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 C57BL/6J mice (5-week-old) 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 have 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 have also longitudinally measured body weight and blood glucose, and at study completion performed intraperitoneal glucose tolerance testing (GTT) and nerve conduction

velocity (NCV) assessments for PN. Subsequently, we sacrificed all mice and harvested sciatic nerves and colons for downstream molecular analysis. Though all planned animal work is complete, we have requested a 6-month no-cost extension to conclude the final planned analyses and publish our results. Study progress and findings to date are discussed below.

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**): (i) "control" mice without antibiotics or FMT; (ii) "no FMT" antibiotic-treated mice gavaged with phosphate buffered saline vehicle; (iii) "C57 FMT" mice receiving FMT of fecal pellets derived from untreated control SD mice; (iv) "select microbiota" mice receiving FMT of a mixture of Lactobacillus acidophilus, Anaerorhabdus furcosa, Lachnoclostridium phytofermentans, and Ruminococcus bromii; (v) "HFD FMT" mice receiving FMT of fecal pellets derived from animals fed a HFD for 16 weeks; (vi) "MUFA FMT" mice receiving FMT of fecal pellets derived from animals feed a MUFA diet for 16 weeks; and (vii) "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.

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,

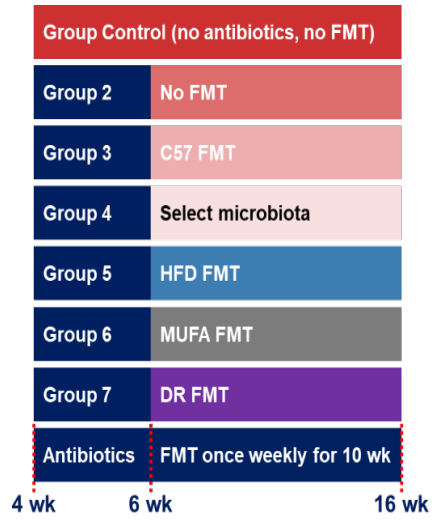

Figure 1: Study groups. Seven groups of mice (5-weeks old) were included in this study. The first group served as a control group (Group 1). All the other groups received an antibiotics cocktail for 10 days to deplete their endogenous microbiota. Subsequently, the microbiota-depleted mice were divided 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).

the C57 FMT group showed a slightly better insulin sensitivity relative to the other groups (**Fig. 2C**). 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. However, microbiota may induce small fiber neuropathy, which we will investigate by assessing intraepidermal nerve fiber density (IENFD) on harvested footpad tissue, which was collected from all mice. IENFD staining and quantification are ongoing.

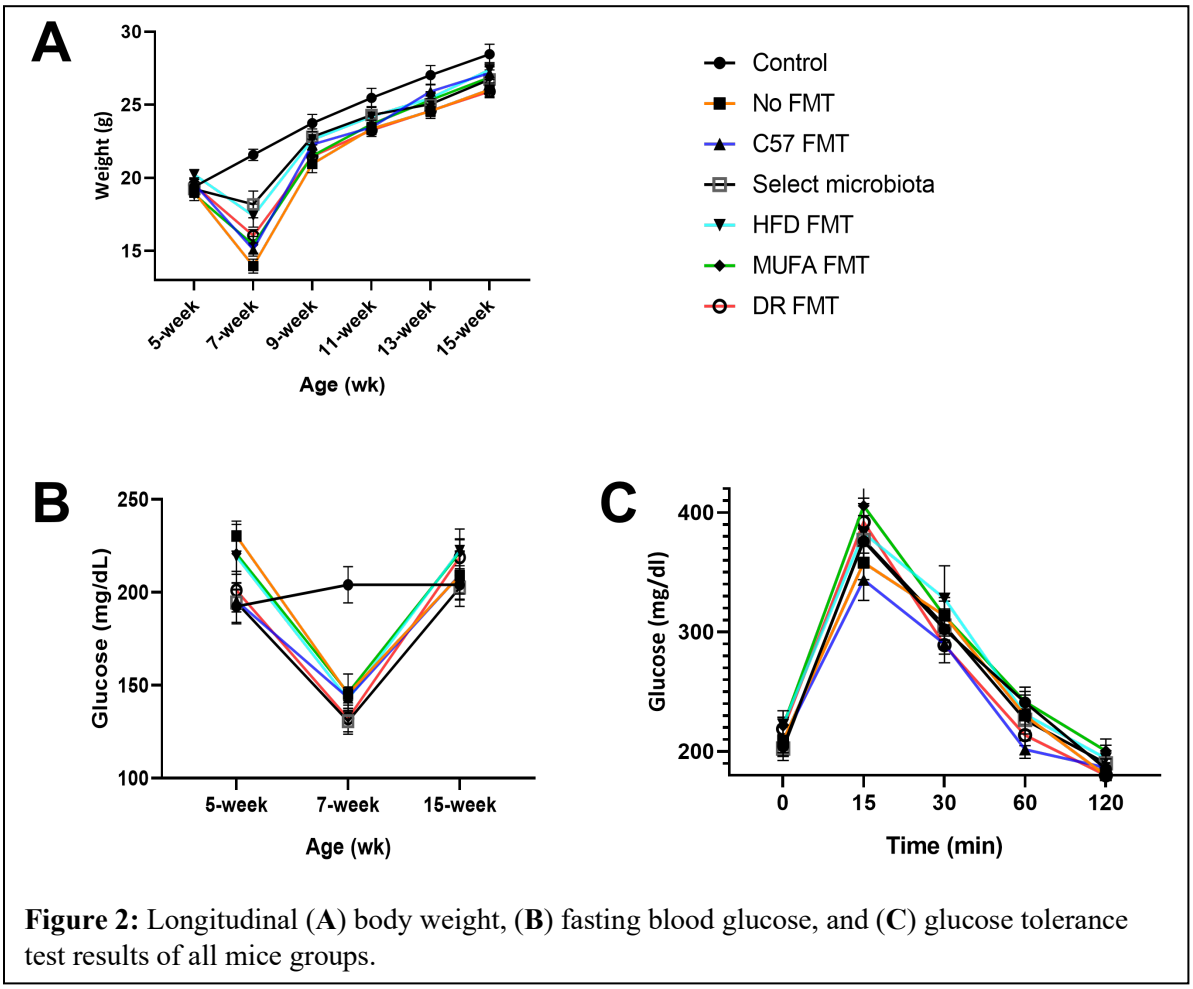

Figure 2: Longitudinal (**A**) body weight, (**B**) fasting blood glucose, and (**C**) glucose tolerance test results of all mice groups.

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. 3A**). 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. 3B**).

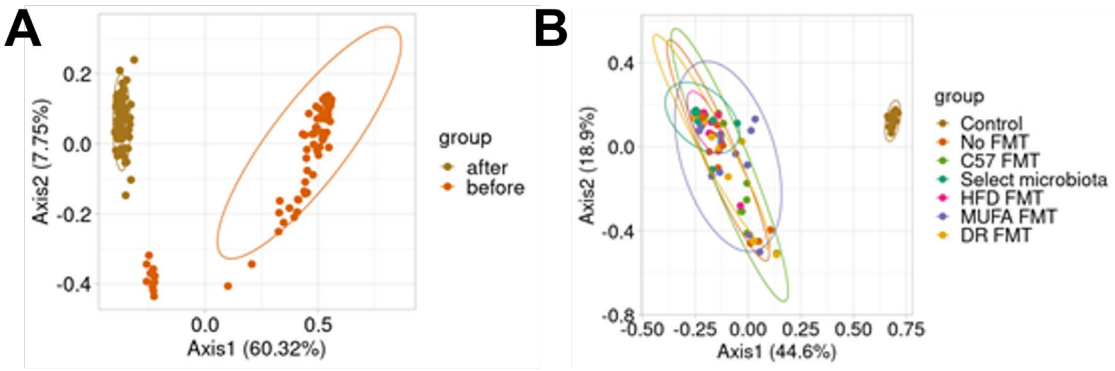

Figure 3: PCoA of microbiota (**A**) before and after FMT inoculation, and (**B**) for the different mouse groups after antibiotic treatment but before FMT inoculation.

In addition, plotting microbial families reveals variations in microbial compositions following microbial depletion and following the different FMT paradigms (**Fig. 4**). Additional detailed analyses are planned, as proposed, and will be completed during the no-cost extension period.

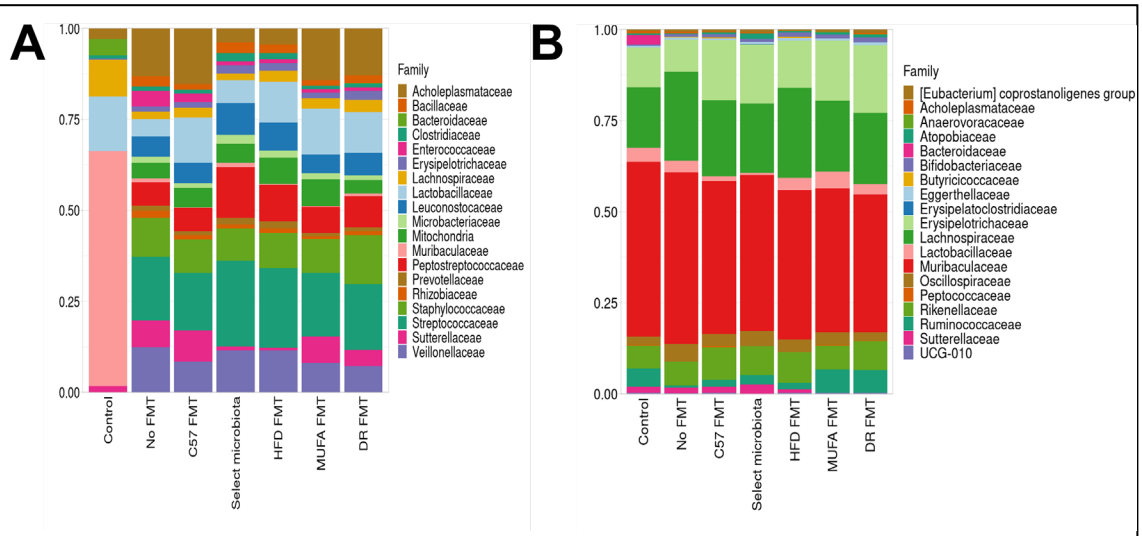

Figure 4: Bar plot of different microbial families in each mouse group after antibiotics treatment (**A**) before or (**B**) after FMT inoculation.

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. Western blot (WB) experiments are ongoing to evaluate the expression of circulating and intestinal fasting-induced adipose factor (Fiaf), proteins involved in fatty acid metabolism in the gut (Gpr41/43, FXR, GPR40, GPR120), and protein involved in fatty acid utilization in

the nerve (MGAT2, DGAT1/2, CYP2C, PKC). Nerve diacylglycerol levels will further be quantified using a commercially available kit (ab242293, abcam).

3. Publications:

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.