Diabetic Complications Consortium

Application Title: A study to explore novel causes of diabetes neuropathy and dysmotility in the gut.

Principal Investigator: Dr. Onesmo B. Balemba

1. Project Accomplishments:

The goal of our project was to determine whether transplantation of stool supernatants from cecum and colon of mice restricted to a high fat diet (HFD) for 8 weeks or from humans with prediabetes and type two diabetes (T2D) into stomachs of healthy mice alter gastrointestinal (GI) motility and impair activities of nitrergic and cholinergic myenteric neurons. Several things were accomplished during the implementation of the project.

By using C57BL/6 mice fed 72% kcal HFD and 45% kcal HFD to model the pathogenesis of GI neuropathy and dysmotility in type 2 diabetes (T2D), we analyzed whether a 2-week HFD regimen disrupts GI motility and elicits damage to myenteric neurons. Standard chow diet (6.2% kcal from fat; SCD) was used in control experiments (n=4-5). We determined whether intragastric gavage of stool supernatants filter-sterilized by 0.2 μm, surfactant-free, lowest protein binding cellulose acetate filters (1:50 dilution) from 2-week HFD mice disrupts GI motility, causes impaired glucose intolerance/insulin resistance and damages myenteric neurons. Supernatants of 8-week HFD and SCD mice were used in controls (n=3-4). Although these experiments have not been completed due to challenges shown below, we are very pleased to report that:

- We discovered that two weeks of HFD ingestion did not cause glucose intolerance and insulin resistance in male and female mice.
- Injury to the enteric nervous system (ENS) —mainly nitrergic myenteric neurons, and intestinal dysmotility occurred in 2-week HFD. This indicated that these GI abnormalities precede impaired glucose tolerance and T2D, independently of sex.
- Accumulation of neuronal damaging and antimotility toxins in stools of HFD occurred before 2 weeks matching the start of decreased GI motility and injury to nitrergic myenteric neurons.

We enrolled 64 human subjects aged 18 years and older and collected data related to diet history, blood glucose, lipid panel, Hemoglobin A1C and stool samples from 60 subjects because 4 subjects did not complete the study. The group categories of these human subjects are Cohort 1- 21 healthy subjects (8 male, 13 females). Cohort 2- 10 subjects diagnosed with prediabetes but had a normal HbA1c (reversed prediabetes, 3 males, 7 females). Cohort 3- 12 subjects diagnosed with prediabetes (4 male, 8 females). Cohort 4- 14 subjects diagnosed with T2D without neuropathy (8 males, 6 females). Cohort 5- 3 subjects diagnosed with T2D and diabetes neuropathy (2 male; 1 females).

We analyzed the composition of the stool microbiota by long-read DNA sequencing technology of 16S-ITS-23S rRNA genes and full-length 16S rRNA genes instead of sequencing

the variable V1-V3 regions of bacterial 16S rRNA genes as originally planned. The reason for the change was to enable us to analyze species and strain level changes. Data concerning the diversity of the gut microbiota (operational taxonomic units (OTUs), Shannon Index, and Fisher's Alpha) were correlated with sedentary behavior, screen time, and physical activity via surveys. I am happy to report that:

- We published one paper highlighting the relative abundance of bacteria strains associated with type 2 diabetes using data obtained by 16S-ITS-23S rRNA genes sequencing.
- We are re-analyzing these data by using computational and machine leaning and improved algorithms to determine the association between the relative abundancy of specific bacteria species and strains with reversed prediabetes, prediabetes and T2D disease states.
- We will compare results with findings obtained by analyzing full-length 16S rRNA genes sequencing data.
- We will submit a manuscript with these results this year.

In brief, our microbiome sequencing data strongly support the idea that specific species and strains of the gut microbiota could be driving GI neuropathy and dysmotility in reversed prediabetes (RPD), prediabetes (PD), and T2D patients. Therefore, we have obtained *Odoribacter laneus* (YIT_12061) and *Flavonifractor plautii* to identify whether their exudates into culture media impair contractions and damage myenteric neurons in cultured duodenojejunal muscle preparations. We will expand this list after completing the analysis outlined above.

We performed several animal and tissue culture experiments to identify the effects of stool supernatants from mice fed HFD or RPD or PD and T2D humans on GI motility, intestinal muscle contractility and activities of nitrergic and cholinergic myenteric neurons. The results of these experiments are unpublished because we need to increase the number of experiments and complete analysis of data. I am pleased to report that:

- We secured support from the University of Idaho to enable us to conclude key experiments and publish results by next year.
- In brief, we tested 37 stool filtrates samples from 19 male subjects and 18 female subjects in sex-matched, healthy ChAT-GCaMP6f mice. We tested 22 stool filtrates samples from 11 male subjects and 11 female subjects in sex-matched, healthy nNOS-GCaMP6f mice.
- We have completed intragastric gavage of LTA+LPS mixtures in male and female ChAT-GCaMP6f mice (4 each sex) and male and female nNOS-GCaMP6f mice (2-3 each sex).
- Experiments underway and future experiments are aimed at testing the remaining 20 stool samples.

We are very excited by the unpublished results from 8-week stool supernatants experiments, which showed that stool supernatants from RPD, PD, and T2D patients have molecules that can decrease GI motility and trigger inflammation in male and female mice and impair glucose tolerance in male mice after 8 weeks. Molecules in stool supernatants of T2D male patients and LPS+LTA mixture cause glucose intolerance and insulin resistance in male mice after 8 weeks. This was not observed in mice gavaged with supernatants of healthy people. Compared with stool supernatants of healthy subjects, stool supernatants of T2D patients significantly enhanced the activities of ChAT myenteric neurons, but damage and decrease activities of nNOS

myenteric neurons. This reciprocity could explain intestinal hypercontractility reported in T2D patients, and decreased GI motility and ectopic contractions observed in our study.

We performed *ex vivo* experiments (n=4-5) to determine whether stool filtrates (1:50 dilution) filter-sterilized stool supernatants from males and females diagnosed with PD and T2D inhibit contractions of duodenojejunal muscularis from sex matched healthy mice, in culture. For control, tissues were treated with stool supernatants of healthy human stools or a mixture of LTA+LPS or left untreated. We also analyzed whether sequestering endotoxins and other negatively charged molecules from supernatants by using 0.2 μm Mustang E Acrodisc filters protects muscularis from supernatants' injurious effects.

- Results from these experiments were reported in an abstract to be presented at the American Neurogastroenterology and Motility Association (ANMS) annual meeting, this August (August 11 - 13, 2023) in Austin, Texas. The abstract will be published in Neurogastroenterology and Motility.

In analyzing tissue sections to determine effects of stool supernatants from RPD, PD, and T2D patients on epithelial barriers and GI inflammation, we have discovered that these stool supernatants disrupt mucosal barrier and GI inflammation. We will complete the analysis of epithelial barriers and GI inflammation using stained tissue sections by the end of this year.

- I am pleased to report that when taken together, the pilot award successfully enabled us to collect ample data to support an R01 grant application entitled: "Identification of Triggers of Diabetic Gastrointestinal Neuropathy and Dysmotility" submitted to NIH, June 22, 2023.
- We will submit a grant application titled "The Role of Water-Soluble Post-Biotic Molecules in the Pathogenesis of Motility Disorders in Prediabetes and Type 2 Diabetes" to Mather's Foundation Grant Program in the Fall Cycle, 2023.

Challenges that limited our productivity.

In 2021, the PI contracted COVID-19 and was out from January 10 to April 1, 2021. At that time, the PI had an on-going experiment consisting of a total of 36 mice, which could not be completed because he had no trained personnel who could complete the experiment. Subsequently, we lost a cohort of nNOS- GCaMP6f mice (n=16) and a cohort of ChAT-GCaMP6f mice (n=18) on 8-week, 45%HFD feeding regimen and intragastric gavage of supernatants from 45%HFD and SCD mice.

From October 2021 to Feb 2022, the laser launcher for the spinning disk confocal microscope had to be shipped to for repair. As a result, we could not perform calcium imaging experiments for five and a half months. Fortunately, we were able to utilize our ChAT-GCaMP6f mice by shipping them to our colleague Dr. Keith Sharkey, at the University of Calgary. The results of this collaboration were published in the Journal of Physiology this year.

2. Specific Aims:

Our project has one Specific Aim and five Sub-Aims. These include:

Specific Aim1: Determine whether transplantation of fecal supernatants from cecum and colon of mice restricted to HFD for 8 weeks or from prediabetic and T2D humans into stomachs of healthy mice alter GI motility and impair nitrergic and cholinergic myenteric neurons.

Aim 1.1- Produce supernatants of feces from cecum and colon of mice fed HFD.

Aim 1.2- Produce supernatants of human stool.

Aim 1.3- Transplant fecal supernatants from HFD mice, pre-diabetes and T2D patient donors into stomachs of healthy mice.

Aim 1.4- Determine whether HFD and fecal supernatant transplantation impair transmission in nitrergic and cholinergic myenteric neurons via Ca^{2+} transient analysis.

Aim 1.5- Accomplish other key analyses including GI mucosa barrier, GI inflammation and microbiota analysis.

2.1. Results

In this section we will outline unpublished results from experiments done to implement the Specific Aims above.

2.1.1. Toxins capable of triggering ENS neuropathy and GI dysmotility accumulate in stools of HFD mice before glucose intolerance. We previously showed that female mice fed a HFD for 8 weeks do not develop glucose intolerance and insulin resistance but have GI dysmotility, ENS neuropathy and altered gut microbiota(1). We also reported that filter-sterilized stool supernatants (1:100 dilution) and stool slurry from these non-diabetic HFD female mice as well as diabetic male mice inhibit propulsive motility in isolated duodenums and colons, and contractility of cultured duodenal and distal colon muscularis from healthy mice. In addition, compared with SCD mouse stool supernatants, supernatant from HFD male mice (with diabetes conditions) and HFD female mice (without diabetes conditions) inhibited duodenal and colon propulsive motility, blocked muscularis contractions after 6-12 hours, and caused loss of nNOS neurons after 24 hours of culture(2). We wished to determine whether palmitate (30 µM), endotoxins from Gram-negative bacteria, lipopolysaccharide (LPS; 0.5 ng/ml) and Gram-positive bacteria, lipoteichoic acid (LTA; 0.5 ng/ml) and short-chain fatty acids (acetate, propionate, butyrate, @ 30 mM) mimic effects of supernatants. We reported that LPS, LTA as well as palmitate, and LPS + palmitate decreased nNOS myenteric neurons, but did not affect muscularis contractions after 24 hours of culture. Short-chain fatty acids did not affect myenteric neurons and contractility of muscularis preparations(3). These results suggested that there are unidentified toxins in the supernatant. To identify the toxins involved, we used bioactivity-guided solid phase extraction (SPE) and HPLC to concentrate and purify supernatants samples into fractions. The aqueous SPE fraction and two of its twenty HPLC sub-fractions, subfractions 10 and 11, blocked muscularis contractions and caused a loss of nNOS myenteric neurons through ganglionitis and nitrosative stress after 24 hours of culture(2). When taken together, results from testing supernatants from HFD and SCD mice prompted us to postulate that intestinal contents from mice fed a HFD have unidentified antimotility and neuron-damaging toxins and their accumulation precedes glucose intolerance. We hypothesized that toxins generated from complex diet–microbiota–host interactions trigger damage to the ENS and dysmotility before glucose intolerance sets in. In implementing our project, we tested whether like female mice, male mice develop neuropathy and dysmotility before glucose intolerance by feeding them 72% kcal HFD for 2-weeks. We discovered that this decreased GI motility and

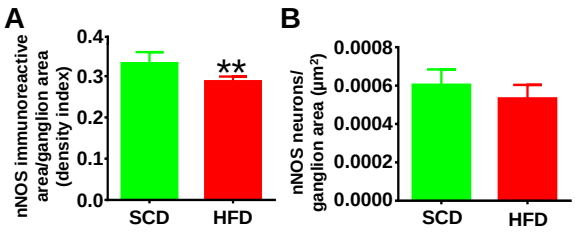

Figure 1. 2-week 72% kcal HFD reduced duodenal myenteric nNOS immunoreactivity per ganglion in male mice (A) but the number of nNOS neurons per ganglion (B) was not significantly affected. n= 5-6. Unpaired T-test.

nNOS immunoreactive varicosities in the myenteric plexus before glucose intolerance, like in female mice. The total number and number of nNOS neurons per ganglion was not altered **[Figure 1]**. These results correspond with clinical studies suggesting peripheral neuropathy and GI dysmotility occur in humans with central obesity and prediabetes(4,5). They indicate a critical need for comprehensive studies to determine triggers of GI neuropathy and dysmotility occurring before T2D and show that the HFD mouse is relevant and suitable for studying the pathogenesis of ENS neuropathy and dysmotility associated with T2D.

To test this idea, we used stool supernatants of male and female mice fed 45% kcal HFD for 2 weeks to identify whether the accumulation of antimotility and neurotoxic molecules precede glucose intolerance. We used 45% kcal HFD because it more relevant to the composition of kcal from fat in the western diet(6–8). Supernatants from male and female mice inhibited contractions of cultured duodenal muscularis after 6-24 hours. In sum, our findings demonstrate an accumulation of unknown, water-soluble toxins in the intestine of 45% and 72% HFD mice before glucose intolerance. These toxins can be concentrated and isolated by HPLC.

2.1.2. Transplanting stool supernatants of non-diabetic HFD mice into healthy mice disrupts GI motility. In preliminary studies to translate the *ex vivo* supernatants findings, supernatants (1:50 dilution,10 ml/kg; sex matched) from mice fed 45% and 72% kcal HFD for 2 weeks were infused (transplanted) by intragastric gavage (3x per week) into healthy mice for 2 weeks. Control mice were gavaged with supernatants from SCD mice. We used a higher concentration assuming stomach acid could denature and reduce levels of toxins in the supernatants. GI transit time measured with 6% Evans Blue + 0.5% methyl cellulose was used to assess upper GI motility. Supernatants from male mice fed HFD delayed GI transit but supernatants from control mice had no affect GI. We are analyzing whether myenteric neurons were damaged **[Figure 2]**. These results firmly support our hypothesis that neurotoxic and antimotility molecules accumulating in GI contents of HFD mice before glucose intolerance trigger ENS neuropathy and GI dysmotility.

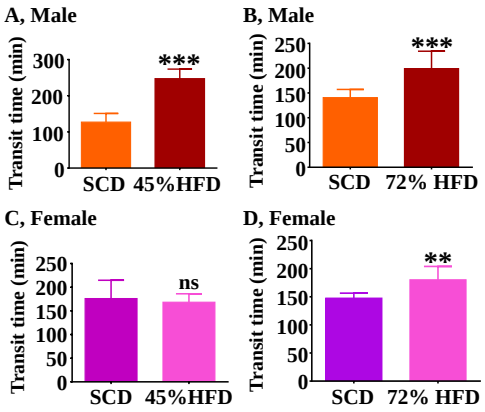

Figure 2. Stool supernatants of 2-week HFD mice delayed GI transit time in recipient mice after 2 weeks. Unpaired T-test; n = 4 mice/group.

2.1.3. Stool supernatants of RPD, PD, and T2D patients contain toxins that disrupt smooth muscle contractions, *ex vivo*. To determine whether results from mouse supernatant studies above translate to humans, we analyzed whether filter-sterilized stool supernatants (1:50 dilution) of males and females diagnosed with RPD or PD or T2D inhibit contractions of duodenojejunal muscularis from sex-matched healthy mice, in culture. Supernatants of healthy human stool, and LTA+ LPS (@0.5 ng/mL) were used for control. We also analyzed whether sequestering endotoxins and other negatively charged molecules from supernatants by using 0.2 µm Mustang E Acrodisc filters (Pall Corporation, MSTG25E3), protects muscularis from supernatants' injurious effects. Compared with healthy subjects, supernatants from RPD, DP, and T2D donors inhibited of contractions duodenojejunal muscularis after 6-12 hours and damaged neurons and smooth muscle cells (SMCs) after 24 hours. LTA+LPS did not inhibit contractions. Sequestering negatively charged molecules from supernatants preserved tissue

health and contractility for up to 36 hours **[Figure 3]**. We performed heat denaturing

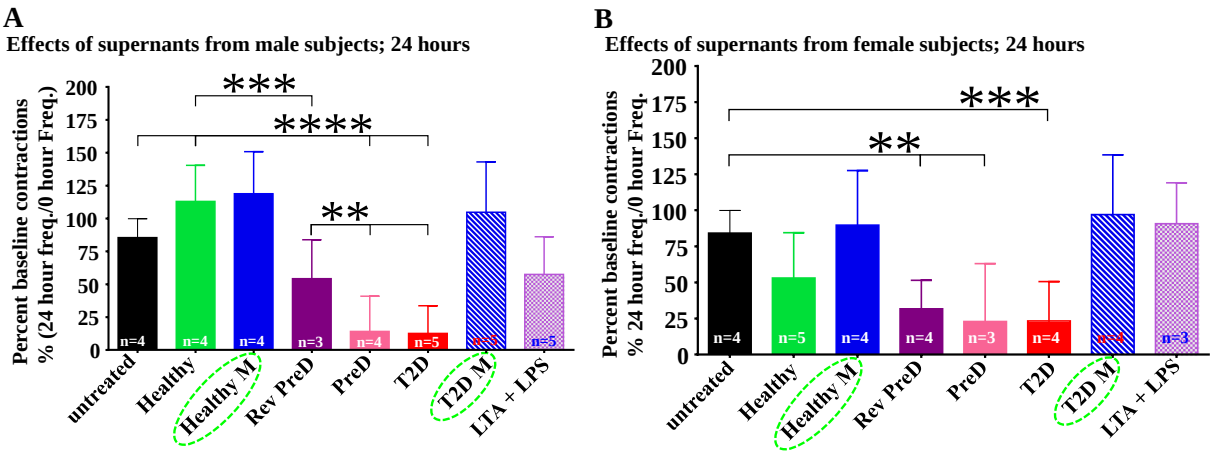

Figure 3. RPD, PD, and T2D supernatants from male and female subjects inhibited muscularis contractions after 24 hours of culture but RPD supernatants' effects were less dramatic. Sequestering endotoxins and other negatively charged molecules from supernatants of healthy and T2D persons (hatched green lines) preserved contractions! One-Way, Anova.

experiments and discovered that heating stool supernatants at 55° Celsius for 15 minutes does not alter the effects of stool supernatants on contractility of cultured duodenojejunal muscularis. In contrast, heating stool supernatants at 100° Celsius for 5 minutes does decreased the effects of stool supernatants on contractility of cultured duodenojejunal muscularis. <u>These discoveries provide further support to our hypothesis that nerve damaging and antimotility toxins accumulate in the gut in HFD mice and humans before T2D. Our findings firmly suggest that the culprit toxins are stable at room temperature and negatively charged molecules.</u>

2.1.4. Stool supernatants of RPD, PD, and T2D patient alter activities of both nNOS and ChAT myenteric neurons. Our preliminary data strongly suggest that toxins from GI contents disrupt motility before development of T2D by damaging myenteric neurons. Likely, activities of myenteric neurons are altered before we can identify microscopic injuries. We postulated that transplanting stool supernatants of RPD, PD, and T2D patient into healthy mice will disrupt GI motility by altering activities of nNOS and ChAT myenteric neurons without causing loss of neurons. We used healthy mice expressing genetically encoded Ca^{2+} indicator protein, GCaMP6f in nNOS or cholinergic (ChAT) myenteric neurons. Mice were bred by crossing female Ai95D (C57BL/6J) mice, which express the Cre-dependent GCaMP6f protein with either male B6;129S6-Chat$^{tm2(cre)Lowl/Mwar}$/J or male B6;129S-Nos1$^{tm1.1(cre/ERT2)Zjh/J}$ /J mice. Cre-ERT2 fusion gene activity and neuronal NOS gene were activated by tamoxifen (50 mg/kg) at 5-6 weeks. For convenience, these F1 crosses are referred to as nNOS-GCaMP6f or ChAT- GCaMP6f mice. At 9 weeks, stool supernatants were transplanted by intragastric gavage into sex matched healthy nNOS-GCaMP6f mice or ChAT-GCaMP6f mice for 8 weeks. For control, mice were gavaged with stool supernatants of healthy human stools or saline or a mixture of LTA+LPS or left untreated. We used submucosa-muscularis preparations from the glandular stomach and distal colon of nNOS-GCAMP6f and ChAT-GCAMP6f mice to study the effects supernatant from RPD, PD and T2D patients have on Ca^{2+} transient activities in the myenteric nNOS and ChAT neurons in recipient mice. Tissues were pinned-stretched between sylgaard blocks. A block was mounted in a tissue bath on an inverted Nikon TiE microscope and superfused with Krebs at

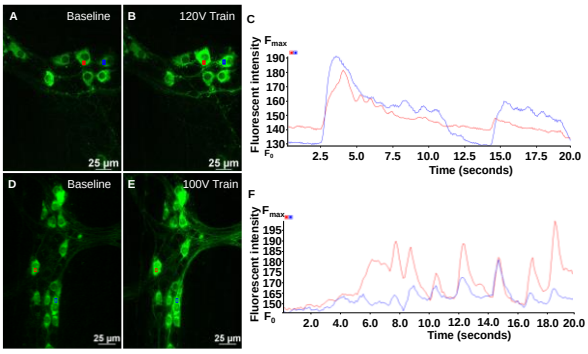

Figure 4. Demonstration of Ca^{2+} transients elicited by stimulation of nNOS myenteric neurons in stomach (A-B) and distal colon (D-F) of a female mouse treated with supernatants from T2D female. Respective traces of fluorescence intensities are shown at right (C and D). For brevity, illustrations of healthy control are not shown.

36.5ºC. Ca^{2+} imaging was performed with a Yokogawa X1 Spinning Disk Confocal system and Prime BSI sCMOS Camera by recording videos at 60 msec. frame rate for 20 seconds using a x40 objective. For stomach preparations, a small window without the submucosa in the middle of the preparation was used for placing a bipolar electrode on the muscle to evoke Ca^{2+} transients (train duration: 500 ms; frequency: 10 Hz; pulse duration: 0.8 ms; voltage: 120 V). For distal colon preparations, we studied myenteric ganglia located ~ 0.5 cm from the oral and aboral ends of ~ 1.5 cm long preparation. Ca^{2+} activities were evoked by electrical field stimulation (train duration: 500 ms; frequency: 10 Hz; pulse duration: 0.8 ms; voltage: 100 V) using previously published procedure(1) **[Figure 4]**. We recorded baseline Ca^{2+} activity before recording evoked Ca^{2+} transients. Ca^{2+} transients in myenteric neurons were inhibited by 1.0 µM tetrodotoxin after 2.5 to 5 minutes indicating that they were mediated by neurotransmitters. Compared with supernatants of healthy humans and normal saline, stool supernatants from both male and female T2D patients increased the number of activated nNOS myenteric neurons but decreased the amplitude of Ca^{2+} transients in soma and nerve fibers in the stomach and distal colon. L-name (200µM) increased basal Ca^{2+} intensity in nNOS myenteric neurons and elicited spiking on plateau of Ca^{2+} transients ("bursting") in preparations from recipients of supernatants from T2D patients. Contrary to our expectations, supernatants from T2D patients increased the number of activated ChAT myenteric neurons per ganglion and the amplitude of Ca^{2+} transients in soma and nerves in the stomachs

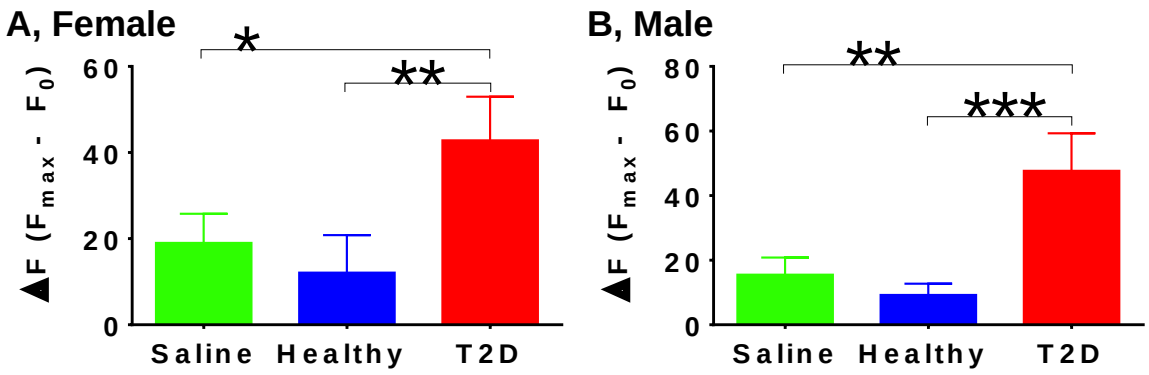

Figure 5. T2D supernatants increased the amplitude (F_{max}-F_0) of evoked Ca^{2+} transients in soma of ChAT-GCAMP6f myenteric neurons in the stomach. n= 3-4; One-way ANOVA.

and colons of recipient mice **[Figure 5]**, and the half maximum duration of Ca^{2+} transients ChAT neurons in distal colon. In addition, supernatants T2D females decreased nNOS immunoreactivity in female recipient mice, which is contrary to our hypothesis but, it indicates that toxins in supernatants damage nNOS neurons in the absence of glucose intolerance. HFD

mice, RPD, and PD patient supernatants are not discussed because they were tested in 1 or 2 mice. We are excited by these pilot results suggesting that toxins in T2D patients enhance the activities of ChAT myenteric neurons, but damage and decrease activities of nNOS myenteric neurons. This reciprocity could explain intestinal hypercontractility reported in T2D patients(9,10) and ectopic contraction seen in our pilot study.

2.1.5. Stool supernants from RPD, PD, and T2D patients trigger inflammation, decrease GI motility, and impair glucose tolerance. We determined whether stool supernatants from humans with RPD, PD, and T2D cause disruption of epithelial barrier, GI inflammation, and T2D conditions because "leaky" epithelial barrier and inflammation are linked with causing insulin resistance and T2D(11). H&E staining was used to analyze inflammation in the stomach, duodenum, ileum, and proximal colon.

Stool supernatants of T2D male patients and LPS+LTA mixture caused glucose intolerance and insulin resistance in male mice. This was not observed in female mice gavaged with stool supernatants from T2D females or male and female mice gavaged with supernatants of healthy people. Supernatants from all groups of humans and LPS+LTA reduced the rate of weight gain in mice. For this reason, we analyzed colon motility by computing the defecation rate (number of pellet expulsions in 3 hours) instead of using bead expulsion, which can be stressful due to

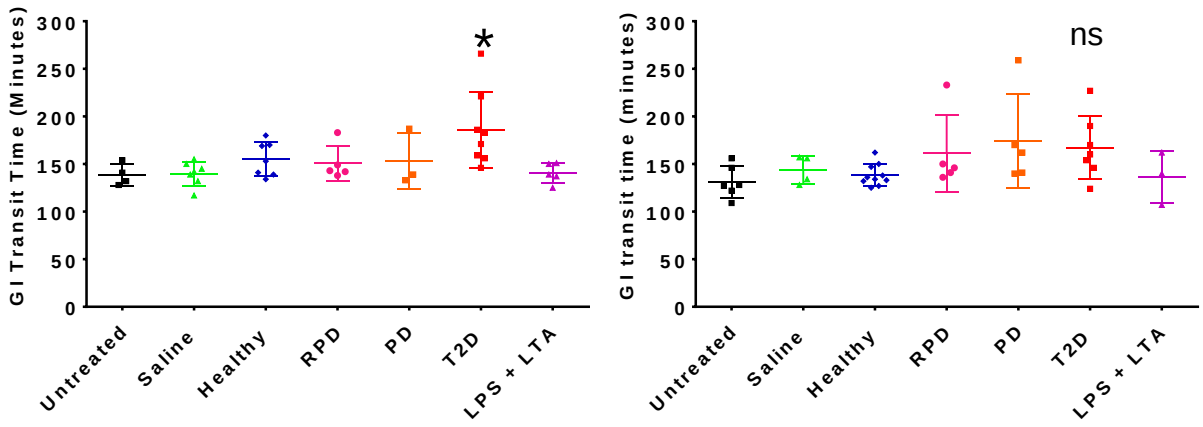

Figure 6. Supernatants from T2D patients significantly decreased GI transit in female mice compared untreated mice or mice gavage with saline (A). Supernants did not significantly reduce GI transit in male mice (B). n=3-8; One-Way ANOVA.

anesthesia. Only supernatants from female T2D decreased GI transit time **[Figures 6]**. Supernatants of RPD, PD, and T2D significantly reduced defecation rate in male mice only. However, we observed decreased Evan blue clearance from stomach in some of the mice treated with supernatants from RPD, PD, and T2D patients in both sexes when mice were euthanized two to three days after transit time measurements. Occasionally, we saw ectopic contractions causing stasis of stool in mice with delayed GI transit. LPS+LTA combined did not affect motility.

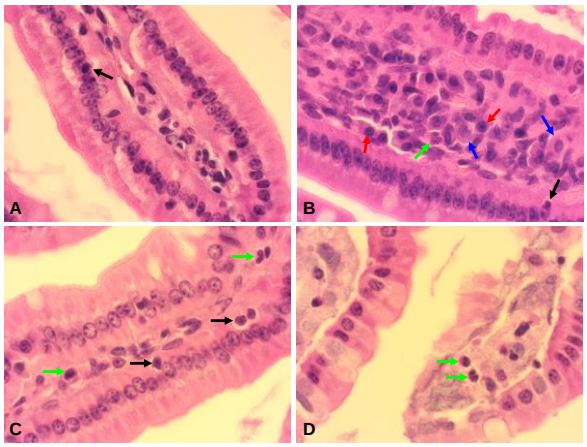

Figure 7. Duodenal H&E sections from female mice. Compared with supernatants of healthy people (A), supernatants of T2D patients (B) increased mucosal macrophages (blue arrows) and plasma cells (red arrows). LTA + LPS (C-D) increased neutrophils (green arrows) and intraepithelial lymphocytes (black arrows). X100 objective.

Stool supernatants from RPD, PD, and T2D patients caused inflammation characterized by increased macrophages and plasma cells in the lamina propria. LPS+LTA mixture elicited a neutrophil-predominant inflammation and more intraepithelial lymphocytes **[Figure 7]**. PD and T2D stool supernatants reduced gap junction staining (ZO-1 and occluding) in the duodenum and ileum. These results suggest that stool supernatants of RPD, PD and T2D have toxins that disrupt GI motility and epithelial barrier. Macrophages, lymphocytes, and neutrophils could have a role in GI neuropathy and dysmotility caused by toxins from GI content. These data indicate a critical need for extensive analyses of gastric emptying and intestinal transit and elucidating confounding factors in order to increase rigor and substantiate our findings.

2.1.6. Specific strains of the gut microbiota could be driving GI neuropathy and dysmotility in RPD, PD, and T2D patients. A recent study suggested that transplanting specific consortia of synthetic bacteria randomly isolated from T2D patients into healthy mice could trigger the onset of T2D via inflammation(12). To identify bacterial species/strains associated with T2D and whether they are linked with production of toxins in the supernatants tested above, we performed deep full-length 16S, ITS, and partial 23S rRNA gene sequencing of bacteria in stools from human donors and from mice, which received supernatants from human stool. We published a pilot study showing several strains that were more abundant in patients with PD and T2D(13). We have obtained *Odoribacter laneus* (YIT_12061) and *Flavonifractor plautii* to investigate the effects of their metabolites on duodenojejunal muscularis contractility, *ex vivo*. In addition, we used full-length 16S sequence data and the GTDB database (r207) to analyze data. By using raw abundance counts of bacteria species, we identified 13 species that discriminate T2D patients from healthy. However, due to large variation within groups, only those bacteria that attained a threshold of mean / SD ratios above 0.5 are being considered for screening for endotoxins and other toxic metabolites **[See Table 1]**.

Table 1. Raw abundance counts showing bacteria species which were found to be abundant in T2D patients compared to healthy humans.

Species	Healthy subjects			Type 2 diabetes patients		
	Mean	SD	Mean/SD	Mean	SD	Mean/SD
Negativibacillus massiliensis	8.8	11.95	0.736	200	348.508	0.574
Blautia A caecimuris	17.15	34.641	0.495	309.889	555.999	0.557
Mediterraneibacter torques	17.45	29.251	0.597	283	405.645	0.698
Schaedlerella glycyrrhizinilytica_A	4.2	7.259	0.579	64	65.827	0.972
Bacteroides ovatus	132.8	195.227	0.680	921.556	989.687	0.931
Phocaeicola vulgatus	907.75	1232.451	0.736	3686.778	3556.632	1.037

Since our last report, we have established collaboration with a new faculty in the PI department of Biological Sciences, Dr. Klas Udekwu. Dr. Udekwu is an experienced microbiologist with strong expertise in both GI microbiome and bioinformatics including machine learning. We are very thrilled by this new collaboration because it will enable to grow monocultures and mixed cultures of anaerobes, which will be identified to be abundant in RPD and PD, T2D and to have strong association with PD and T2D in his laboratory in future studies. We will then test bacterial culture supernatants on cultured duodenojejunal muscularis preparations tissue to identify bacterial cells whose transudates and cell components disrupt muscle contractions and damage tissue components. This will enable us to harvest live bacterial cells for intragastric gavage to confirm their effects through *in vivo* studies and to use bacterial culture to identify candidate toxins.

2.1.7. Sedentary Behavior and Screen Time are Associated with Human Gut Microbiome Diversity of PD and T2D patients. Physical activity and sedentary behavior affect the composition of the gut microbiome and glycemic control. We determined the associations of sedentary behavior and screen time with alpha diversity of the human gut microbiome in adults with and without impaired glycemic control. Domain specific sedentary behavior and physical activity, device specific screen time, and dietary intake data were collected via survey from 47 adults (mean ± SD: 51 ± 16 years; 38% with impaired glycemic control) who were free from gastrointestinal related illness. Hemoglobin A1c, glucose and lipid levels were collected via finger stick blood draw. Alpha diversity of the gut microbiome was determined by sequencing 16S-ITS-23S rRNA genes using stool samples. Results were expressed as observed operational taxonomic units (OTUs), Shannon Index, and Fisher's Alpha. Linear regression analyses, controlling for moderate-to-vigorous physical activity (MVPA), body mass index (BMI), blood glucose, high-density lipoprotein cholesterol (HDL), and triglycerides were used to assess associations between sedentary behavior, screen time and alpha diversity **[Figure 8]**. We found

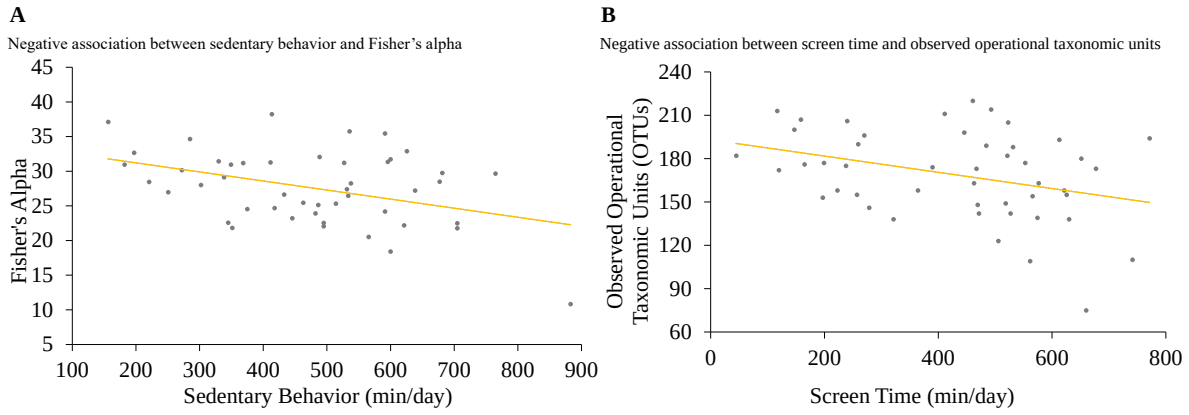

Figure 8. Higher levels of sedentary behavior and screen time are associated with lower alpha diversity of the gut microbiome.

that sedentary behavior was negatively associated with the number of observed OTUs and Fisher's Alpha. These associations were slightly attenuated but remained significant when controlling for MVPA, BMI, glucose, HDL, and triglycerides. Screen time was negatively associated with the number of observed OTUs, Shannon Index, and Fisher's Alpha; however, only the association with observed OTUs was independent of all covariates. Taken together, our findings suggest that higher levels of sedentary behavior and screen time are associated with

lower alpha diversity of the gut microbiome. This may be a potential mechanism linking sedentary behavior to increased risk of PD, T2D and cardiovascular disease. A manuscript reporting these findings will be submitted this year.

In conclusion, DIACOMP financial support enabled us to successfully implement a pilot research project, whose discoveries firmly indicate that:1) there are toxins in stool supernatants from non-diabetic HFD mice and humans with RPD, PD and T2D capable of disrupting GI motility and ENS function, and causing inflammation and glucose intolerance; 2) these toxins accumulate in gut contents before glucose intolerance, corresponding with the start of ENS injury and dysmotility; 3) chemically, the toxins are stable at room temperature and are most likely negatively charged; 4) specific species and strains of the gut microbes could be linked with these toxins and the development of T2D. There is an urgent and critical need to perform extensive studies to validate these important discoveries, identify the toxins, and their mechanisms of action. This could improve the diagnosis, prevention, and treatment of diabetes GI neuropathy and dysmotility, and diseases characterized by extra gastrointestinal neuropathy and smooth muscle injuries, which are linked to the gut microbiome or toxins from the gut.

2.8. Experiments in Progress

2.8.1 ChAT-GCaMP6f mice and nNOS-GCaMP6f mice

We have 16 ChAT-GCaMP6f mice (9 female, 7 male) undergoing stool supernant gavage and one breeding pair. We have 15 nNOS-GCaMP6f mice (10 female, 5 male) undergoing stool supernant gavage and three breeding pairs. We need to complete the analysis of videos with calcium imaging data. This includes quantifying the number of fluorescent myenteric neurons per ganglionic area, fluorescence amplitudes (Fmax- F0), fluorescence ratios (Fmax/F0), and durations of Ca^{2+} transients using established methods (14). We will also need to complete post hoc identification of imaged neurons by using immunohistochemistry (IHC) staining of nNOS, ChAT, and all neurons, and compute the total number of myenteric neurons, nNOS and ChAT neurons and density indexes.

We collected duodenal samples fixed and embedded them for Transmission Electron Microscopy. We need to complete sectioning, staining, and imaging.

2.8.2. Microbiota analysis

The analysis to be completed was highlighted above. We collected samples from all mice gavaged with stool supernatants from human subjects. A few samples were sequenced with human stool samples using the full-length 16S rRNA gene sequencing. Most of these samples have not been processed for sequencing. We will perform the analysis if we are able to secure funding.

2.8.3. Junction Potential Measurements. In the request for the extension, we aimed to determine the effects of stool transplantation on neuromuscular transmission. We did not do these experiments to focus on calcium imaging. The graduate student on the project is fully trained to independently conduct calcium imaging, which will allow the PI to do junction potential measurements beginning from July, this year.

2.8.4. Identification of toxic molecules. We shipped 6 samples to our colleague Dr. Timo Stark at Technical University Munich. Dr. Stark could not do the planned experiments because his technician left the lab. at the time when he was extensively engaged in teaching. He has a new technician who will perform initial chemical analyses in August and September, this year.

2.8.5. Immunohistochemistry staining and tight junction analysis. We collected paraffin sections at stored them at 4°C. Tissues for mRNA were collected and stored at -80°C. We will complete the staining and analysis of epithelial tight junction proteins by using ZO-1 and occludin expression by immunohistochemistry and mRNA concentrations this fall.

3. Publications:

1. Hendricks SA, Vella AC, New DD, Aunjum A, Antush M, Geidl R, Andrews RK, Balemba OB. High-Resolution Taxonomic Characterization Reveals Novel Human Microbial Strains with Potential as Risk Factors and Probiotics for Prediabetes and Type 2 Diabetes. Microorganisms 2023, 758; 11(3):758. doi: 10.3390/microorganisms11030758. PMID: 36985331 PMCID: PMC10051885.
2. Cavin JB, Wongkrasant P, Glover JB, Balemba OB, MacNaughton WK, Sharkey KA. Intestinal distension orchestrates neuronal activity in the enteric nervous system of adult mice. J Physiol. 2023 Volume 601, Issue 7, Pages 1183-1206. doi: 10.1113/JP284171. PMID: 36752210.

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2. Nyavor Y, Brands CR, Nicholson J, Kuther S, Cox KK, May G, et al. Supernatants of intestinal luminal contents from mice fed high fat diet impair intestinal motility by injuring enteric neurons and smooth muscle cells. Neurogastroenterol Motil. 2021;33(1):e13990.
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11. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56(7):1761–72.
12. Liaqat I, Ali NM, Arshad N, Sajjad S, Rashid F, Hanif U, et al. Gut dysbiosis, inflammation and type 2 diabetes in mice using synthetic gut microbiota from diabetic humans. Brazilian J Biol. 2023;83:1–14.
13. Hendricks SA, Vella CA, New DD, Aunjum A, Antush M, Geidl R, et al. High-Resolution Taxonomic Characterization Reveals Novel Human Microbial Strains with Potential as Risk Factors and Probiotics for Prediabetes and Type 2 Diabetes. Microorganisms. 2023;11(3):758.
14. Cavin JB, Wongkrasant P, Glover JB, Balemba OB, MacNaughton WK, Sharkey KA. Intestinal distension orchestrates neuronal activity in the enteric nervous system of adult mice. J Physiol. 2023;7:1183–206.