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INTERVENTIONAL RESEARCH PROTOCOL TEMPLATE

(HRP-503a)

STUDY INFORMATION

- **Title of Project:**
  Effect of a high-fiber supplement in Multiple Sclerosis
  IND 153352 (NBT-NM108)

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  Version 6 – 25May2022

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1.0 Research Design

1.1 Purpose/Specific Aims

A. Objectives

1) To assess the effect of a high-fiber supplement (HFS) diet on gut microbiota and immune cells in Multiple Sclerosis (MS).

2) To identify biomarkers of disease exacerbation in relapsing remitting (RR)-MS.

B. Hypotheses / Research Question(s)

Increase in consumption of high-fiber diet could be beneficial for neurodegenerative diseases including MS. Recently, our collaborator, Dr. Liping Zhao, developed a HFS and his study suggests an association between intake **of** HFS and reduced gut dysbiosis and the growth of SCFA-producing gut bacteria in Type-II diabetes mellitus (T2DM). Since MS is associated with gut dysbiosis and decreased production of SCFA, we will investigate the effect of HFS on MS associated gut dysbiosis. Currently, there is no patent present, but the submission of this patent is in process.

1.2 Research Significance

It has been suggested that dysbiosis of gut commensal bacteria is associated with increased risk of autoimmune diseases including MS. However, there is no viable intervention available to correct dysbiosis. Since, intake of HFS is associated with the growth of healthy bacteria in the gut, we propose to investigate the effect of HFS on the growth of SCFA-producing gut bacteria and development of regulatory cells in MS. If this study is successful, consumption of HFS could be beneficial therapeutic supplement to disease modifying drugs in halting MS progression and would justify the launch of a clinical trial with this HFS.

Additionally, it is necessary to find a non-invasive biomarker for gut dysbiosis-mediated CNS autoimmunity in MS. Since we found that fecal Lcn-2 is a biomarker of gut dysbiosis-mediated CNS autoimmunity in MS animal models, we will investigate the association of fecal Lcn-2 levels with disease activity in RR-MS. If this result is positive, fecal Lcn-2 could become one of the biomarkers for disease exacerbation in RR-MS.

1.3 Research Design and Methods

RR-MS patients will be recruited from the Robert Wood Johnson Center for MS under the direction of Dr. Suhayl Dhib-Jalbut. The patients will be asked to participate in the study during their regular clinical visit to the MS Center.

Inclusion criteria includes patients aged 21-65 Relapsing Remitting Multiple Sclerosis (RRMS) who met the 2017 McDonald criteria.

The first group **(Group A)** of MS patients **(n=25)** will receive a dietary fiber supplementation. They will consume 60 g/day of HFS for 12 weeks (High-fiber supplement group, Group-A). The supplement consists of a mixture of soluble fibers (from inulin and fibersol-2) and insoluble fibers (from oat bran, corn bran, wheat bran and sorghum bran) and it will be consumed as a drink.

The second group of MS patients **(Group B)** (n=25) will not consume any high fiber supplement.

The first group (**Group C**) of healthy volunteers **(n=25)** will receive a dietary fiber supplementation. They will consume 60 g/day of HFS for 12 weeks. Healthy volunteers will not have MS.

The second group **(Group D)** of healthy volunteers **(n=25)** will not consume any high fiber supplement**.**

For Groups A, B, C & D stool, and serum samples will be collected at 3 time points (baseline; Week 8; & Week 12).

Group E (n=25) will consist of 25 subjects with MS who experience an MS flare up.

These 25 subjects can be those participants with MS who are already part of the above study (Group A or B) and consent to 2 extra blood and 2 extra fecal samples collected at the time of the acute flare up and upon resolution of symptoms.

Screening visits will typically take place during routine MS follow up visit at the Robert Wood Johnson Center for MS. During this time, eligibility will be reviewed, and consent will be obtained once the patient has had time to review the study, have questions answered, and agree to participate. During this screening visit, details regarding the patients MS history, food allergies/intolerances, medical history, and medications will be recorded. Eligible participants will be asked to provide blood and stool samples for baseline assessment.

A. Research Procedures

During the baseline visit, all MS patients and HDs will provide a blood and stool sample. For MS patients and HDs who are in the HFS group, they will be provided with supplies and instructions to take the supplement (Appendix 5). All the participants will be provided with stool kits and instruction on stool collection. Stool samples will be collected at home and transported to the laboratory by the participant in Piscataway for analysis.

Group A and Group C: MS patients in Group A and HDs in Group C will consume supplement orally as a drink two times a day to ensure fiber intake of 60 g/day for 12 weeks (High-fiber supplement group, Group A), as shown in Dr. Zhao's T2DM study (1). Participants will gradually increase the dosage of supplement over several days to mitigate the potential tolerability issues,

starting with daily consumption of 30g/day for first 5 days and then increasing to targeted daily consumption of 60g/day.

A member of the research team will follow up by phone at the end of the first week to check for compliance and adverse effects and then every month thereafter (Appendix 6). Patients will receive the fiber supplement in individual sachet (30g/sachet) by mail every two weeks, and they will be asked to return any unused supplement in their next study visit. Since the risks of study drug on embryofetal development are unknown, participants in Group A and Group C will be required to use contraception methods. Please refer section 5 special considerations for more details.

At baseline, Week 8 and week 12, 6 samples (3 blood and 3 fecal sample) will be collected from each subject.

All MS patients will be followed prospectively, and one more blood and one more fecal sample will be collected during relapse (before steroid treatment) and upon symptom resolution, and examined for intestinal inflammation, permeability, and microbial composition. The concentration of intestinal inflammation biomarkers in feces including Lcn-2, Calprotectin, and Lactoferrin, and a biomarker for intestinal permeability, serum zonulin, will be measured by ELISA assay.

As a control we will also analyze these biomarkers in the fecal samples isolated from healthy donors (n=25) in **Group C** and **Group D.** Healthy donors will be asked to provide 3 blood and 3 stool samples over the course of 12 weeks (baseline, week 8, and week 12).

Group E will consist of 25 subjects with MS who experience an MS flare up.

These 25 subjects will be those participants with MS who are already part of the above study (Group A or B) and consent to 2 extra blood and 2 extra fecal samples collected at the time of the acute flare up and upon resolution of symptoms. These subjects will be asked to sign a separate consent form agreeing to be part of the MS flare up group (n=25). Since around 50% of RRMS patients relapse at least once per year, we anticipate recruiting 25 relapsing RRMS patients from Group A and B in 1 years (2). RRMS flare up and resolution will be determined by Dr. Suhayl Dhib-Jalbut.

Table-1. Schedule of specimen collection

Screening/Baseline assessment	**8 weeks post HFS**	**12 weeks post HFS**	**Unscheduled visit at MS Relapse**	**Unscheduled visit at MS remission**
MS history Medical history Medication history Food Allergy Consent Blood draw* Fecal collection*	Blood draw Fecal collection	Blood draw Fecal collection	Blood draw** Fecal collection**	Blood draw Fecal collection

*Sample collection will be done on the day of screening or before high fiber supplement.

** Sample collection will be done at MS relapse before steroid treatment.

Sample collection:

1. Stool Sample: Subjects will be asked to collect a single-stool sample without any laxative or dietary restrictions using a sample collection kit (Appendix 1). The collected stool sample will be brought to the MS research laboratory by the participants. If participants cannot bring the stool sample to the MS research laboratory, our lab staff will pick up the stool sample. The stool sample will be examined for the composition of gut bacteria. The stool sample will also be transplanted into an animal model of MS and examined for the development of experimental autoimmune encephalomyelitis (EAE).

2. Blood Draw: All participants will visit RWJ MS center to provide a blood specimen. Each subject will have a blood draw (80ml – about 5 tablespoons) as shown in Table-1. The immune cells will be isolated from each blood specimen and the presence of pathogenic and regulatory immune cells will be analyzed.

*NOTE: It is requested that all blood work be fasting. Nothing to eat or drink 10-12 hours before blood draw. Subjects may have water &/or black coffee /tea.

Fasting is very important to check the levels of short-chain fatty acid in the serum.

B. Experiment

1. Examine the effect of HFS on production of SCFA, intestinal microbial composition, and intestinal permeability

Multiple sclerosis is associated with gut dysbiosis. Notably, gut dysbiosis in MS is associated with the loss of bacterial taxa involved in fermentation of dietary fiber and the production of SCFA (3-5). Since SCFA can promote the differentiation of Foxp3Tregs, a decrease in SCFA-producing bacteria in gut microbiota would be a risk factor for MS. Recently, our collaborator, Dr. Liping Zhao at our institution, developed a HFS. His recent study suggests an association between intake of HFS and reduced gut dysbiosis and increased abundance of short chain fatty acid (SCFA)-producing gut bacteria in T2DM (1). Since increase in SCFA-producing bacteria may be beneficial for inflammatory diseases including MS, we will investigate whether HFS can promote the growth of SCFA-producing bacteria in MS patients and promote the development of regulatory immune cells.

The stool and serum will be collected at 3 time points (pre-HFS; 8 and 12 weeks after consumption of HFS) from MS patients. Similar samples will be obtained from the HFS treated and non-treated control group at similar time points. Fecal DNA will be extracted using the QIAamp PowerFecal DNA Kit (QIAGEN). DNA quality and quantity will be measured using the NanoDrop™ One microvolume UV-Vis Spectrophotometer (Thermofisher) and Qubit 1x dsDNA HS assay (ThermoFisher) respectively. Gut bacterial composition will be examined by 16S rRNA gene sequencing using the Ion GeneStudio S5 (Thermofisher) in Dr. Liping Zhao's lab. QIIME 2 will be used to analyze the sequencing data to identify the amplicon sequence variants (ASVs). ASVs will then be grouped into guilds based on their abundance correlations. Biomarkers and microbiome-based prediction models will be explored at both the ASV and guild level using statistical methods such as Random Forest and Partial Least Squares regression models. Samples of potential significance will be shortlisted for subsequent whole genome metagenomic sequencing. Concentration of fecal and serum SCFAs will be measured by Rutgers metabolomics core facility. Since Vitamin-D (Vit-D) is one of the most important nutrients that affect immune modulation (6, 7), we will also measure serum Vit-D levels.

Next, we will assess the effect of HFS on intestinal permeability. MS is associated with gut dysbiosis and intestinal permeability (3-5, 8). Therefore, we will examine gut intestinal permeability (IP) by measuring the serum levels of zonulin, which is a biomarker of IP (9), using a zonulin ELISA kit

PBMCs will be stained with anti-CD3, CD4, Foxp3, CD25, and CD127 mAbs and CD3+CD4+CD25+CD127lo Foxp3+population in a CD4 T cell compartment will be analyzed by flow cytometry using Kaluza software. Development of Th1, Th17, and Th-GMCSF will be analyzed by flow cytometry with anti-CD4, CD3, IFN-γ, IL-17A, and GM-CSF mAbs. Since low expression of E3 ubiquitin ligase genes is associated with MS disease exacerbation, we will examine the expression of E3 ubiquitin ligase genes. Naïve and memory/effector T cells will be isolated from PBMCs by naïve-memory isolation MACS beads and expression of CBLB, ITCH, and GRAIL genes will be examined by q-PCR. Since PD-1:PD-L1 signaling is important to prevent CNS autoimmunity and a fiber rich vegetarian diet can promote PD1:PD-L1 signaling (10-12), we will also study the expression of PD-1 on CD4+ T cells and PD-L1 on monocytes/macrophage and DCs using flow cytometry. PBMCs will be stained with anti-CD4, CD3, CD11c, CD11b, CD14, PD-1 and PD-L1 mAbs, and CD3+CD4+PD-1+ population in CD4+ T cells; and CD11c+PD-L1+ (DC), CD11b+PD-L1+ (macrophage) and CD14+PD-L1+ (monocyte) cell compartment will be analyzed by flow cytometry. To examine the production of inflammatory cytokines in antigen presenting cells, DCs and macrophages isolated from PBMCs will be cultured in the presence or absence of exogenous lipopolysaccharides (LPS) for 3 days and production of IL-6, IL-12, IL-1, and IL-23 will be examined by ELISA.

2. Examine the effect of HFS-induced gut microbiota on EAE in a 3A6/DR2a MS animal model:

Human microbiota-associated (HMA) mice, in which the human gut microbiota is established in germ-free mice via fecal transplantation, has been used for evaluation of pathogenic and beneficial role of gut microbiota in human health (13). Several studies have shown the usefulness of this model that can mimic the pathophysiology of human diseases such as asthma, pregnancy-induced adiposity, and obesity (14-18).

We will establish a germfree 3A6/DR2a at Taconic Inc. and expand them in our facility. Since 3A6/DR2a mice are prone to develop EAE between the age of 6 to 10 week-old, 4 week-old mice will be used for creating HMA-3A6/DR2a mice. Fecal samples will be collected from HDs and MS patients on regular diet and HFS at 3 time points (pre-treatment; 8, and 12 weeks after treatment) and the fecal sample will be transplanted into 3A6/DR2a mice (20 mice in each group). Each fecal sample (0.5 g) will be diluted in 25 mL of a sterile Ringer working buffer in an anaerobic chamber. The fecal material will allowed to settle by gravity and the clarified supernatant will be mixed with an equal volume of 20% (w/v) skimmed milk (Oxoid). Mice will be orally administered with 100 µL of fecal suspension inoculum. The development of spontaneous EAE will be monitored until the age of 20 weeks.

We will also assess the microbial composition and intestinal permeability in the transplanted mice. The fecal samples will be collected every 7 days post fecal transplantation and gut microbiota composition will be examined by sequencing of V4 region of 16S rRNA gene using iontorrent sequencer (Thermofisher) as described earlier. To measure the intestinal permeability, the transplanted mice will be administered with 150 µl of FITC dextran by oral gavage and appearance of FITC-dextran in the blood will be measured 4 h after oral inoculation. The serum FITC-dextran concentration will be measured using a fluorimeter. Since serum levels of endotoxin is also a biomarker of intestinal permeability, serum endotoxin activity will be measured using Pierce™ Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit.

We will next assess the development of pathogenic and regulatory immune cells. Three mice in each group will be euthanized at the age of 6-10 week-old and development of pathogenic Th1, Th17, and Th-GMCSF cells in intestinal lamina propria, mesenteric lymph nodes, spleen, and CNS will be examined by flow cytometry with anti-CD4, Vβ5.1, CD3, IFN-γ, IL-17A, GM-CSF mAbs. Development of Foxp3 Tregs will be examined by flow cytometry with anti-Foxp3, GITA, CD4, and Vβ5.1 mAbs. Based on our earlier findings (19), down-regulation of E3 ubiquitin ligase, CBLB and ITCH, will be examined by q-PCR.

We will also examine the development of inducible EAE in C57BL/6 mice transplanted with fecal samples. Germ-free C57BL/6 mice will be purchased from Taconic Inc. and expanded for the experiment. The fecal samples will be collected from regular diet- and HFS-treated HDs and MS patients as described above and transplanted into the germ-free C57BL/6 mice (10 in each group). The transplanted mice (7-9 week-old) will be immunized with MOG35-55 at 200 ug/mouse and injection with pertussis toxin at 100 ng/mouse. Development of EAE will be examined for 40 days. Demyelination and axonal loss in the brain and spinal cord will be examined by immunohistology

with anti-MBP and -Neurofilament Abs, respectively. Microbial composition, intestinal permeability, and development of Th-1, Th-17, Th-GMCSF and Foxp3 Tregs will be examined as described above.

3. Explore biomarkers of MS-associated gut dysbiosis and response to HFS:

RR-MS and healthy donors (21-55 year-old) from RWJ MS Centers will be asked to participate in this study according to institutional IRB guidelines. In this study, fecal and blood samples will be collected from RR-MS patients during relapse (before steroid treatment) and later during remission and examined for intestinal inflammation, permeability and microbial composition. We will enroll 25 patients based on sample size calculation using related previous studies with a statistical power of 80% (8, 20). The fecal samples (200 mg) will be dissolved in 0.5 ml PBS containing proteinase inhibitor and the supernatant will be collected. The concentration of intestinal inflammation biomarkers including Lcn-2, Calprotectin, and Lactoferrin, and a biomarker for intestinal permeability, serum zonulin, will be measured by ELISA assay. We will examine the association between increase in intestinal inflammation/permeability and disease activation.

The fecal samples collected from HFS-treated and un-treated MS patients at the 3 time points described in Aim-1, will be analyzed for intestinal inflammation and permeability as described above. We will simultaneously examine the microbial composition in the fecal samples as described in Aim-1 to determine if an association between MS relapse and gut dysbiosis exists.

C. Study Duration

Subjects participation will be 12-week period.

Study Timetable:
Upon IRB approval-Sept. 2023 Collection of blood and fecal specimens
Sept. 2021-Sept. 2023 Analysis of blood and fecal specimens
Sept. 2021-Aug. 2023 Fecal transplantation in germ free mice and analysis for EAE

D. Endpoints

The primary endpoint will be detection of effect of HFS on intestinal microbial composition, intestinal permeability, production of SCFA, intestinal permeability, and immune cells in 25 MS patients between before and after intake of high-fiber supplement.

The secondary endpoint will be detection of biomarkers for intestinal inflammation-associated disease exacerbation in 25 MS patients.

1.4 Preliminary Data

***A.* A high-fiber supplement selectively promotes a group of SCFA producing bacteria.**

Recently, our collaborator, Dr. Liping Zhao at our institution, developed a high-fiber supplement (HFS). His study suggests an association between intake of HFS and reduced gut dysbiosis and

increased abundance of short chain fatty acid (SCFA)-producing gut bacteria, and demonstrated that HFS can ameliorate type-2 diabetes mellitus (T2DM) (1). This beneficial effect was associated with increased levels of SCFA, butyric acid (Fig.1B). Furthermore, HFS promoted the growth of 15 SCFAs-producing bacteria which mostly peaked at day 28 and remained stable afterward till the end of the trial (Day 84), while only 3 acetate producers among the 15 SCFA-producing bacteria were promoted in controls (Fig.1C). To test whether HFS-promoted microbiota can contribute to glucose homeostasis, fecal samples isolated from HFS-treated (W group) and non-treated (U group) T2DM patients were transplanted into germ-free mice and glucose homeostasis was examined in the transplanted mice. As shown in Fig.2, the transplanted microbiota from HFS-treated group improved glucose homeostasis. Since increase in SCFAs-producing bacteria may be beneficial for inflammatory diseases including MS, we will investigate whether HFS can promote the growth of SCFAs-producing bacteria in MS patients and promote the development of regulatory immune cells.

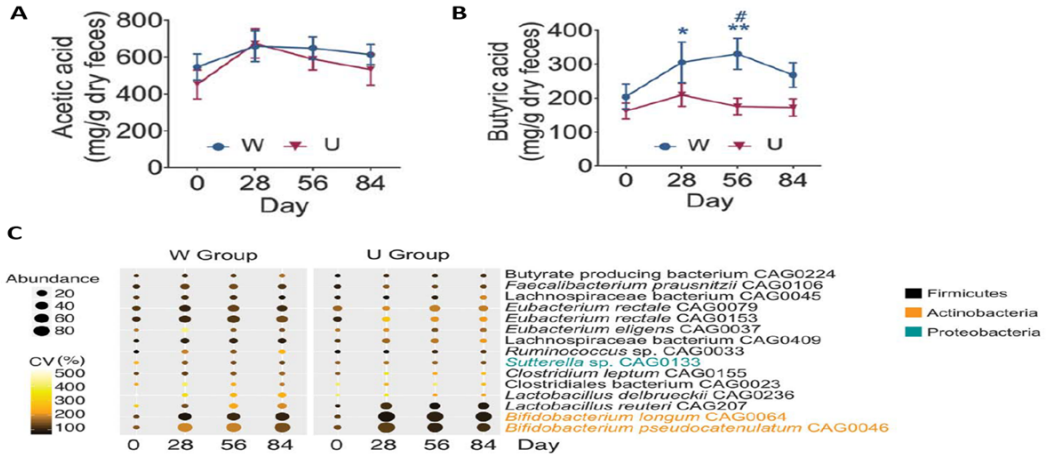

Fig. 1. A high-fiber diet selectively promotes a group of SCFA producers as the major active producers. (A and B) Changes in fecal concentrations of acetic acid and butyric acid. SCFAs were measured using gas chromatography, and amounts are expressed as milligrams per gram of dry feces (± SE). (C) Time-course changes in the abundance of the active SCFA producers. The sizes and colors of the circles indicate the average abundance and the coefficient of variance (CV) of the abundance of the strains, respectively. W group: intervention group on high-fiber diet composed of whole grains, traditional Chinese medicinal foods, and prebiotics. U group: control group consuming diet recommended based on the 2013 Chinese Diabetes Society guidelines for T2DM. Two-way repeated-measures ANOVA with Tukey's post hoc test was used for intra- and intergroup comparisons. *P < 0.05, **P < 0.01, and ***P < 0.001 for comparison with day 0; #P < 0.05 for comparison with the U group at the same time point. n = 27 patients in the W group and 16 patients in the U group.

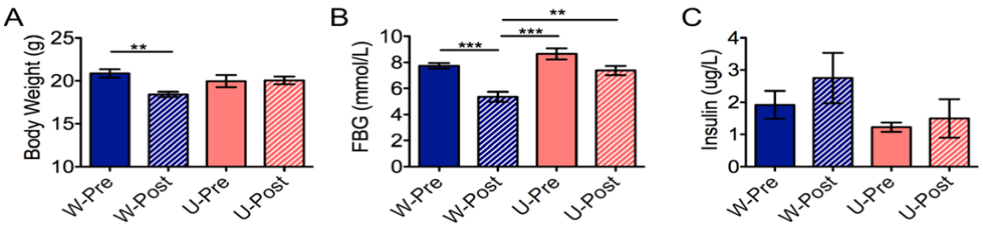

Fig. 2. Transplantation of fiber-altered gut microbiota improves glucose tolerance in mice. Fecal samples isolated from HFS-treated (W group) and non-treated (U group) T2DM patients were transplanted into germ-free mice before (W-pre and U-pre) and 84 days after treatment (W-post and U-post). Body weight (A), fasting blood glucose (FBG) (B) and fasting insulin (C) were examined 4 weeks after transplantation. Mice receiving transplant: n = 5 for W-Pre, W-Post, U-Pre and n = 4 for U-Post. *P <0.05, **P <0.01 and ***P <0.001 using one way ANOVA with a Tukey's post-hoc test for intra- and inter-group comparisons.

B. Gut-dysbiosis promotes spontaneous EAE in 3A6/DR2a mice

To examine the effect of gut microbiota on CNS autoimmunity, we created transgenic (Tg) mice that express the MS-associated HLA-DR2a gene and TCR gene specific to MBP87-99/HLA-DR2a that had been isolated from a 3A6 T cell clone derived from an MS patient (21) (Fig.3). The 3A6TCR/HLA-DR2a Tg mice were further crossed with MHC class II-KO mice to replace endogenous MHC class II with HLA-DR2a (referred to as 3A6/DR2a Tg mice). Interestingly, the MBP-specific 3A6 TCR Tg T cells do, in fact, spontaneously differentiate into GM-CSF^{+} Th1 and Th17 cells, and 20–30% of the 3A6/DR2a Tg mice develop spontaneous EAE in our SPF animal facility (Table 2) (19). Microbial infection has been suggested to be a possible etiology for MS; therefore, we examined the effect of gut microbiota on the development of spontaneous EAE. As shown in Fig. 4A, antibiotic treatment abrogated the development of spontaneous EAE, suggesting that the microbiota plays an essential role in the development of CNS autoimmunity. Gut dysbiosis, which alters the gut bacterial composition, has been associated with MS (3-5). Since fecal IgM levels increase in response to pathogenic enteric bacteria that lead to gut dysbiosis (22, 23), we examined IgM levels in fecal samples isolated from EAE and non-EAE mice. We found that increases in fecal IgM levels were associated with decreases in Foxp3 and CBLB gene expression and a subsequent development of spontaneous EAE (Fig.4B.C, and D). We then examined the microbial composition of fecal samples by 16S RNA gene analysis and found a marked expansion of *B. vulgatus, B. acidifaciens,* and *B. xylanisolvens* in the gut, which was associated with dysbiosis and the development of spontaneous EAE (Fig4.E) (19). These data suggest that gut dysbiosis is highly associated with a reduction in peripheral tolerance and the subsequent development of CNS autoimmunity. We will use this MS animal model to investigate the effect of HFS on CNS-autoimmunity.

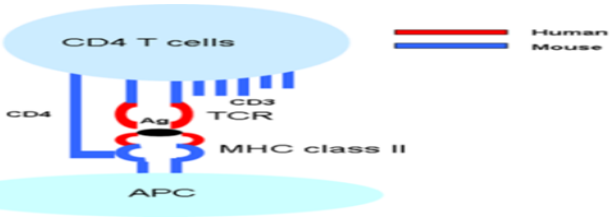

Fig.3. Schematic diagram showing the expression of MS-associated HLA-DR2a gene and TCR gene specific to MBP87-99/HLA-DR2a in Transgenic (Tg) mice.

Gender	Ascending EAE Score							Atypical EAE	Severity (Mean+/-SD)	Frequency	Onset Age (Week-old)
	1.0	1.5	2.0	2.5	3.0	3.5	4.0+				
Male	20	1	15	1	16	3	0	5	2.0+/-0.9	61/213 (28.6%)	7.3+/-2.3
Female	17	1	9	4	23	2	1	7	2.2+/-0.9	63/254 (24.8%)	8.2+/-3.1
Total	37	2	24	5	39	5	1	12	2.1+/-0.9	124/467 (26.5%)	7.8+/-2.7

Table-2. Development of spontaneous EAE in 3A6/DR2a Tg mice.

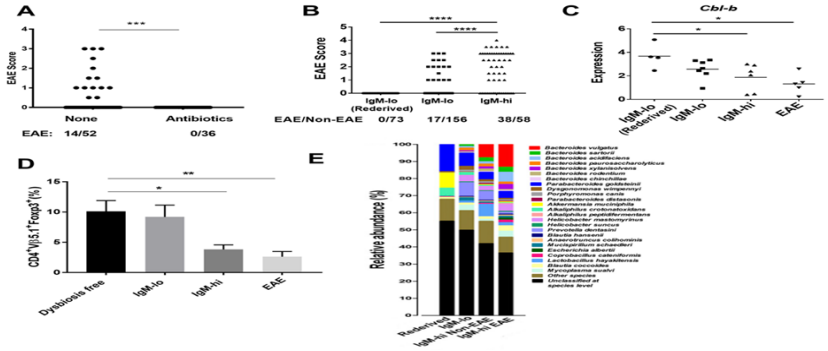

Fig. 4. Gut-dysbiosis promotes spontaneous EAE in 3A6/DR2a mice. (A) Antibiotic treatment effect on the development of spontaneous EAE. Breeding pairs of 3A6/DR2a Tg mice were treated with a cocktail of ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml), and their offspring were continuously treated with antibiotics. The development of EAE was examined until 20 weeks of age (***P<0.001). (B) Development of spontaneous EAE in fecal IgM-lo (Rederived), fecal IgM-lo, and IgM-hi mice. IgM-lo and -hi mice were determined by average fecal IgM concentration during the period from 5 weeks to 10 weeks of age. IgM-lo mice: <2.0 ng/mg total fecal protein, IgM-hi mice: ≥2.0 ng/mg total fecal protein. 3A6/DR2a Tg mice (n=214) were divided into fecal IgM-hi (n=58) and fecal IgM-lo (n=156) groups. Development of EAE was examined in fecal IgM-lo (Rederived), fecal IgM-lo, and fecal IgM-hi mice. (****P<0.0001). (C) Down-regulation of E3 ubiquitin ligase genes in $CD4^+CD25^-$ T cells is correlated with the up-regulation of the complement C3 gene in whole spleen cells. Expression of E3 ubiquitin ligase *Cbl-b* and complement C3 in $CD4^+CD25^-$ T cells and whole spleen cells, respectively, were measured by qPCR. (D) Frequency of $CD4^+$ $V\beta5.1^+$ $Foxp3^+$ T cells in the $CD4^+$ $V\beta5.1^+$ T cell compartment of 5 to 10-week-old mice is shown. Mean+/- SEM (n=3-4). (*P<0.05, **P<0.01). (E) Fecal IgM levels were examined by ELISA and fecal DNA was isolated from rederived, fecal IgM-lo (<2.0 ng/mg total protein) non-EAE, fecal IgM-hi (≥2.0 ng/mg total protein) non-EAE, and IgM-hi EAE 3A6/DR2a Tg mice. Enteric bacterial species were identified by Illumina 16S rRNA sequence analysis. n=5~7.

C. Fecal Lcn-2 is a biomarker of spontaneous EAE

We had identified a biomarker for EAE-associated gut dysbiosis. IgM is produced in response to expanded enteric bacteria in the gut (22, 23); therefore, we examined the correlation between gut dysbiosis and fecal IgM levels in 3A6/DR2a Tg mice. As shown in Fig.4B, increase in fecal IgM was associated with development of spontaneous EAE. Fecal Lcn-2 is a well-known biomarker for gut dysbiosis in IBD as described in the background (20, 24); therefore, we also examined the levels of fecal Lcn-2 in healthy and EAE 3A6/DR2a Tg mice. As we expected, there was a high association between fecal IgM and fecal Lcn-2 (Fig. 5A) and increase in Lcn-2 levels was associated with development of spontaneous EAE (Fig.5B). Interestingly, fecal IgM levels increased 1-2 weeks before onset of spontaneous EAE; while fecal Lcn-2 levels increased 3-4 weeks before disease onset (Fig. 5C), suggesting that fecal Lcn-2 is an earlier and more sensitive biomarker for CNS autoimmunity than fecal IgM. We also detected the increase in fecal Lcn-2

levels in C57BL/6 mice upon induction of EAE (Fig. 5D). These spontaneous and inducible EAE data suggest that fecal Lcn-2 may be a possible biomarker for CNS autoimmune diseases including MS. Therefore, we will investigate the effect of HFS on intestinal inflammation and association between intestinal inflammation and disease exacerbation in MS using intestinal biomarkers including Lcn-2.

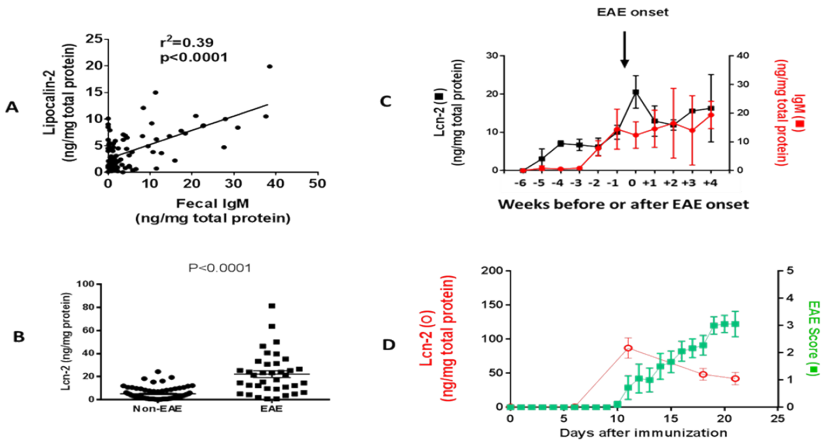

Fig.5. Fecal Lcn-2 is a biomarker of spontaneous EAE. (A)Association between fecal IgM levels and lipocalin-2 production**.** Fecal samples were collected from healthy and EAE mice, and concentration of IgM and lipocalin-2 was measured by ELISA. (B) Association between fecal Lcn-2 levels and spontaneous EAE. (C) Fecal Lcn-2 is a more sensitive biomarker for spontaneous EAE. Fecal Lcn-2 levels before and after clinical EAE onset are shown. Mean ± SEM (n = 10). (D) Increase in Lcn-2 upon EAE induction in C57BL/6 mice. EAE was induced in C57 BL/6 mice by Immunization with Mog35-55/CFA and injection with pertussis toxin. Fecal Lcn-2 levels were analyzed by ELISA.

1.5 Sample Size Justification

Our collaborator, Dr. Liping Zhao employed n = 27 in the HFS-treated group and n=16 in non-treated group and observed a significant effect of HFS on SCFA production in type 2 diabetes mellitus (T2DM) study (1). Since the therapeutic effect of HFS may be different between MS and T2DM, we sought the assistance of Dr. Yong Lin who is a Biostatistician at Rutgers University. We selected the published MS studies that showed the effect of high vegetable diet on development of Tregs (n=10 in each group in this study) (12), and calculated a reasonable sample size using mean and standard deviation of the data, assuming a mean difference of 5% type I error and 80% power. Our sample size calculation indicated that n=20 in each group is sufficient to observe the statistical difference in the development of regulatory cells. Although there is no published data on the effect of diet on intestinal permeability in MS, a study of intestinal permeability in MS (n=14) vs healthy donor (HD) (n=16) showed statistically significant increase in intestinal permeability in MS patients compared to HD (8). Our sample size calculation also indicated that n=20 in each group is sufficient to observe a statistical difference. Based on our previous HFS-data and sample size calculation, we propose to enrol 25 patients in each group in Aim-1. Regarding the sample size in Aim-3, there is no published intestinal inflammation biomarker data in MS. Therefore, we used the data from an IBD study (20) where n=20 was sufficient to detect

differences in Lcn-2, lactoferrin, and zonulin between IBD vs HD. From our sample size calculation we propose to enroll 25 patients in each group in Aim-3.

Summary:

Stool, and blood samples isolated from 50 MS patients and 50 healthy controls will be used for gut permeability, bacteria DNA sequencing, immunology experiments, and MS animal experiments.

1.6 Study Variables

A. Independent Variables, Interventions, or Predictor Variables

Experiment 1: Four groups (HDs and MS patients with and without HFS treatment).
Experiment 2: Four groups (Fecal samples from HDs and MS patients with & without HFS treatment).
Experiment 3: Two groups (MS patients with MS relapse and healthy volunteers).

B. Dependent Variables or Outcome Measures

Experiment-1: Percentage of pathogenic and non-pathogenic bacteria, intestinal permeability, and percentage of pathogenic and regulatory immune cells.
Experiment-2: Incidence and severity of EAE development.
Experiment-3: Intestinal inflammation and intestinal permeability.

1.7 Drugs/Devices/Biologics

We will treat HDs and MS patients with HFS consisting of a mixture of soluble (inulin and fibersol-2) and insoluble (oat bran, corn bran, wheat bran and sorghum bran) fibers. MS patients will consume HFS as drink to achieve a dietary fiber intake of > 60 g/day for 12 weeks.

The HFS that will be used in this study is a mixture of brans and xanthan gum (49.8% oat bran, 12.8% corn bran, 18.7% sorghum bran, 14.8% wheat bran and 4% xanthan gum w/w). The brans are the major ingredients in the wholegrain, traditional Chinese medicinal foods and prebiotic (WTP) diet that we used in reference #4 and therefore the HFS also contains a large amount of fiber of diverse physicochemical structures. This high fiber supplement has been tested in a clinical study approved by the New Brunswick/Piscataway Arts and Sciences Institutional Review Board of Rutgers University (protocol # 18-074m) and registered on ClinicalTrials.gov (NCT03334643). We showed that the HFS was well-tolerated and induced minimal changes in blood glucose in individuals who had normal glycemic control, prediabetes or type 2 diabetes. A sample of the HFS was tested for nutrient profiling, microbial load check, and stability and shelf life by an ISO 17025 accredited food nutritional laboratory (Eurofins Nutrition Analysis Center). The supplement will be produced in large quantities by an industrial partner (a food ingredient supplier). The supplement will be stored at a food-grade facility. The supplement will be contracted to a food company to be produced in large quantities and packaged into sachets (one sachet per fiber drink) by an industrial partner (a food ingredient supplier). A nutritional label will be printed on the sachets.

1.8 Specimen Collection

A. Primary Specimen Collection

A. ***What types of specimen will be collected, where, and by whom?***

1. Blood specimen will be collected by Yaritza Rosario, APN at RWJ MS center (Rutgers-Robert Wood Johnson Medical center at 125 Paterson Street Suite 6100 New Brunswick NJ 08901).

All subjects (MS patients and healthy donors) will have 3 blood samples collected at baseline, 8 weeks and 12 weeks. The amount of each blood sample will be approximately 80ml.
For subjects with MS who experience a relapse, there will be 2 more blood samples that pertain to the relapse. The first blood sample will be collected during the acute phase of the MS relapse and the second blood sample will be drawn once the patient has recovered from the symptoms of the relapse. This second blood sample can range anywhere from 1-6 months and will be at the discretion of the PI, Dr. Suhayl Dhib-Jalbut.

2. Fecal samples will be collected by participants at home. There will be 3 fecal samples collected at baseline, 8 weeks and 12 weeks.

For subjects with MS who experience a relapse, there will be 2 specific fecal samples that pertain to the relapse. The first fecal sample will be collected during the acute phase of the MS relapse and the second fecal sample will be collected once the patient has recovered from the symptoms of the relapse. This second fecal sample can range anywhere from 1-6 months and will be at the discretion of the PI, Dr. Suhayl Dhib-Jalbut.

If an enrolled MS participant has a flare up during or after study completion, they will be asked to participate in this MS relapse group (Group E) and have 2 additional blood samples and 2 additional stool samples collected. They will be asked to sign the MS relapse consent form.

B. ***How will the specimens be transported and by whom?***

1. Blood will be transported to the laboratory (Department of Neurology, 683 Hoes Lane West, Rm 166, Piscataway, NJ 08854) from RWJ MS center in New Brunswick in a private vehicle by Dr. Sudhir Kumar Yadav (IATA Refersher Completed 8/23/21) or Ms. Naoko Ito (IATA Refresher Completed 10/12/19).
2. Fecal samples will be transported by study participants to our laboratory in Piscataway (Department of Neurology, 683 Hoes Lane West, Rm 166, Piscataway, NJ 08854), and received by Dr. Sudhir Kumar Yadav. If the study participants cannot transport the fecal samples, Dr. Sudhir Kumar Yadav or Ms. Naoko Ito will pick up the fecal sample at their home.

C. ***Who will process the specimens?***

Blood and fecal samples will be processed by Dr. Sudhir K. Yadav and Ms. Naoko Ito. Aliquots of the fecal samples will be further processed for 16S rRNA gene sequencing by graduate student Emily Hanselman in Dr. Zhao's laboratory:
Lipman Hall – 76 Lipman Drive Rm 326
New Brunswick, NJ 08901-8525.

D. ***How long will the specimens be kept?***

Kept in a -80^{o}C freezer for 3 years.

The morning of the scheduled visit, subjects will be asked to collect a single-stool sample using a special container. The container containing the stool sample will be then placed in a plastic bag containing a disposable oxygen-absorbing /carbon dioxide-generating packet and then placed in an airtight box with ice packs. The container, ice packs, a disposable oxygen-absorbing /carbon dioxide-generating packet and box will be provided to each subject prior to stool collection. The collected stool sample will be brought to the MS research laboratory at Piscataway by a patient. If MS patient cannot bring the stool sample to the MS research laboratory, our lab staff will pick up the stool sample. Enough ice packs will be provided to preserve the stool in the event of delay during transport.

E. ***How will the specimens be destroyed upon study completion?***

By autoclave.

F. ***If specimens will be banked for future use, what will be the process for providing investigators with access to the bank and how will this be tracked?***

Frozen specimens will be stored at -80°C freezer and the specimens will be tracked by Dr. Yadav's computer.

1.9 Data Collection

A. Primary Data Collection- N/A

B. Secondary Data Collection – N/A

1.10 Timetable/Schedule of Events

Upon IRB approval-Sept. 2023 Collection of blood and fecal specimens.
Sept. 2021-Sept. 2023 Analysis of blood and fecal specimens.
Sept. 2021-Aug. 2023 Fecal transplantation in germ free mice and analysis for EAE.

2.0 Project Management

1.10 Research Staff and Qualifications

Dr. Suhayl Dhib-Jalbut is professor and chair of Neurology at Rutgers University. He is a neuro-immunologist who has been studying MS and its animal models for over 30 years.
Dr. Kouichi Ito is an associate professor and has been studying animal and clinical studies of Multiple Sclerosis for over 20 years.
Dr. Sudhir K Yadav is a Research Associate and has been working on gut-neuroimmunology for 10 years.
Yaritza Rosario is an advanced practice nurse and has been working on Multiple Sclerosis clinical studies for over 15 years.
Ms. Naoko Ito is a technical supporting staff and has been working on projects of clinical and animal studies for over 10 years.
Dr. Liping Zhao is the Eveleigh-Fenton Chair of Applied Microbiology in the School of Environmental and Biological Sciences and the Director of the Center for Nutrition, Microbiome and Health at the New Jersey Institute for Food, Nutrition and Health. Dr. Zhao contributes to the experimental design and leads the development of the fiber supplement. He will also oversee data analysis and interpretation.
Dr. Yan Lam is an Assistant Research Professor at the Department of Biochemistry and Microbiology and the Director of the Microbiome Core at the New Jersey Institute for Food, Nutrition and Health. With background training in clinical dietetics, Dr. Lam contributes to the experimental design and research material development. She will also oversee sample measurement and assist with data analysis and interpretation.
Dr. Guojun Wu is a Postdoctoral Research Associate of the Zhao Lab at the Department of Biochemistry and Microbiology. Dr. Wu will oversee bioinformatics and statistical analysis of the gut microbiota data.

Emily Hanselman is a graduate student of the Zhao Lab at the Department of Nutritional Sciences. She will lead sample processing and data analysis for gut microbiota profiling.

2.2 Research Staff Training

All study staff participants are familiar with research protocol, study procedures and maintaining subject confidentiality. They have each reviewed the research protocol and are familiar with study procedures due to experience from previous studies.

2.3 Resources Available

For study related purposes, the Department of Neurology, has medical resources available to collect the specimens, research laboratory to process the specimens, and animal facility to transplant the human fecal specimens to mice.

Research laboratory to process fecal sample and perform next-generation sequencing for gut microbiota profiling is available at Department of Biochemistry and Microbiology.

2.4 Research Sites

Specimen collection site: Rutgers-Robert Wood Johnson Medical School, 125 Paterson Street Suite 6100, New Brunswick NJ 08901.

Research laboratory: Rutgers-Robert Wood Johnson Medical School, Department of Neurology, 683 Hoes Lane West, Rm 166, Piscataway, NJ 08854. School of Environmental and Biological Sciences, Department of Biochemistry and Microbiology, 76 Lipman Dr, NJ 08901.

Animal facility: Rm 83, 683 Hoes Lane West, Piscataway, NJ 08854.

3.0 Multi-Center Research

N/A

4.0 Subject Considerations

4.1 Subject Selection and Enrollment Considerations

A. Method to Identify Potential Subjects

2017 McDonald criteria will be used to select relapsing remitting MS patients. Age and sex matched healthy donors will be recruited from Rutgers students/staff volunteers or New Jersey local resident using e-mail with study flyers.

B. Recruitment Details

The study will recruit both participants who have MS and Healthy Volunteers.
Subjects will be recruited at RWJ Center for Multiple Sclerosis ambulatory clinic.

Patients will be recruited from the outpatient neurology clinic of the Rutgers Robert Wood Johnson Center for MS. The patients will be asked to participate in the study during their regular clinical visit to RWJ MS Center. Only if the patient agrees to participate and meets enrollment criteria will they be enrolled in the study.

Healthy volunteers will be recruited from Rutgers students/staff volunteers or New Jersey local resident. There will be no undue influence on students / staff. Participation is completely voluntary. The healthy donor recruitment flyer will be emailed to students / staff. Those who are interested can reach out to obtain further information and be screened for eligibility.

C. Subject Screening

Subjects will be screened by Dr. Suhayl Dhib-Jalbut at RWJ MS center based on inclusion and exclusion criteria laid out in this study.

- **Inclusion Criteria**

1. Patients who meet 2017 McDonald criteria for RRMS.
2. Both males and females (age 21-65 year old).
3. Newly diagnosed and RRMS patients who are taking non-oral MS medications like interferon, Copaxone and Tysabri.
4. RRMS patients who refuse to take MS medications but are interested in other interventions.
5. Healthy volunteers without any chronic inflammatory or autoimmune diseases will be included in the study as controls.

Exclusion Criteria

1. Primary or secondary progressive MS.
2. Patients with autoimmune comorbidities.
3. Having received prior chemotherapy.
6. Pregnant women.
7. Cognitively impaired.
8. Antibiotic use within last 6 months.
9. Probiotic use within the last 2 months.

10. Self-reported allergy or intolerance to any ingredients in the fiber supplement.

110. Self-reported or diagnosed gastrointestinal disorders (such as; Irritable Bowel Syndrome, Diverticulitis, Chron's Disease)

121. Active or history of malignant tumors

4.2 Secondary Subjects

N/A

4.3 Number of Subjects

A. Total Number of Subjects

50 MS patients and 50 healthy donors will be enrolled in this study. Please refer to section "1.5 Sample Size Justification" for calculation regarding sample size.

B. Total Number of Subjects If Multicenter Study – N/A

C. Feasibility

RWJ MS Center evaluates enough newly diagnosed MS patients every year to be able to recruit 50 MS patients for the study over 1 years. 50 healthy volunteers will be recruited from Rutgers students/staff and New Jersey local resident..

4.4 Consent Procedures

A. Consent Process

- **Location of Consent Process**
  Rutgers – Robert Wood Johnson Medical Group
  RMJ Multiple Sclerosis Center,
  125 Paterson Street Suite 6100
  New Brunswick NJ 08901

- **Individual Roles for Researchers Involved in Consent**
  Dr. Suhayl Dhib- Jalbut &/ or Yaritza Rosario, APN, MSCN (study coordinator) will be the only 2 individuals who will obtain informed consent from study participants.
- **Consent Discussion Duration**
  Consent discussion will take place until the subject is fully aware of all study details and all questions have been answered. There is no set time for consent discussion.
- **Coercion or Undue Influence**
  Subjects will not be coerced to participate. Participation is voluntary and will not interfere with any current or future medical care at the MS Center.
- **Subject Understanding**

Subjects will be able to read / review the consent in its entirety. Study participation and expectations will be fully and clearly explained. The timeline of study will also be explained. Subjects will have all questions answered prior to signing of the informed consent. Subjects will receive a copy of the signed / dated informed consent.

B. Waiver or Alteration of Consent Process

N/A

C. Documentation of Consent

- **Documenting Consent**

  The study will be explained to the subject by the investigator and/or study coordinator. The subject will be allowed time to read the consent. All subject's questions will be answered. The subject and investigator and/or study coordinator will sign the consent form. A dated and signed copy will be given to the subject.

4.5 Special Consent/Populations

N/A

4.6 Economic Burden and/or Compensation for Subjects

A. Expenses

Subjects will not incur any expense

B. Compensation/Incentives

Both MS patient and healthy volunteers will be compensated in the amount of $100 for travel reimbursement.

C. Compensation Documentation

A dated log will be kept to document subject compensation.

4.7 Risks of Harm/Potential for Benefits to Subjects

A. Description of Risks of Harm to Subjects

- **Reasonably Foreseeable Risks of Harm**
  - Risk of potential loss of subject privacy due to collection of medical information.
  - The most encountered side effect of venipuncture is bruising at the site of the needle puncture up to an excessive bleeding and infection, all of which will be minimized by using a sterile technique.
  - Collection of fecal samples at home may impose inconvenience for the participants and/or their families.

- Participants may experience gastrointestinal symptoms (e.g. bloating, flatulence, diarrhea, constipation or cramping) when they consume HFS. These discomforts are common; however, these discomforts are minor and they should not last longer than a few hours.

B. Procedures which Risk Harm to Embryo, Fetus, and/or Pregnant Subjects

Pregnant females will be excluded from the study.

- **Provisions to Protect the Privacy Interests of Subjects**
  The study investigators will be utilizing Rutgers University REDCap (research electronic data capture). REDCap is a secure web application designed to support data capture for clinical studies. REDCap is a suitable method for data entry, editing, and quality assurance. This web application will be used to keep patient information secure

C. Potential Benefits to Subjects

HFS can promote the growth of healthy bacteria including SCFAs-producers in the gut, and the growth of SCFAs-producing gut bacteria can help the development of regulatory cells. Therefore, consumption of high-fiber supplement could be beneficial therapeutic supplement to disease modifying drugs in halting MS progression.

5.0 Special Considerations

5.1. Life style consideration

Since the risks of study drug on embryofetal development are unknown, participants in Group A and Group C will be required to use contraception methods.

Men and women of childbearing potential participating in this study will be required to use contraception from study recruitment until 30 days after the last day of study participation. Acceptable forms of contraception:

For men with female partners of childbearing potential:

1. Agree to use male condom.

2. Abstinence is an acceptable method only if it is consistent with the preferred and usual lifestyle of the patient

For women of childbearing potential:
1. Agree to use highly effective contraception method below:
1-1. Combined (estrogen and progesterone-containing) hormonal contraception by oral, intravaginal, or transdermal method.
1-2. Progesterone-only hormonal contraception by oral or injectable method.
1-3. Intrauterine device.
1-4. Bilateral tubal occlusion.

1-5. Vasectomized partner.
2. Abstinence is an acceptable method only if it is consistent with the preferred and usual lifestyle of the patient.

Men should refrain from donating sperm and women from donating eggs during the specified duration as well.

5.2 Health Insurance Portability and Accountability Act (HIPAA)
To gather information in regard to a subject's medical history the following information may be necessary to access. This will pertain to subjects with Multiple Sclerosis.

Information in your medical record
- Hospital discharge summaries
- Radiology records or images (MRI, CT, PET scans)
- Medical history or treatment
- Medications
- Laboratory/diagnostic tests or imaging

Healthy donors will also be asked name, gender, date of birth, and medical history (to make sure they are eligible for the study).

5.3 Family Educational Rights and Privacy Act (FERPA)
N/A

5.4 NJ Access to Medical Research Act (Surrogate Consent)
N/A

5.5 General Data Protection Regulation (GDPR)
N/A

5.6 Code of Federal Regulations Title 45 Part 46 (Vulnerable Populations)

A. Special Populations
N/A

6.0 Data Management Plan

6.1 Data Analysis

6.2 Data Security
1. The study investigators will be utilizing Rutgers University REDCap (research electronic data capture). REDCap is a secure web application designed to support data capture for clinical studies.
2. Data will be stored in password protected computers and only authorized person involved in study will have access.
3. Data will be transferred via encrypted emails and password protected portable storage device.

6.3 Data and Safety Monitoring

Data/Safety Monitoring Plan

A. Periodic Data Evaluation
Data will be accessed every month.

B. Type of Data Evaluated
1. Fecal sample 16S rRNA sequencing.
2. Gut permeability.
3. Cytokine production.
4. Development of pathogenic and regulatory immune cells.

C. Collection of Safety Information

- Details regarding allergic reactions and intolerance to ingredients of the fiber supplement.
- MS patients and HDs on HFS will be monitored for side effects such as nausea, excessive gas, loose stools, and diarrhea during first week of intervention and every month thereafter throughout the intervention.

D. Frequency of Data Collection
We will collect the research and safety data every two weeks. We will start the safety data collection when patients visit our clinical center for this study.

E. Reviewer of Data
Dr. Suhayl Dhib-Jalbut, Dr. Kouichi Ito, and Dr. Sudhir K Yadav

F. Schedule Of Review Of Cumulative Data
Data will be accessed every month.

G. Tests for Safety Data
Potential side effects by ingestion of HFS such as nausea, excessive gas, loose stools, and diarrhea will be monitored.

Dr. Dhib-Jalbut and Yaritza Rosario, APN, MSCN will analyze safety data to determine whether harm is occurring. Appropriate medical referrals will be made if necessary.

H. Suspension of Research

In the event a study participant develops a severe allergic reaction or any other adverse reaction to HFS, this individual will be required to no longer participate in the study. Also, if any personal study subject information is leaked, they will be removed from the study.

A. Data/Safety Monitoring Board Details
N/A

6.4 Reporting Results

A–C. Subjects' Results
After data analysis is complete, a scholarly paper will be submitted for publication and presented in scientific meetings

No individual results will be given.

D. Clinical Trials Registration, Results Reporting and Consent Posting-
N/A

6.5 Secondary Use of the Data
N/A

7.0 Research Repositories – Specimens and/or Data

(1) Specify the data elements and/or type(s) of specimens to be stored.

1. Specimens: Stool samples and peripheral blood mononuclear cells (PBMCs) isolated from MS patients and HDs will be stored.

2. Data: bacterial composition of the stool samples and composition of immune cell types of PBMCs will be stored.

8.0 Approvals/Authorizations

Approval from Institutional Biosafety Committee to process fecal samples in Zhao Lab has been obtained under Dr. Zhao's program of work titled "Personalized gut microbiota-targeted dietary intervention for patients with type 2 diabetes" (#17-051). Please see attached Appendix 7.

IACUC (Gut dysbiosis in MS) was approved (PROTO201900129). Appendix 8

Biosafety protocol (Isolation of peripheral blood mononuclear cells) was approved (15-006). Appendix 9

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