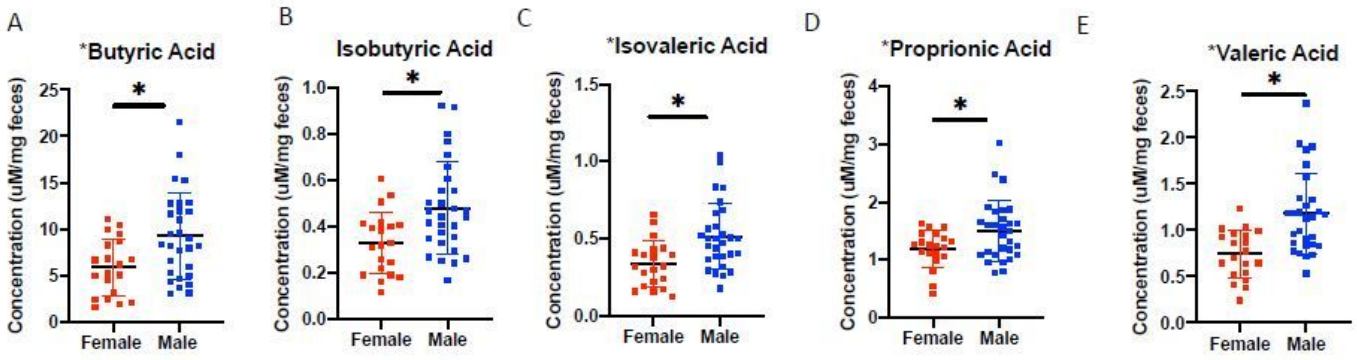


Figure 3

Baseline levels of SCFA in fecal samples from males and females: A) butyric acid B) isobutyric acid, C) isovaleric acid D) proprionic acid, E) valeric acid. In all cases, males had higher levels of SCFA. N=20-29; *: p<0.05

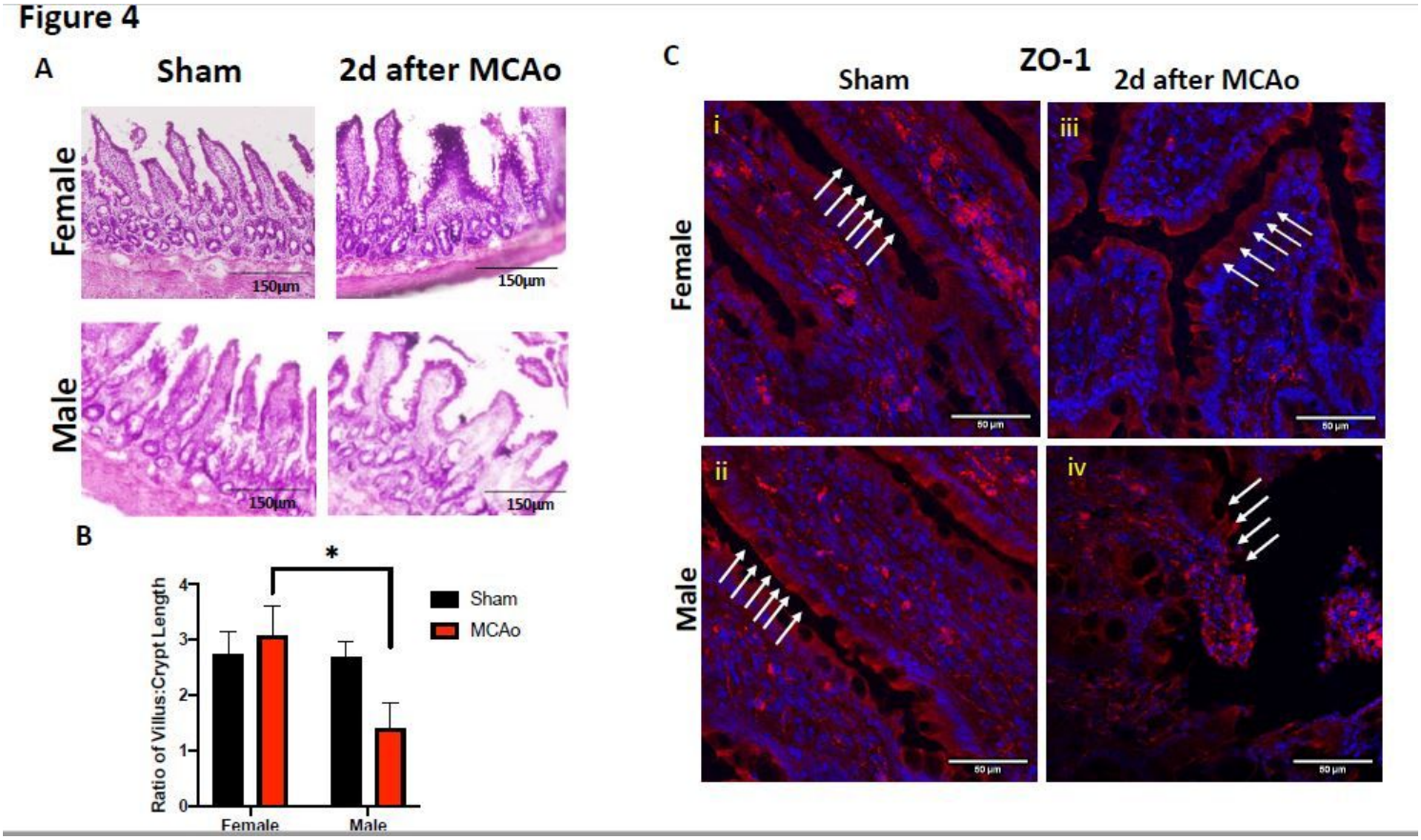


Figure 4

Histological analysis of the gut in sham and stroke animals. A) H&E stained sections of the ileum from sham and MCAo males and females. B) Histogram depicting the mean (+/- SEM) ratio of villus:crypt height for each group. N=7-9 per group, *: p<0.05. C) Immunohistochemistry for the tight junction protein ZO-1 in thin sections of the ileum from male and female sham and MCAo groups. White arrows indicate the location of the epithelial barrier. Inter-epithelial expression of ZO-1 was virtually absent in male rats 2d after MCAo compared to the other groups.

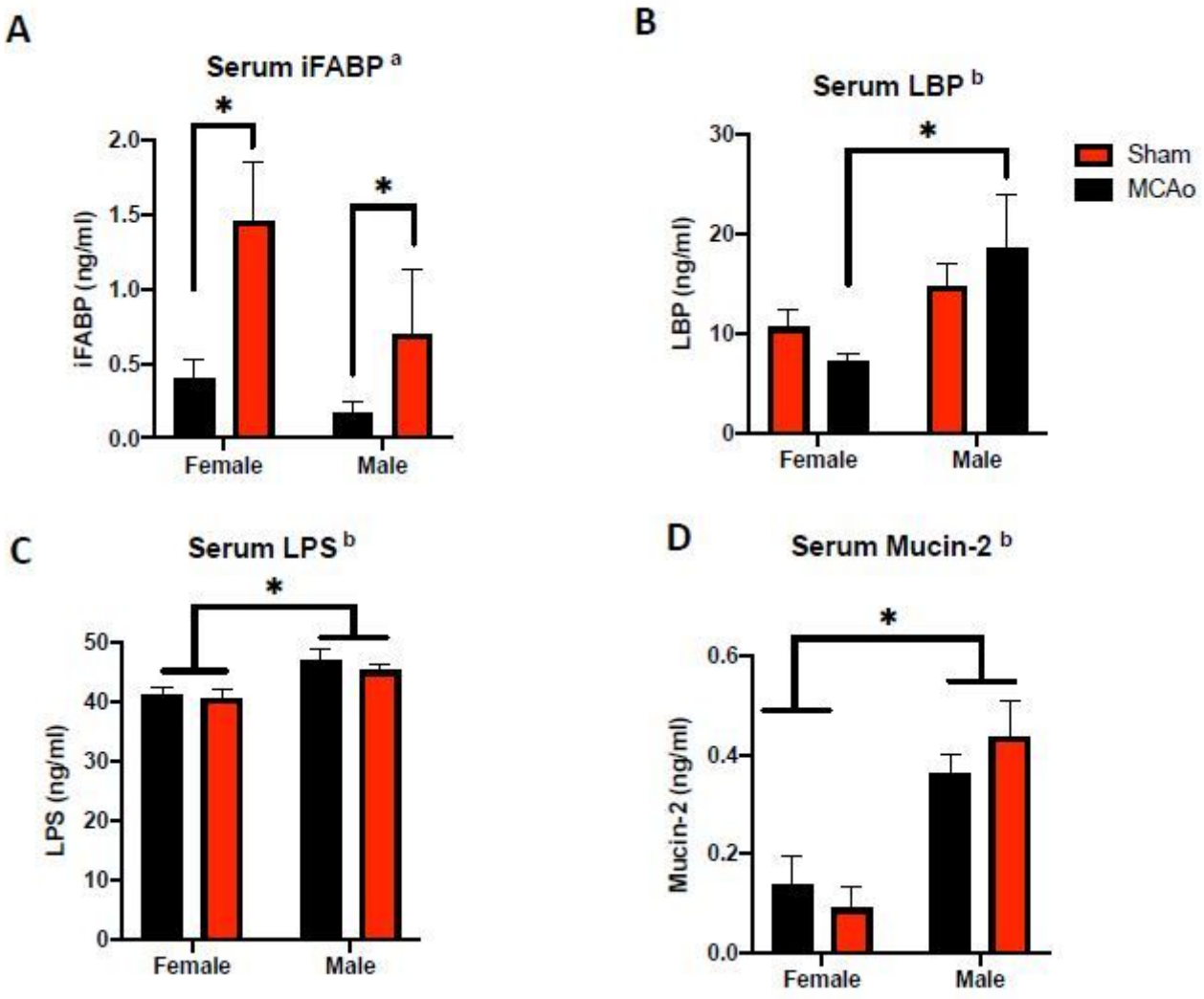


Figure 5

Serum levels of gut proteins/metabolites: Gut proteins/metabolites levels were assayed in serum as a surrogate measure of gut permability. A) Serum iFABP (15kDa) was elevated after stroke in both males and females. B) Serum LBP (60kDa) show a sex difference which was mainly due to increased levels of the protein in male rats after MCAo compared to females. Levels of serum C) LPS and D) Mucin-2 was significantly elevated in males irrespective of stroke/sham group. Key: a: main effect of stroke, b: main effect of sex, *: p<0.05

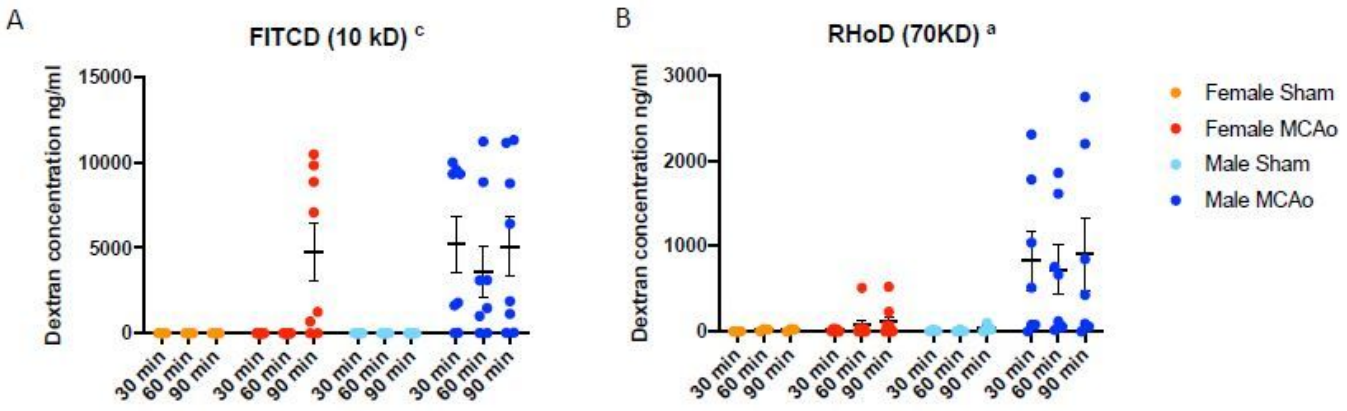


Figure 6

Functional analysis of gut permeability in the hyperacute phase of stroke: Sham and stroke males and females received an oral gavage of fluorescently labeled dextran prior to surgery. Serum levels of oral-gavage (A) FITC-labeled dextran (FITCD, 10kD) and (B) Rhodamine-labeled dextran (RhoD, 70kD) were sampled 30, 60 and 90 min after MCAo (or Sham). n=3 for shams, n=8 ET-1 groups. a: main effect of sex, c: interaction effect (sex and time).

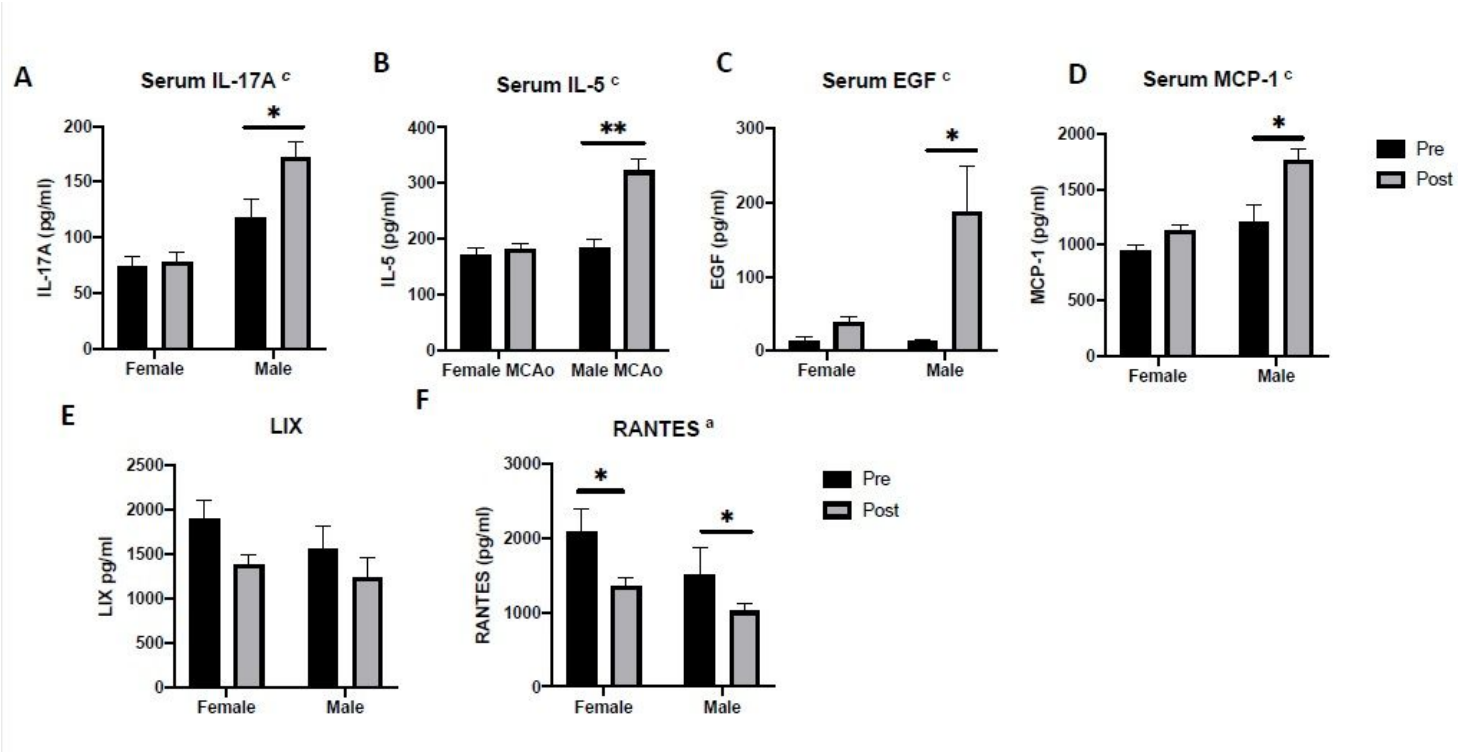


Figure 7

Serum levels of inflammatory cytokines pre and post MCAo in males and females. Levels of (A) IL-17A, (B) IL-5, (C) EGF, D) MCP-1 were significantly elevated after MCAo only in males. Levels of (E) LIX were not regulated by stroke, while expression of (F) RANTES was decreased in both males and females after stroke. Histograms represent mean +/- SEM, n=3-6 in sham groups, n=5-11 in MCAo groups. Key: a: main effect of stroke, c: interaction effect (stroke and sex), *: $p<0.05$.