Diabetic Complications Consortium

Application Title: Role of Microbiota in the Pathogenesis of Diabetic Neuropathy

Principal Investigator: Dr. Eva Feldman

1. Project Accomplishments:

1.1. Introduction

Metabolic alterations associated with diabetes, dyslipidemia, prediabetes, and metabolic syndrome lead to serious neurological complications, including peripheral neuropathy (PN). Much effort has been undertaken to understand the pathophysiology of PN and the link between metabolic dysregulation and peripheral nerve injury. Work by our group and others using a high fat diet (HFD)-mouse model has shown that microbiota of the HFD-fed mice are significantly different than those of control littermates. This disruption in microbiota is referred to as dysbiosis and has been correlated with PN. Moreover, dietary reversal (DR) of the HFD to a standard diet (SD), or an oleate-rich monounsaturated fatty acid (MUFA) diet, rectifies the disruption in microbiota and reverses PN. Apart from a handful of association studies, the role of microbiota in PN has not been well studied or characterized. There is also a gap in our understanding of the mechanisms by which HFD-associated dysbiosis imparts nerve injury and predisposes to PN.

Our goals for the current study are to: (1) investigate the role of microbiota in mediating PN, and (2) determine the effect of microbiota on fatty acid absorption and metabolism in the gut and nerves as a candidate pathway for nerve injury. We are using 5-week-old C57BL/6J mice that receive an antibiotic cocktail for 10 days to deplete their microbiota, followed by fecal microbial transplant (FMT) from animals fed a variety of diets (**Fig. 1**). Mice are then phenotyped for any PN abnormalities. After 10 weeks of FMT inoculation (16 weeks of age), feces collected from all mouse groups undergo 16S rRNA sequencing to analyze microbiota, determination of fecal fatty acid content, and measurement of metabolic parameters (glucose, lipid profile, fatty acids, fatty acid metabolites). Nerves, colons, and ileum are harvested to determine expression of signaling proteins involved in fatty acid absorption and metabolism (Mogat2, PLA2g2e, Cyp2c). The results of this study have potential to provide novel insight into the role of microbiota in PN and the mechanism(s) by which microbiota impact nerve injury or protection. This understanding will ultimately allow us to optimally target microbiota to restore nerve function in prediabetic, diabetic, and obese subjects with PN.

1.2. Accomplishments

We successfully generated the animal model, depleted its microbiota using the proposed antibiotic protocol, and inoculated the mice with different FMT. Study groups are outlined in **Fig. 1**. We also longitudinally measured body weight and blood glucose, and at study completion performed intraperitoneal glucose tolerance testing (GTT) and nerve conduction ve-

locity (NCV) assessments for PN. Subsequently, we sacrificed all mice and harvested sciatic nerves and colons for downstream molecular analysis. Study progress and findings to date are discussed below.

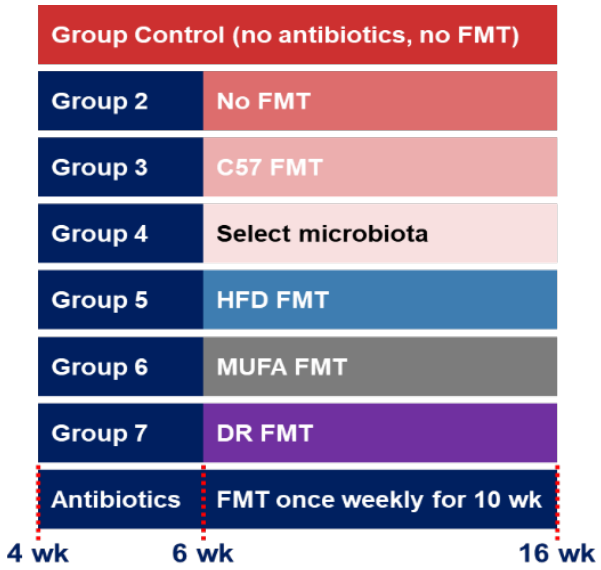

Figure 1: Study groups. Seven groups of mice (5-weeks old) were included in this study. The first group served as a control group (Group 1). All the other groups received an antibiotics cocktail for 10 days to deplete their endogenous microbiota. Subsequently, the microbiota-depleted mice were divided into 6 groups as follows: "no FMT" mice with no FMT inoculation (Group 2); "C57 FMT" mice receiving FMT of fecal pellets derived from control SD-fed mice (Group 3); "select microbiota" mice receiving FMT of a mixture of Lactobacillus acidophilus, Anaerorhabdus furcosa, Lachnoclostridium phytofermentans, and Ruminococcus bromii (Group 4); "HFD FMT" mice receiving FMT of fecal pellets derived from animals fed a HFD for 16 weeks (Group 5); "MUFA FMT" mice receiving FMT of fecal pellets derived from animals feed a MUFA diet for 16 weeks (Group 6); and "DR FMT" mice receiving FMT of fecal pellets derived from animals fed a HFD for 8 weeks followed by dietary reversal to a SD for 8 weeks (Group 7).

2. Specific Aims:

2.1. Specific Aim 1: Investigate the role of microbiota dysbiosis in mediating neuropathy.

Results: To deplete endogenous microbiota, six cohorts of mice were supplied with drinking water containing an antibiotic cocktail composed of amoxicillin (0.5 mg/mL), vancomycin (2.5 mg/mL), metronidazole (0.5 mg/mL), amphotericin B (0.025 mg/mL), and streptomycin (0.025 mg/mL) for 10 days. After depletion of microbiota, mice were divided into the following groups (**Fig. 1**): (1) "control" mice without antibiotics or FMT; (2) "no FMT" antibiotic-treated mice gavaged with phosphate buffered saline vehicle; (3) "C57 FMT" mice receiving

FMT of fecal pellets derived from untreated control SD mice; (4) "select microbiota" mice receiving FMT of a mixture of Lactobacillus acidophilus, Anaerorhabdus furcosa, Lachnoclostridium phytofermentans, and Ruminococcus bromii; (5) "HFD FMT" mice receiving FMT of fecal pellets derived from animals fed a HFD for 16 weeks; (6) "MUFA FMT" mice receiving FMT of fecal pellets derived from animals feed a MUFA diet for 16 weeks; and (7) "DR FMT" mice receiving FMT of fecal pellets from animals fed a HFD for 8 weeks followed by dietary reversal to a SD for 8 weeks. Each group included 12 mice.

Longitudinal body weight measurements showed an initial loss of body weight following microbiota depletion, but mice rapidly regained lost weight after termination of the antibiotic protocol and there were no significant differences in body weight between groups at the end of the study (**Fig. 2A**). There was similarly an initial reduction of fasting blood glucose (FBG) following microbiota depletion that recovered after termination of the antibiotics protocol, but again no significant differences in FBG between groups at the end of the study (**Fig. 2B**). Terminal GTT results were also not significantly different between groups; however, the C57 FMT group showed a slightly better insulin sensitivity relative to the other groups (**Fig. 2C**).

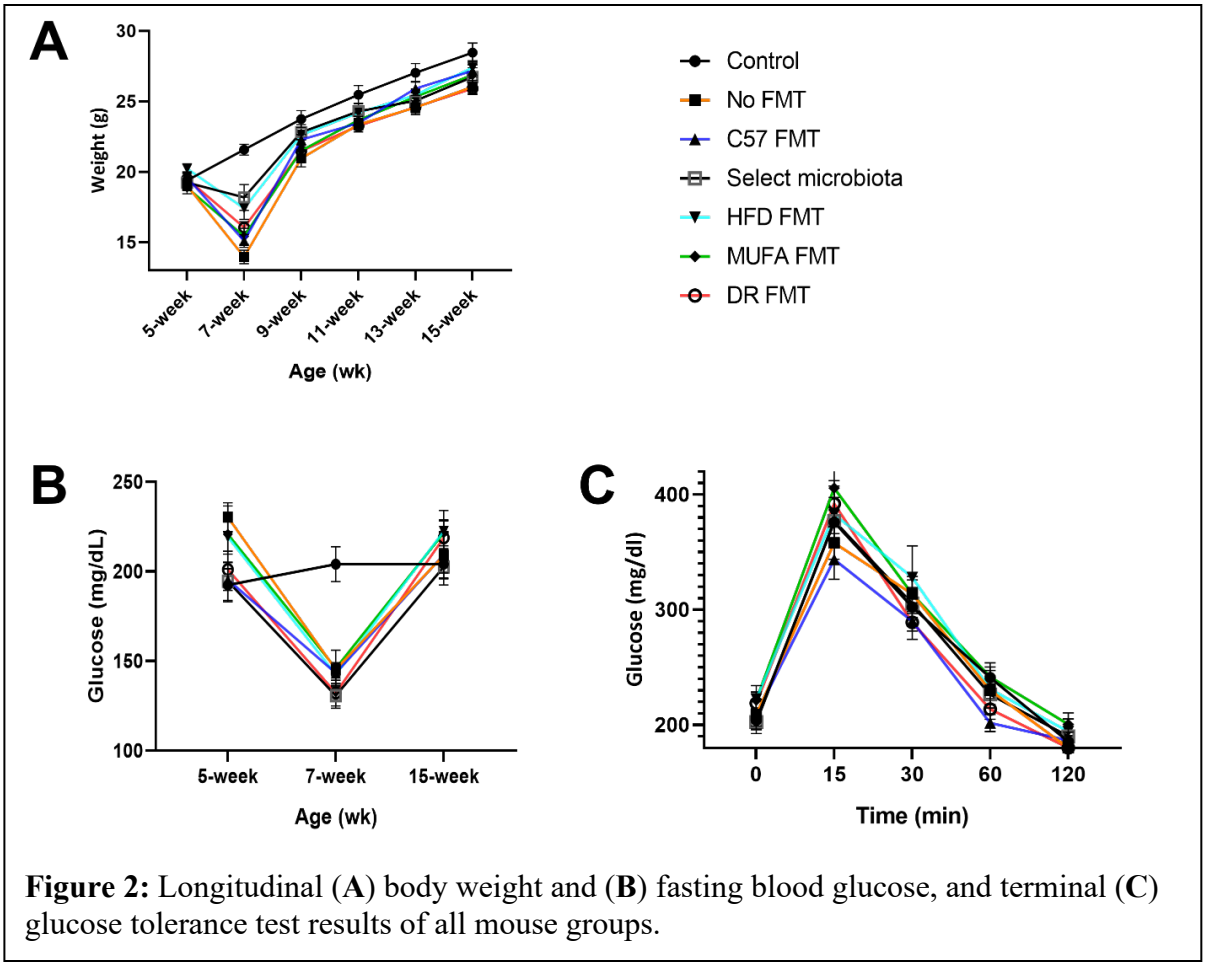

Figure 2: Longitudinal (**A**) body weight and (**B**) fasting blood glucose, and terminal (**C**) glucose tolerance test results of all mouse groups.

NCV was measured for both sensory and motor nerves, and analysis of these data is still in progress. Of note, preliminary NCV findings do not reflect significant changes between groups, suggesting that microbiota are not able to induce large fiber neuropathy (**Fig. 3**). However, microbiota may induce small fiber neuropathy, which we are investigating by assessing intraepidermal nerve fiber density (IENFD) on harvested footpad tissue that was collected from all mice. IENFD staining and quantification are ongoing. Data from the first 3 animals/group did not reflect any significant changes (**Fig. 4**), but analysis of the remaining footpads is underway and results will be reanalyzed one the dataset is complete.

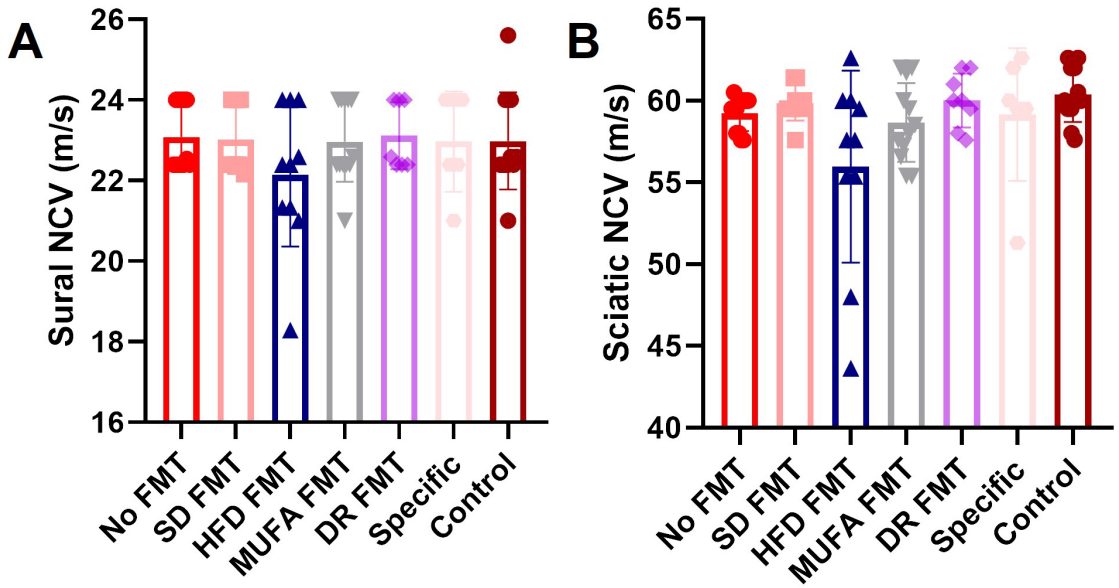

Figure 3: (**A**) Sural sensory and (**B**) sciatic motor nerve conduction velocity (NCV) for all mouse groups at 15 weeks of age.

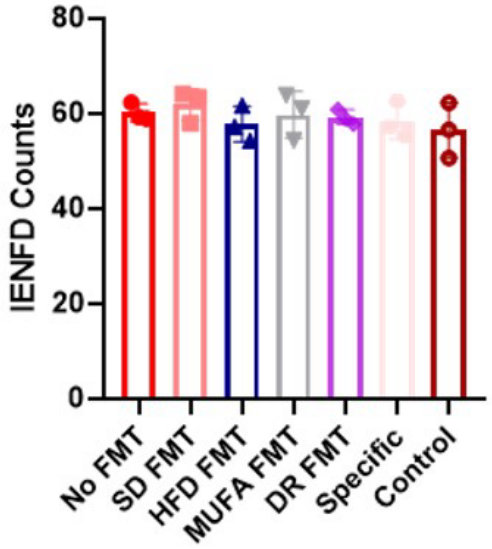

Figure 4: Intraepidermal nerve fiber density (IENFD) for n=3 mice per group.

Fecal pellets collected after microbiota depletion and at the termination of the study were submitted for 16S rRNA sequencing for microbiota identification. These data are in-hand and analysis is underway. Initial principal coordinate analysis (PCoA) results show differences in microbial species before and after FMT inoculation (**Fig. 5A**). PCoA analysis between the different mouse groups immediately after antibiotic treatment but before FMT inoculation likewise shows differences in composition in the microbiota-depleted groups versus the control non-depleted group (**Fig. 5B**). In addition, plotting microbial families reveals variations in microbial compositions following microbial depletion and following the different FMT paradigms (**Fig. 6**). Additional detailed analyses are planned, as proposed, and we additionally performed differential expression testing for microbial genera in the HFD FMT group compared to control group (**Fig. 7**) and SD FMT group (**Fig. 8**). These data show that most microbial changes induced by HFD microbiota belongs to Lachnoclosteridia and Lachnospiraceae.

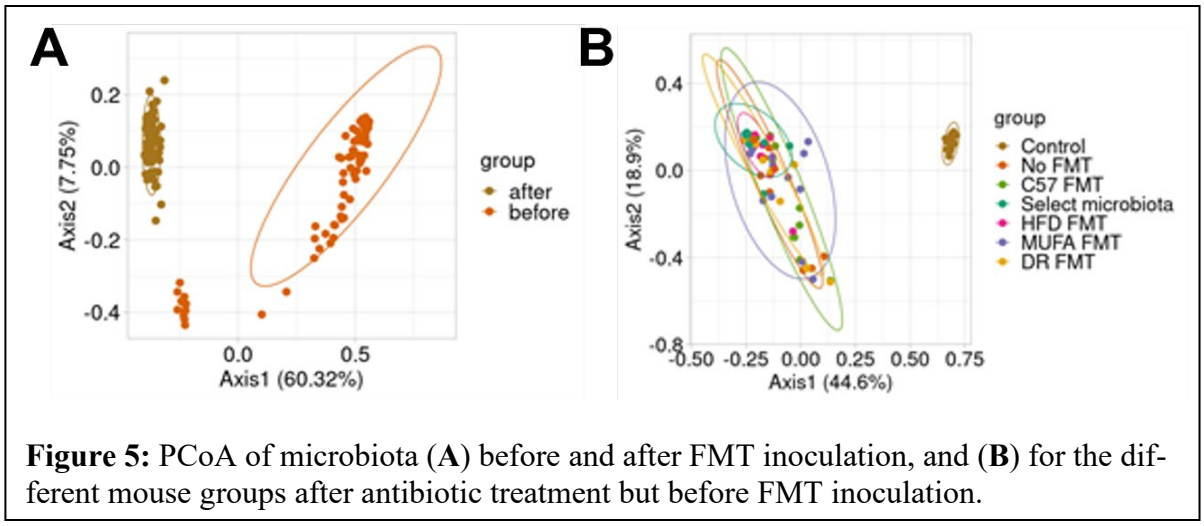

Figure 5: PCoA of microbiota (**A**) before and after FMT inoculation, and (**B**) for the different mouse groups after antibiotic treatment but before FMT inoculation.

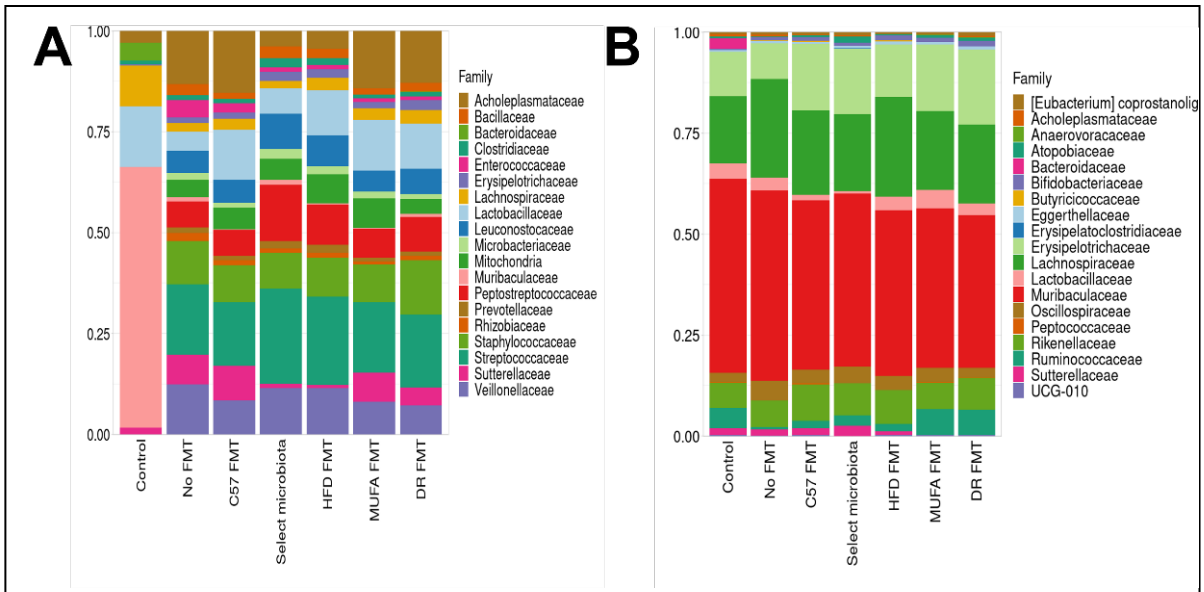

Figure 6: Bar plot of different microbial families in each mouse group after antibiotics treatment (**A**) before or (**B**) after FMT inoculation.

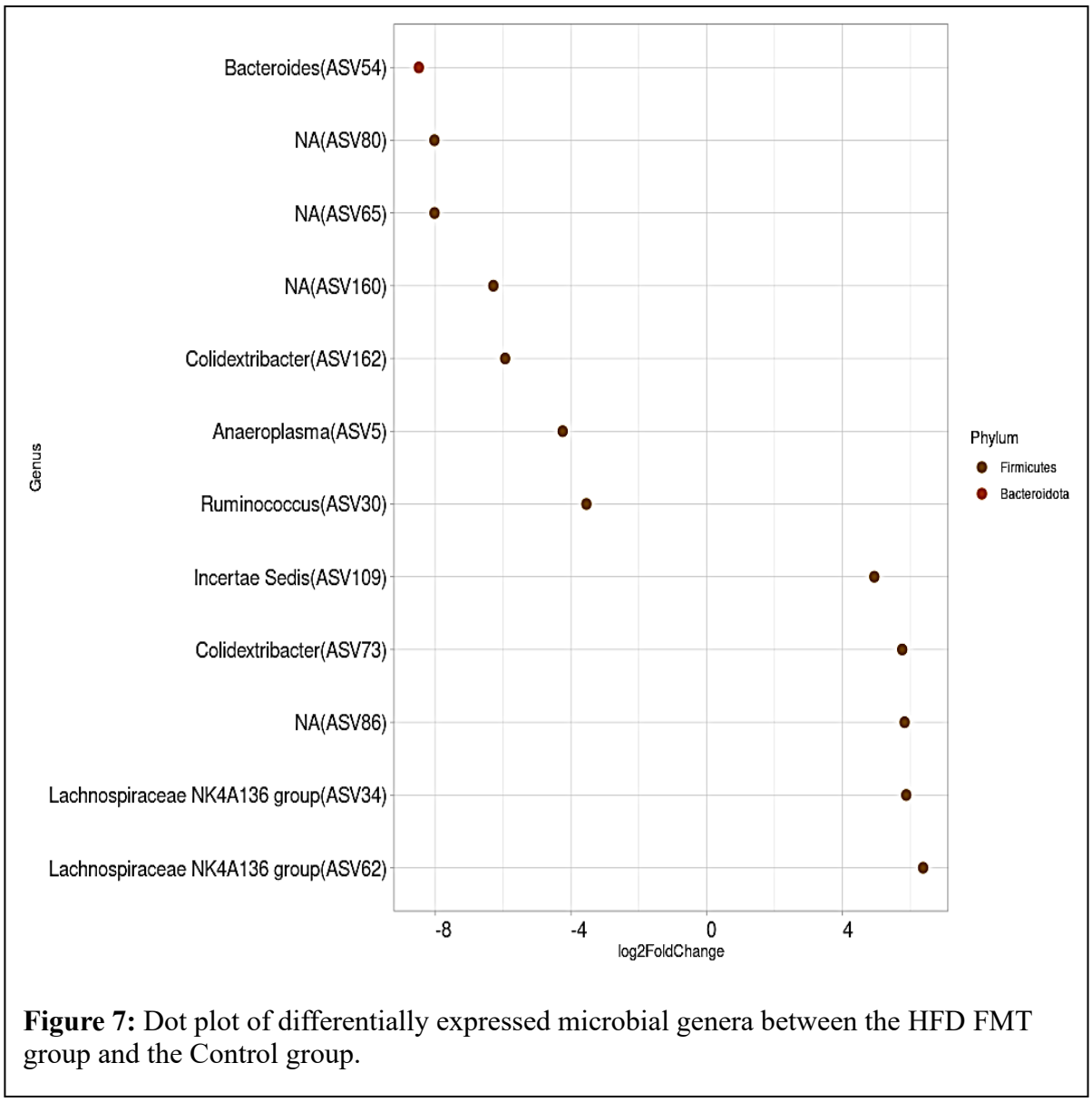

Figure 7: Dot plot of differentially expressed microbial genera between the HFD FMT group and the Control group.

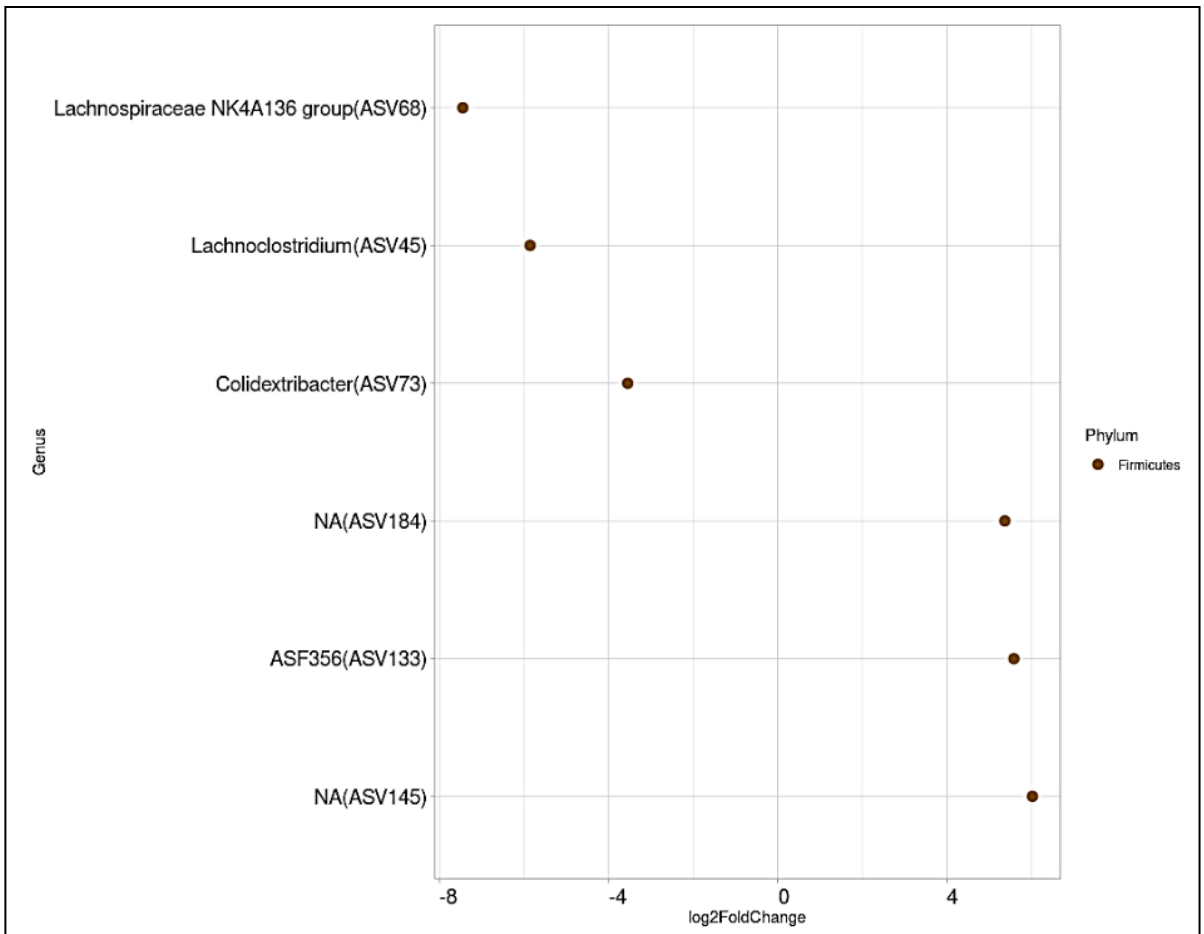

Figure 8: Dot plot of differentially expressed microbial genera between the HFD FMT group and the SD FMT group.

2.2. Specific Aim 2. Evaluate fatty acid metabolism as the mediator of microbiota dysbiosis on neuropathy.

Results: In support of Specific Aim 2, we harvested sciatic nerves and colons from all mice. We investigated the expression of proteins involved in fatty acid metabolism in the gut and sciatic nerve (Gpr41/43, FXR, GPR40). Our analysis shows that GPR40 is not well expressed in the colons or sciatic nerve; however, Gpr41/43 (**Fig. 9**) and FXR (**Fig. 10**) exhibited apparent differential expression in the sciatic nerve between different groups, although it didn't reach statistical significance. Western blot experiments on FXR protein expression in colon tissues were also completed (**Fig. 11**) and quantification is ongoing. As we are facing some technical difficulties in assessing Gpr41/43 protein expression experiments in the colons, we are alternatively using qPCR to detect Gpr41/43 mRNA colon expression using previously reported primers:

Gene	Forward	Reverse	Reference
Gpr43	5'-GGCTTCTACAGCAGCATCTA-3'	5'-AAGCACACCAGGAAATTAAG-3'	PMID: 23652017
Gpr41	5'-GTGACCATGGGGACAAGCTTC-3'	5'-CCCTGGCTGTAGGTTGCATT-3'	PMID: 23652017

Additional Western blot experiments are also ongoing to evaluate the expression of circulating and intestinal fasting-induced adipose factor (Fiaf) and proteins involved in fatty acid utilization in the nerve (e.g., MGAT2, DGAT1/2, CYP2C, PKC). Nerve diacylglycerol levels will further be quantified using a commercially available kit (ab242293, abcam).

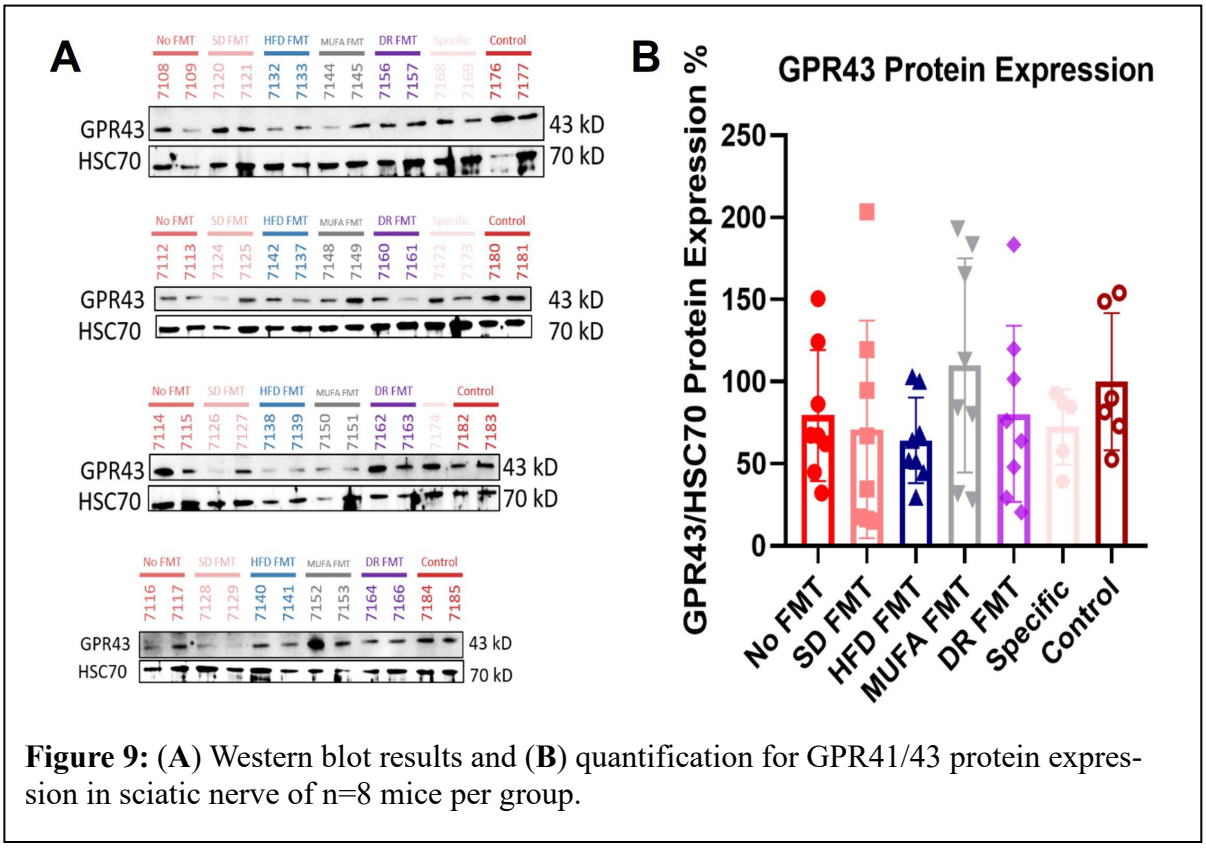

Figure 9: (**A**) Western blot results and (**B**) quantification for GPR41/43 protein expression in sciatic nerve of n=8 mice per group.

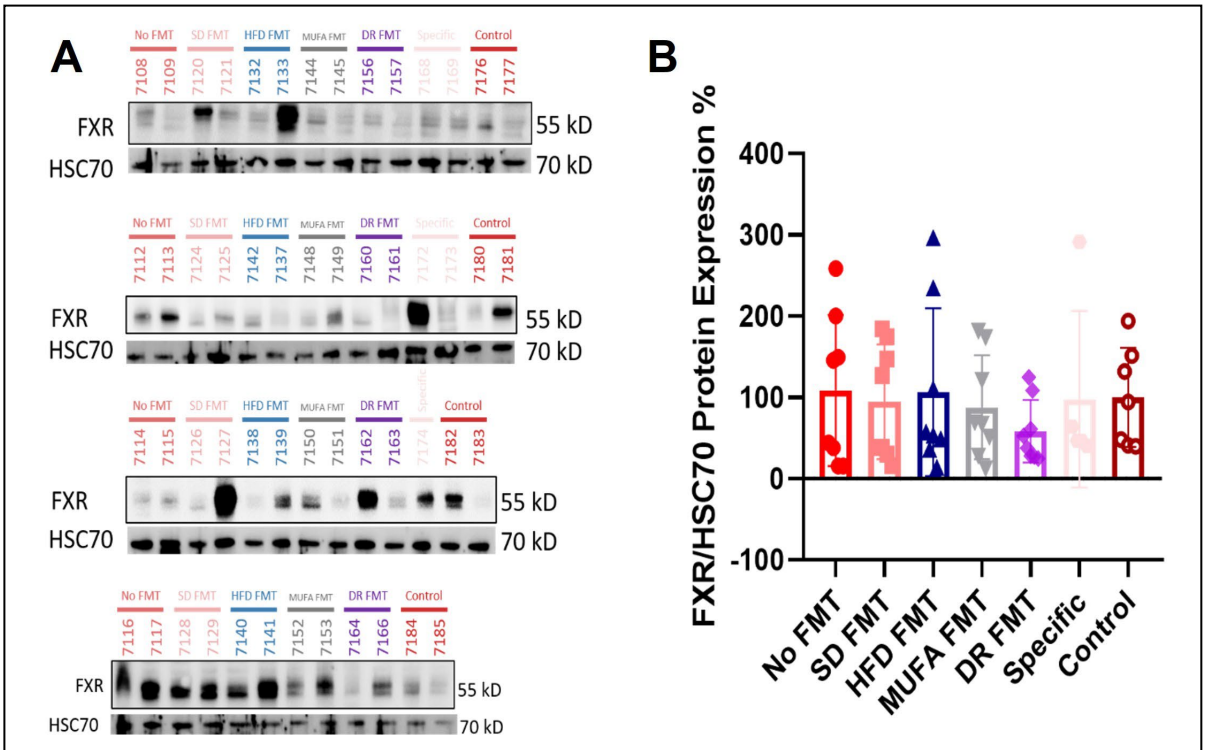

Figure 10: (**A**) Western blot results and (**B**) quantification for FXR protein expression in sciatic nerve of n=8 mice per group.

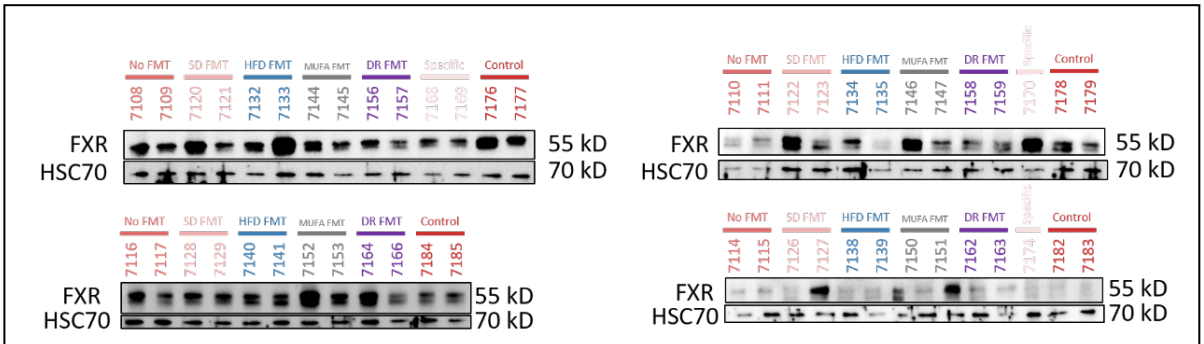

Figure 11: Western blot of FXR protein expression in colons of n=8 mice per group.

3. Publications:

Guo K, Figueroa-Romero C, Noureldein M, Hinder LM, Sakowski SA, Rumora AE, Petit H, Savelieff MG, Hur J, Feldman EL. Gut microbiota in a mouse model of obesity and peripheral neuropathy associated with plasma and nerve lipidomics and nerve transcriptomics. *Microbiome*. 11(1):52, 2023.

Henn RE, Guo K, Elzinga SE, Noureldein MH, Mendelson FE, Hayes JM, Rigan DM, Savelieff MG, Hur J, Feldman EL. Single-cell RNA sequencing identifies hippocampal microglial dysregulation in diet-induced obesity. *iScience*. 26(3):106164, 2023.

Reynolds EL, Watanabe M, Banerjee M, Chant E, Villegas-Umana E, Elafros MA, Gardner TW, Pop-Busui R, Pennathur S, Feldman EL, Callaghan BC. The effect of surgical weight loss on diabetes complications in individuals with class II/III obesity. Diabetologia. 2023 Mar 14:1-16. doi: 10.1007/s00125-023-05899-3. Online ahead of print.
PMID: 36917280

Henn RE, Elzinga SE, Glass E, Parent R, Guo K, Allouch AA, Mendelson FE, Hayes J, Webber-Davis I, Murphy GG, Hur J, Feldman EL. Obesity-induced neuroinflammation and cognitive impairment in young adult versus middle-aged mice. Immun Ageing. 2022 Dec 22;19(1):67. doi: 10.1186/s12979-022-00323-7.
PMID: 36550567

Ang L, Mizokami-Stout K, Eid SA, Elafros M, Callaghan B, Feldman EL, Pop-Busui R. The conundrum of diabetic neuropathies-Past, present, and future. J Diabetes Complications. 2022 Nov;36(11):108334. doi: 10.1016/j.jdiacomp.2022.108334. Epub 2022 Oct 7. PMID" 36306721

Savelieff MG, Chen KS, Elzinga SE, Feldman EL. Diabetes and dementia: Clinical perspective, innovation, knowledge gaps. J Diabetes Complications. 2022 Nov;36(11):108333. doi: 10.1016/j.jdiacomp.2022.108333. Epub 2022 Oct 5. PMID: 36240668

Elzinga SE, Henn R, Murdock BJ, Kim B, Hayes JM, Mendelson F, Webber-Davis I, Teener S, Pacut C, Lentz SI, Feldman EL. cGAS/STING and innate brain inflammation following acute high-fat feeding. Front Immunol. 2022 Sep 29;13:1012594. doi: 10.3389/fimmu.2022.1012594. eCollection 2022.
PMID: 36248795

Henn RE, Noureldein MH, Elzinga SE, Kim B, Savelieff MG, Feldman EL. Glial-neuron crosstalk in health and disease: A focus on metabolism, obesity, and cognitive impairment. *Neurobiol Dis.* 170:105766, 2022.