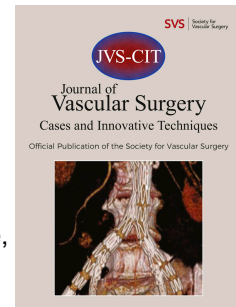# Journal Pre-proof

Chronic Mesenteric Ischemia Intestinal Dysbiosis Resolves after Revascularization

Jennifer A. Munley, MD, Ravinder Nagpal, PhD, Nancy C. Hanson, BA, Amin Mirzaie, BS, Liza Laquian, MD, Alicia M. Mohr, MD, Philip A. Efron, MD, Dean J. Arnaoutakis, MD, Michol A. Cooper, MD, PhD

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1 **Chronic Mesenteric Ischemia Intestinal Dysbiosis Resolves after Revascularization**
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1   **ARTICLE HIGHLIGHTS**

2   **Type of Research:** Single center, prospective observational cohort study

3   **Key Findings:** Eight patients with chronic mesenteric ischemia demonstrated intestinal dysbiosis

4   characterized by decreased alpha-diversity (p=0.03) and increased abundance of potentially

5   pathogenic bacteria compared to healthy controls. Open or endovascular revascularization

6   altered intestinal microbial composition and increased diversity, with resolution of dysbiosis

7   post-operatively.

8   **Take home Message:** Chronic mesenteric ischemia induces a 'pathobiome' state that returns to

9   homeostasis after endovascular or open mesenteric revascularization.

10

1   **<u>TABLE OF CONTENTS SUMMARY</u>**

2   This prospective observational cohort study of eight patients with chronic mesenteric ischemia

3   demonstrated a baseline intestinal pathobiome. Open or endovascular mesenteric

4   revascularization results in an increase in microbial diversity and commensal bacterial

5   abundance with resolution of intestinal dysbiosis.

6
7

1   **ABSTRACT**

2

3   **Objectives**: Chronic mesenteric ischemia (CMI) is a debilitating condition arising from intestinal

4   malperfusion from mesenteric artery stenosis/occlusion. Mesenteric revascularization is the

5   standard of care but carries substantial morbidity/mortality. The majority of perioperative

6   morbidity is secondary to postoperative multiple organ dysfunction, potentially from

7   ischemia/reperfusion injury. The intestinal microbiome is a dense community of microorganisms

8   in the gastrointestinal tract that help regulate pathways ranging from nutritional metabolism to

9   the immune response. We hypothesized that patients with CMI have microbiome perturbations

10   that contribute to this inflammatory response and potentially normalize in the postoperative

11   period.

12

13   **Methods**: A prospective study of CMI patients who underwent mesenteric bypass/stenting was

14   conducted between 2019-2020. Stools were collected at 3 timepoints: pre-operative clinic,

15   perioperatively within 14 days, and post-operative clinic >30 days post-revascularization. Stool

16   samples from healthy controls were used for comparison. Microbiome was measured by 16S

17   rRNA sequencing on an Illumina-MiSeq® sequence platform and analyzed using qiime2-dada2

18   bioinformatics pipeline with SILVA database. β-diversity was analyzed using principal

19   coordinates analysis and permutational analysis of variance. α-diversity (microbial richness and

20   evenness) was compared using non-parametric Mann-Whitney-U test. Microbial taxa unique for

21   CMI patients versus controls were identified using the linear discriminatory analysis effect size

22   (LEfSe) analysis. $P<0.05$ was considered statistically significant.

23

1   **Results**: Eight patients underwent mesenteric revascularization (25% male; average age: 71).

2   Nine healthy controls were analyzed (78% male; average age: 55). Bacterial α-diversity (number

3   of OTUs) was dramatically reduced in preoperative group versus controls (p=0.03); however,

4   revascularization partially restored the species richness and evenness in perioperative and

5   postoperative phases. Beta-diversity was only different between perioperative and post-operative

6   groups (p=0.03). Further analyses revealed increased abundance of *Bacteroidetes* and *Clostridia*

7   taxa pre-operatively and perioperatively compared to controls, which was reduced during post-

8   operative period.

9

10   **Conclusion**: Patients with CMI have intestinal dysbiosis that resolves after revascularization.

11   This is characterized by loss of alpha-diversity, which is restored perioperatively and is

12   maintained postoperatively. This microbiome restoration demonstrates the importance of

13   intestinal perfusion to sustain gut homeostasis and suggests that the microbiome modulation

14   could be a possible intervention to ameliorate acute and sub-acute post-operative outcomes in

15   these patients.

16

17   **KEYWORDS**: chronic mesenteric ischemia, microbiota, revascularization

18

23

1   **INTRODUCTION**

2   Chronic mesenteric ischemia (CMI) is a debilitating disease characterized by abdominal

3   pain and weight loss secondary to mesenteric artery stenoses or occlusions.[1] Due to the non-

4   specific abdominal complaints, patients often have a significant delay in diagnosis leading to

5   significant weight loss and malnutrition.[1] In addition to known complications associated with

6   frailty and malnutrition, treatment with open or endovascular mesenteric revascularization can

7   result in a systemic inflammatory state with a protracted postoperative course and high

8   perioperative morbidity.[2, 3] One factor potentially contributing to this inflammatory state is

9   alterations in the intestinal microbiome in this patient population.

10   The intestinal microbiome consists of trillions of microbial organisms and plays a critical

11   role in immune system regulation.[4] The term "pathobiome" has been established in critical

12   illness as a decrease in intestinal microbial diversity and an overabundance of pathogenic

13   organisms.[5] The role of the microbiome in acute mesenteric ischemia has been studied in

14   preclinical models. These studies demonstrate intestinal dysbiosis, or disruption of the normal

15   gut microbiota, and injury characterized by decreased alpha-diversity (microbial species

16   richness) as well as changes in beta-diversity (inter-individual variability) and microbial

17   community composition, suggestive of a pathobiome state.[6-11] Animal studies of acute

18   mesenteric ischemia have even shown that depletion of commensal bacteria prior to mesenteric

19   ischemia attenuates the initial post-revascularization inflammatory response, despite the presence

20   of these bacteria generally being beneficial to homeostasis.[12-14] In other models, administration

21   of *Lactobacillus plantarum* or *Bifidobacterium bifidum* PRL2010 each reduced the inflammatory

22   response after acute mesenteric ischemia and reperfusion.[15, 16] In addition, both human and

23   preclinical studies of acute mesenteric ischemia and reperfusion have shown breakdown of the

1   integrity of the intestinal barrier which resolves after reperfusion.[17-21] Administration of

2   Pravastatin or *Lactibacillus murinus* have each been implicated in the attenuation of this

3   intestinal injury in animal models.[9, 22] Furthermore, pre-operative fasting in a preclinical model

4   of acute mesenteric ischemia was associated with decreased intestinal injury and intestinal

5   dysbiosis.[23] Together, these studies suggest a critical role of the microbiome on the inflammatory

6   milieu seen after revascularization for acute mesenteric ischemia.

7   While studies of acute mesenteric ischemia support the role of the microbiome in the

8   systemic inflammatory response associated with revascularization, it's role in CMI patients

9   remains poorly understood. It is likely that there is a more significant impact of the microbiome

10 in the CMI population given the more prolonged preoperative course and weight loss, significant

11 alterations in diet and malnutrition as well as prolonged alterations in intestinal blood flow. In

12 this study, we sought to characterize the microbiome composition and diversity in patients with

13 chronic mesenteric ischemia in the preoperative, perioperative, and postoperative periods. We

14 hypothesized that patients with CMI would have baseline microbiome perturbations which could

15 contribute to the initial inflammatory response after revascularization and potentially normalize

16 in the postoperative period.

17

18 **METHODS**

19 <u>Study Population</u>

20   A single center, prospective observational cohort study was conducted between 2019-

21 2020 at the University of Florida comparing two patient populations: healthy controls (n = 9) and

22 patients with chronic mesenteric ischemia (n = 8). This sample size was chosen to serve as a pilot

23 study which would lead to a refined effect size analysis to determine the number of patients

1   necessary to adequately power a larger scale study. Stool samples were obtained from patients

2   with chronic mesenteric ischemia at three time points: pre-operatively (CMI-A); perioperatively

3   within 14 days when not being treated with antibiotics and tolerating a regular diet (CMI-B); and

4   at least 30 days post-operatively (CMI-C). One stool sample was obtained from each healthy

5   control. Inclusion criteria for patients with CMI included: diagnosis of chronic mesenteric

6   ischemia as defined by the American College of Radiology Appropriateness Criteria and

7   undergoing open mesenteric bypass or endovascular intervention, ability to obtain patient

8   informed consent, and age greater than 18.[24] Exclusion criteria for patients with CMI were as

9   follows: acute mesenteric ischemia, inability to obtain informed consent, pregnancy, evidence of

10   multi-organ failure on presentation, and inability to follow-up at our institution. This study was

11   approved by the University of Florida Institutional Review Board (IRB 201802545, IRB

12   201400611). All subjects gave informed consent to participate in the study.

13   Clinical Data

14   Clinical data including baseline characteristics such as age, sex, race, weight, body mass

15   index (BMI), medical co-morbidities, and surgical history associated with CMI were collected.

16   Management and outcome parameters included: post-operative complications, antibiotic

17   administration, diet, intensive care unit (ICU) length of stay, hospital length of stay, and

18   mortality. Patients were seen in follow-up at four weeks and at least twelve weeks post-

19   operatively.

20   Stool Collection and Processing

21   After collection, stool was immediately frozen and stored in a -80°C freezer until

22   processing. Microbial DNA extraction from stool samples and 16S library construction were

23   performed blinded at the University of Florida Interdisciplinary Center for Biotechnology

1   Research Gene Expression Core (Research Resource Identifier SCR_019145) and sequencing

2   was performed at NextGen Sequencing (Research Resource Identifier SCR_019152). High-

3   quality genomic DNA was extracted from fecal samples using the QIAmp PowerFecal Pro DNA

4   Kit (Qiagen, Hilden, Germany) per manufacturers protocol and quantified using a Nanodrop

5   spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3-4 hypervariable

6   region of the bacterial 16S rRNA gene DNA (1ng) was amplified using the QIAseq 16S/ITS

7   Region Panel (Qiagen, Hilden, Germany) for 220 cycles of polymerase chain reaction (PCR)

8   amplification (95°C for 2 minutes, (95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 2

9   minutes for 12 cycles), with 4°C hold). The PCR products were then purified with QIAseq Beads

10 (Qiagen, Hilden, Germany) according to manufacturer's protocol. Barcode addition was

11 performed with 19 cycles of PCR amplification and the library clean-up performed with QIAseq

12 Beads (Qiagen, Hilden, Germany). Finally, the individual library was quantified using the KAPA

13 library quantification kit (Kapa Biosystems, Boston, MA, USA) and run on the Bio-Rad CFX96

14 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The purified library was

15 pooled in equimolar concentrations and sequenced on an Illumina MiSeq platform (Illumina Inc.,

16 San Diego, CA, USA) using a 2x300 base pairs reagent kit (MiSeq reagent kit v3; Illumina Inc.)

17 for paired-end sequencing.

18   Microbiome profiles were analyzed per our previously described methods.[25] Briefly,

19 resulting sequences (.fastq files) were processed using the Quantitative Insights Into Microbial

20 Ecology (QIIME2) bioinformatics software suite (version 2.2021.2; https://qiime2.org/) in a

21 miniconda environment.[26] Sequences were subjected to de-multiplexing based on unique

22 barcodes assigned to each sample. Subsequent quality-filtering, adapter-trimming, denoising, and

23 removal of non-chimeric amplicons was performed with the DADA2 pipeline using the q2-

1 dada2-plugin using default parameters, as described in our previous reports.[27, 28] Alpha-

2 rarefaction was performed at the lowest sequencing depth to avoid the bias of sequencing depth.

3 Bacterial taxonomy was assigned to the amplicon sequence variants (ASV) by implementing the

4 Naïve-Bayes classifier natively implemented in qiime2-dada2 and pre-trained on SILVA

5 reference database (version 138.1, updated March 2021).[29] The dataset was filtered to omit

6 taxonomic features annotated as 'mitochondria' and 'chloroplast'. Alpha-diversity is defined as

7 microbial community richness, also described as distribution of microbial species in each

8 sample. Community richness (alpha-diversity) metrics included operational taxonomic units

9 (OTU) and Chao1 and Shannon indices. The Chao1 index estimates the richness of microbial

10 species present, while the Shannon index accounts for both richness and evenness of microbial

11 species. Beta-diversity, which represents community dissimilarities between two ecosystems

12 (i.e., inter-individual variability), was quantitatively evaluated by Bray-Curtis distance algorithm

13 within QIIME2 and were represented by a principle coordinate analysis (PCoA) plot. The raw

14 read counts were transformed to relative abundances by dividing each value by the total reads

15 per sample and collapsed to taxonomic levels by summing their respective relative abundances.

16 All samples were batch-processed to avoid any bias of DNA extraction or PCR

17 primers/conditions on community composition obtained by amplicon sequencing.

18 <u>Statistical Analysis</u>

19 Comparisons between cohorts of alpha-diversity, beta-diversity (PCoA), bacterial

20 abundance, and linear discriminatory analysis (LDA) effect size (LEfSe) were analyzed using the

21 'R' statistical package version 4.1.2.[30, 31] Beta-diversity was assessed using the Bray-Curtis

22 dissimilarity index and was visualized by PCoA plots to visually depict global microbiome

23 differences between the samples and groups. Statistical analysis for differential clustering of

1   samples on the PCoA plot was done by permutational analysis of variance (PERMANOVA) test,

2   a permutation-based multivariate analysis of variance to a matrix of pairwise distance to partition

3   the inter-group and intra-group distance. Alpha-diversity data are presented as mean ± standard

4   deviation. Unique bacterial taxa driving specific group-specific differences were determined

5   using the biomarker discovery algorithm LEfSe with parameters set at LDA score of >3.0 and p-

6   value <0.05. The LEfSe analysis performs a non-parametric factorial Kruskal-Wallis sum-rank

7   test to identify taxa with significant differential abundance with respect to the groups, then

8   validates the biological significance using pairwise unpaired Wilcoxon rank-sum test, and finally

9   performs LDA analysis to estimate the effect size of each differentially abundant taxon.[30] The

10   normalization method of taxon abundance data consisted of data transformation and scaling.

11   Data transformation was done by generalized log-transformation (base 2) and data scaling by

12   auto-scaling (mean-centered and divided by standard deviation of each variable). Patient

13   characteristics were analyzed with GraphPad Prism version 9.4.1 (GraphPad Software, La Jolla,

14   CA) using Welch's t-test, Welch's ANOVA with multiple comparisons with Dunnett T3

15   correction, or Fisher's exact test. For all tests, $p<0.05$ was considered statistically significant.

16

17   **RESULTS**

18   *Patient Characteristics*

19   Patient characteristics for CMI patients and healthy controls are listed in Table I. The

20   CMI patients were older than healthy controls. There was no difference in patient race or self-

21   reported sex between healthy controls and CMI patients. Patients with CMI were found to have

22   coronary artery disease (CAD) compared to healthy controls. Otherwise, there were no

23   differences in the following comorbidities between groups: active smoking, hypertension,

1 hyperlipidemia, renal disease (defined by creatinine greater than 1.5mg/dL), diabetes, chronic

2 obstructive pulmonary disease, and congestive heart failure. For the CMI patients, half had a

3 history of prior mesenteric stent placed complicated by failure. The average duration of

4 abdominal symptoms such as pain, diarrhea, and weight loss was 8.2 months.

5   The majority of patients in the CMI group underwent open mesenteric bypass (75%) and

6 of these, half involved bypass of only the superior mesenteric artery (SMA) and the remainder

7 underwent bypass of both the celiac and superior mesenteric arteries. Endovascular stenting for

8 the other patients was only performed in the superior mesenteric artery via brachial artery access.

9 Post-operatively, two patients (25%) received antibiotics prior to the first post-operative stool

10 sample collection: one received a one-time administration for a planned additional surgery

11 during the same admission and the other was treated for a post-operative hospital-acquired

12 pneumonia for a seven-day course. One patient received enteral tube feedings as supplemental

13 nutrition post-operatively and another received both total parenteral nutrition (TPN) and enteral

14 tube feedings post-operatively as before resuming a regular diet. One patient was restarted on her

15 home medication of lactobacillus while inpatient, but the remaining patients were not

16 administered probiotics or any oral dietary supplements during admission. The mean ICU length

17 of stay was 3.8 days ($\pm$2.2) and mean hospital length of stay was 11.5 days ($\pm$7.6). The mean

18 preoperative BMI was 20.6 kg/m^2 ($\pm$4.3); this remained unchanged postoperatively at 4 weeks

19 (19.6 kg/m^2$\pm$3.6) and 12 weeks (19.1 kg/m^2$\pm$3.8) (p=0.76). The postoperative mortality was

20 zero. Three patients were lost to follow-up at 12 weeks despite attempts at re-engagement by the

21 research team. Post-operative stool samples were collected on average 11 days post-operatively

22 (CMI-B) and 23 weeks post-operatively (CMI-C).

23

1   *Baseline microbiome for CMI patients versus healthy controls*

2   Pre-operatively, patients with CMI had lower alpha-diversity (OTUs) compared to

3   healthy controls (CMI-A: 381.6±59.7, Control: 486.4±158.4, p=0.03) (Fig. IA). Patients with

4   CMI also demonstrated lower Shannon index pre-operatively compared to healthy controls

5   (CMI-A: 2.9±0.2, Control: 3.0±0.6, p=0.03) (Fig. IB). Chao1 index was decreased in the CMI

6   group pre-operatively compared to controls, but this did not reach statistical significance (CMI-

7   A: 314.7±42.7, Control: 338.9±61.8, p=0.30) (Fig. IC). Beta-diversity was not different between

8   chronic mesenteric ischemia patients and healthy controls (p=0.33) (Fig. II).

9

10   *Changes in the microbiome in CMI patients post mesenteric revascularization*

11   Once tolerating a diet post-operatively, patients showed an increase in alpha-diversity

12   (OTUs) when compared to their pre-operative sample, although this did not reach statistical

13   significance (CMI-A: 381.6±59.7, CMI-B: 426.1±44.5, p=0.39) (Fig. IA). This increase in OTUs

14   was similar to healthy control patients (CMI-B: 426.1±44.5, Control: 486.4±158.4, p=0.24) (Fig.

15   IA). Surprisingly, the Shannon index decreased slightly post-operatively compared to pre-

16   operative (CMI-A: 2.9±0.2, CMI-B: 2.7±0.7, p=0.77), but were not different compared to

17   healthy controls (CMI-B: 2.7±0.7, Control: 3.0±0.6, p=0.08) (Fig. IB). Chao1 index increased

18   slightly, although this was not significant (CMI-A: 314.7±42.7, CMI-B, 331.8±39.6, p=0.46)

19   (Fig. IC). Beta-diversity was not different between pre-operative samples and initial post-

20   operative samples (p=0.25) or between initial post-operative samples and healthy controls

21   (p=0.41) (Fig. II).

22   At greater than 30 days after revascularization, patients demonstrated similar alpha-

23   diversity to initial post-operative samples and healthy controls. The number of OTUs was similar

1   compared to initial post-operative samples (CMI-B: 426.1±44.5, CMI-C: 411.6±27.9, p=0.80)

2   and healthy controls (CMI-C: 411.6±27.9, Control: 486.4±158.4, p=0.18) (Fig. IA). Shannon

3   index increased further out from mesenteric revascularization (CMI-B: 2.7±0.7, CMI-C:

4   2.85±0.2, p=0.77) and was similar to that seen in healthy controls (CMI-C: 2.85±0.2, Control:

5   3.0±0.6, p=0.06) (Fig. IB). Chao1 index also continued to increase post-operatively (CMI-B:

6   331.8±39.6, CMI-C: 346.3±32.0, p=0.51) and was similar to healthy controls (CMI-C:

7   346.3±32.0, Control: 338.9±61.8, p=0.64) (Fig. IC). Beta-diversity was significantly different

8   between initial post-operative and long term post-operative samples (p=0.03), but not between

9   long-term post-operative patients and healthy controls (p=0.31) (Fig. II).

10

11 *Chronic mesenteric ischemia microbiome composition pre and postoperatively*

12   Microbial composition of patients with CMI was unique compared to healthy controls.

13 Major phyla and genera present in CMI patients at three time points and healthy controls are

14 shown in Figure IIIA-B. Healthy control microbiota was dominated by *Murimonas* (p<0.05)

15 (Fig. IIIC). Pre-operatively, CMI patients had an abundance of *Clostridium*, *Hungatella*,

16 *Citrobacter*, and *Subdoligranulum* (p<0.05) as identified through LEfSe (Fig. IIIC). Within two

17 weeks of revascularization, *Erysipelotrichaeceae*, *Akkermansia*, and *Flavonifractor* were the

18 most prominent genera (p<0.05) (Fig. IIIC). *Anaerostipes* was identified in uniquely elevated

19 numbers long-term after mesenteric revascularization (p<0.05) (Fig. IIIC).

20

21 **DISCUSSION**

22   Chronic mesenteric ischemia is a devastating disease for which standard of care with

23 open or endovascular mesenteric revascularization can result in systemic inflammation and a

1   morbid postoperative course.[2, 3] In our study, we showed that CMI induces intestinal dysbiosis

2   characterized by decreased alpha-diversity and a unique microbial composition. This pathobiome

3   signature begins to resolve within two weeks of open mesenteric bypass or endovascular

4   revascularization with subsequent increases in alpha-diversity and changes in microbial organism

5   composition. Overall, these findings show that revascularization appears to have sudden changes

6   in microbiome diversity and composition which become more similar to healthy controls. These

7   changes occur within two weeks of surgery and are re-demonstrated at a later time point,

8   highlighting the importance of normal mesenteric flow as well as normalization of diet for a

9   homeostatic microbiome.

10   Our findings suggest that at baseline, patients with chronic mesenteric ischemia have

11   drastically fewer bacterial species and reduced evenness, as demonstrated by decreased alpha-

12   diversity, in their intestinal microbiome compared to healthy controls. After revascularization,

13   we found that patients had increased alpha-diversity represented by OTUs and Shannon index.

14   These baseline differences in alpha-diversity suggest that malnutrition and hypoperfusion could

15   contribute to intestinal microbiome diversity. These alpha-diversity results are similar to that of

16   animal studies of acute mesenteric ischemia which investigated the microbiome.[6, 23] Some of

17   these models revealed an initial decrease in alpha-diversity indices hours post-operatively, but

18   this may differ from our post-operative results given that our initial post-reperfusion samples

19   were collected on average seven days after surgery.[9, 23, 32] Wang et al found that acute mesenteric

20   ischemia induced a decrease in alpha-diversity, which increased six hours after revascularization,

21   similar to our results of increasing alpha-diversity over time.[6] Additional animal studies

22   investigating the effects of acute mesenteric ischemia on the small intestine microbiota also

23   demonstrated increases in the number of bacteria after revascularization.[10, 33, 34] A murine study

1 of ischemia/reperfusion showed that subjects which died within one hour had lower baseline

2 alpha-diversity compared to those who survived.[9] Together this elucidates the importance of

3 normal mesenteric flow to maintain the number and evenness of bacterial species in the intestine

4 and contribute to homeostasis.

5   We were surprised to find that beta-diversity was only different between perioperative

6 and post-operative samples after revascularization in our chronic mesenteric ischemia patients. It

7 is possible that these slight differences in beta-diversity reflect the recovery of the microbiome

8 post-operatively as patients begin to resume improved nutrition; however, this requires additional

9 study in larger cohorts. Wang et al found an initial disturbance in beta-diversity in rats after

10 ischemia and reperfusion which normalized over the course of 72 hours.[6] Similarly, Deng et al

11 showed different beta-diversity between mice who underwent ischemia/reperfusion compared to

12 sham mice in the small intestine.[34] Similar findings were also revealed in rat ileum contents after

13 acute mesenteric ischemia and reperfusion.[10] However, these other studies were all of acute

14 mesenteric ischemia and not chronic mesenteric ischemia.

15   We found that the microbial composition of patients with CMI was unique compared to

16 healthy controls. Prominent bacteria in the pre-operative control group consisted of pathogenic

17 bacteria such as *Citrobacter* but also the commensal bacteria *Clostridium XIVa* and

18 *Subdoligranulum*, along with *Hungatella* whose function requires further study.[35-38] While it was

19 surprising to find an abundance of commensal bacteria, preclinical studies of acute mesenteric

20 ischemia and reperfusion have shown that even commensal bacteria contribute to the

21 inflammatory response seen after reperfusion.[12-14] In our study, one week after reperfusion,

22 *Akkermansia, Erysipelotrichaeceae*, and *Flavonifractor* were present in high abundance, all

23 noted to be commensal bacteria.[39-41] Months after revascularization in our patients, *Anaerostipes*

1 dominated the fecal microbiome, also a commensal bacteria.[42] A preclinical study of acute

2 mesenteric ischemia showed an initial bloom in *Escherichia coli* within three hours of

3 reperfusion and *Lactobacillus* further out from reperfusion in rats.[6] Other animal models of acute

4 mesenteric ischemia and reperfusion resulted in abundances of commensal bacteria such as

5 *Ruminococcaceae-UCG-010, Enterobacteriaceae, Lactobacillus*, *Oscillobacter*, and

6 *Lachnoclostridium*.[9, 23, 32] Overall, our study provides insight into the unique microbial

7 composition of patients with chronic mesenteric ischemia and its temporal changes after

8 reperfusion. It is important in future studies to understand the role of commensal and pathogenic

9 bacteria in systemic inflammation after revascularization as well as the interplay between these

10 bacteria.

11   This study has several limitations. One major limitation of this study is the small sample

12 size which precludes subgroup analyses. However, this pilot study demonstrates critical changes

13 in the microbiome in chronic mesenteric ischemia warranting a larger investigation. Despite

14 efforts to enroll a diverse population of patients, the majority of the CMI patients were female

15 and Caucasian; patients with CMI were also older than healthy controls. It has been shown that

16 age, sex and race all influence the microbiome.[43-45] Potential confounders in this study include

17 differences in pre-operative diet part of which may be related to the duration of symptoms and

18 the administration of post-operative antibiotics in two patients; both diet and antibiotics have

19 been shown to affect intestinal microbiota.[46, 47] However, only one patient received post-

20 operative antibiotics for a seven-day course to treat pneumonia while the other received only one

21 dose for a planned second surgery. In addition, this study included patients who underwent either

22 mesenteric stenting or open mesenteric bypass. While not yet investigated, these different

23 revascularization techniques could have different influences on the microbiome and should be

further studied. Finally, three patients were lost to follow-up at 12-weeks despite attempts at re-

engagement by the research team which may have influenced our long-term post-operative

results.

Given the limitations of this pilot study, further investigation of changes in the

microbiome as well as its role in the inflammatory response in CMI patients are warranted using

a larger sample size. Subgroup analyses to evaluate effects of sex, age, smoking status, post-

operative antibiotics and revascularization techniques could provide further insight into how

each of these affects the microbiome in this disease. In addition, multiple studies in acute

mesenteric ischemia and reperfusion have demonstrated the benefits of commensal microbiome

depletion, probiotic administration, and even pre-operative fasting on the attenuation of the post-

operative systemic inflammation.[9, 12-16, 23] Fecal metabolomics to quantify short-chain fatty acid

(SCFA) levels would also be of interest to investigate; in acute mesenteric ischemia and

reperfusion, administration of SCFA decreases inflammation and intestinal injury in acute

mesenteric ischemia and reperfusion.[48-50] These interventions could potentially play a role in

tempering morbidity after revascularization in chronic mesenteric ischemia.

CONCLUSION

In summary, we found that chronic mesenteric ischemia induces intestinal dysbiosis

characterized by decreased overall species abundance and domination of pathogenic bacteria.

This pathobiome signature begins to resolve within two weeks of open or endovascular

revascularization with subsequent increases in alpha-diversity and changes in microbial

community composition. Further from surgery, our subjects had microbiome diversity and

composition most similar to that of healthy controls. The evidence provided in this study

1   suggests that patients with chronic mesenteric ischemia have baseline microbiome perturbations

2   which normalize after revascularization. Future studies are needed to evaluate the influence of

3   race, age, sex, smoking status, and type of revascularization on the microbiome in this unique

4   disease. Additionally, evaluation of links between the microbiome and systemic inflammation in

5   these patients, fecal metabolomics, and the effects of chronic mesenteric ischemia on intestinal

6   barrier integrity are important next steps.

7

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14   **AUTHOR CONTRIBUTIONS**

15   JAM, RN, AMM, PAE, DJA, and MAC contributed to the conception and design of the study.

16   DJA and MAC were involved in obtaining funding for the project. JAM, LL and RN contributed

17   to analysis and interpretation of the data; RN performed the statistical analysis. JAM, NCH, and

18   AM were involved in data collection. JAM drafted the manuscript and all other authors

19   contributed critical revisions to produce the final manuscript. All authors approve of the final

20   manuscript and agree to be accountable for its contents.

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FIGURE LEGENDS

Figure IA-C. Alpha-diversity represented by a) OTUs, b) Chao1, and c) Shannon indices in pre-operative (CMI-A), initial post-operative (CMI-B), and long-term post-operative (CMI-C) patients compared to healthy controls (CTL). CMI – chronic mesenteric ischemia. Significant differences denoted with an * (defined as $p < 0.05$).

Figure II. Principal coordinate analysis (PCoA) plots based on the Bray-Curtis dissimilarity index showing changes in microbiome beta-diversity, the measure of differences in biodiversity across cohorts (CMI-A: pre-operative; CMI-B: initial post-operative; CMI-C: long term post-operative; CTL: healthy control; CMI: chronic mesenteric ischemia, M: Male; F: Female; PERMANOVA: permutational analysis of variance). Percent on each orthogonal axis represents the proportion of overall variance in the data.

Figure IIIA-C. Microbial composition at the level of a) major bacterial phyla b) major bacterial genera and c) LefSE (Linear discriminatory analysis Effect Size) in pre-operative (CMI-A), initial post-operative (CMI-B), and long-term post-operative (CMI-C) patients and healthy controls (CTL). LDA: Linear Discriminatory Analysis; CMI: chronic mesenteric ischemia.

Table I. Patient characteristics.

Patient Group	Healthy Control (n=9)	Chronic Mesenteric Ischemia (n=8)	p-value
Age, mean (SD)	55.6 (12.6)	70.9 ($\pm$7.6)	0.008
Male sex, n (%)	7 (78)	2 (25)	0.06
Race			0.47
White, n (%)	9 (100)	7 (87.5)	
African-American, n (%)	0	1 (12.5)	
Comorbidities, n (%)			
Active Smoker	0	3 (37.5)	0.08
Hypertension	3 (33.3)	6 (75)	0.15
Hyperlipidemia	3 (33.3)	4 (50)	0.63
Renal disease (Cr>1.5mg/dL)	0	2 (25)	0.21
Coronary Artery Disease	0	4 (50)	0.03
Diabetes	1 (11.1)	1 (12.5)	0.99
COPD	0	1 (12.5)	0.47
CHF	0	1 (12.5)	0.47

COPD - Chronic Obstructive Pulmonary Disease; CHF - Congestive Heart Failure; Cr – creatinine.

A

B

C

PCoA [Bray-Curtis]
PERMANOVA *P* = 0.1703