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Title: "Soluble Fiber Supplementation for Asthma"

Authors. Principal Investigator: Matthew Rank, M.D. (PCH); Co-Investigators: Emily Cope, Ph.D. (NAU), Kathryn A. Conn (NAU), Sophia Williams, M.D. (PCH)

Abstract:

Significance: Asthma is a complex inflammatory disease of the airways that is estimated to affect 300 million people worldwide. Incidence of asthma is steadily increasing in Western populations; an additional 100 million asthma diagnoses are anticipated by the year 2025. Asthma is a multifactorial disease affected by genetic and environmental factors. One major and potentially modifiable environmental factor is the Western diet, one high in sugar, saturated fats, and low in fiber. The Western diet influences the microbiome, which in turn, may influence inflammatory airway diseases via a gut microbiome-airway axis. The gut microbiome-airway connection is not well understood and is the focus of this study. We propose a study to modify the gut microbiome of children with asthma using prebiotic fiber supplementation (NOVELOSE™ 3490 soluble fiber-Soluble tapioca starch from Ingredion).

Hypothesis: Our hypothesis is that prebiotic dietary fiber supplementation leads to increased circulating short chain fatty acid production and improvement in asthma disease outcomes.

Key Preliminary data: Gut microbial metabolism of prebiotic dietary fiber results in production of immunomodulatory metabolites, short chain fatty acids (SCFAs; propionate, butyrate, and acetate)[1]. These SCFAs can act locally, in the gut, to promote expansion of CD4+FOXP3+ regulatory T cells (Treg), or peripherally to reduce activation of Th2-polarizing dendritic cell (DCs) in the lung.[1] In mice, microbiome-driven allergic lung inflammation was reduced by short chain fatty acids.[2] A single soluble meal challenge was shown to reduce airway inflammatory markers in a study of 17 adults with asthma.[3] Soluble fiber supplementation at 12 grams/day (about ½ of daily recommended amount of fiber) results in shifts in the gut microbiome and an increase in calcium absorption in a study of pubertal females.[4]

Synopsis of the methods: We will recruit up to 105 children, ages 6-17 years old, with asthma from the Severe Asthma Clinic or General Pulmonary Clinic at Phoenix Children's. To minimize heterogeneity, we will select patients most likely to have type-2 airway inflammation by only enrolling children who have an exhaled nitric oxide of higher than 50 parts per billion or a known history of environmental allergies. Participants will be randomly assigned (1:1) using a simple randomization scheme to ingest NOVELOSE™ 3490 soluble fiber, an amount based on estimated baseline fiber intake using the Automated Self-Administered 24-Hour Dietary Assessment tool ASA24®() in a fruit-flavored beverage or placebo (malodextrin in a similar fruit-flavored beverage) as previously described.[4] Participants will be asked to consume the prebiotic soluble fiber (or placebo) for 4 weeks alongside their normal diet and normal asthma treatments. The amount of prebiotic soluble fiber will be the target ASA24® daily fiber goal minus the estimated baseline fiber intake as estimated by the individual's estimated baseline fiber intake using the ASA24®.

A four-week consumption period was chosen based on prior studies demonstrating increased circulating short chain fatty acids and modification of the gut microbiota in that time frame[4] as well as to allow time for modification of the adaptive immune response to decrease Th2 activity in the airways. Venipuncture will be performed pre- and post-fiber intervention to measure baseline and post-intervention circulating SCFAs. We

will collect ~20 mL (two 10mL tubes) of whole blood at each time point. Serum will be frozen then shipped to PI Cope's lab for SCFA analysis. Participants will be asked to complete a dietary recall (ASA24®) pre- and post-intervention. The ASA24® is self-administered online in six steps or passes (meal-based quick list, meal gap review, detail pass, final review, forgotten foods, last chance) which have been shown to provide the highest quality and least biased self-report dietary data. Stool samples will be collected pre-, during, and post- fiber consumption and nasal wash will be collected pre-, and post- fiber consumption for microbiome and inflammatory analysis. DNA and RNA will be extracted from nasal wash specimens for microbiome analysis (DNA) and gene expression (RNA) for markers of type-2 asthma in the upper airways.[5] Fecal specimens will be collected by individual volunteers at home and mailed directly to PI Cope's lab where they will be extracted for microbial DNA sequencing. **All samples sent to PI Cope's lab will be de-identified – only relevant metadata will be shared (e.g. clinical data, age of participant, and additional relevant non-PHI information).**

Specific Aim(s)/Hypothesis

The human body is host to trillions of microorganisms, collectively termed the human microbiome. Recent technological advances have vastly expanded our understanding of the human microbiome, and it is becoming clear that the human gut microbiome impacts diverse aspects of human health. Gastrointestinal (GI) microbiome dysfunction has been directly linked with illnesses such as obesity and inflammatory bowel disease, and recent evidence suggests its involvement in allergic inflammation of the airways. Of particular interest is asthma, a chronic airway inflammatory disease that incurs a substantial healthcare burden.

Asthma is a complex inflammatory disease of the airways that is estimated to affect 300 million people worldwide. Incidence of asthma is steadily increasing in Western populations; an additional 100 million asthma diagnoses are anticipated by the year 2025. Asthma is a multifactorial disease affected by genetic and environmental factors. The rise of incidence of asthma parallels a shift toward a diet low in fiber, especially in urban low-income populations. High fiber diets have multiple health benefits, and result in reduction of allergic airway disease in mouse models.

One mechanistic hypothesis for how gut microbiota impact remote body sites involves production of metabolites from microbial degradation of food particles that enter circulation and impact remote organs such as the airways. Indeed, gut microbial metabolism of prebiotic dietary fiber results in production of immunomodulatory metabolites, short chain fatty acids (SCFAs; propionate, butyrate, and acetate). These SCFAs can act locally, in the gut, to promote expansion of CD4+FOXP3+ regulatory T cells (Treg), or peripherally to reduce activation of Th2-polarizing dendritic cells (DCs) in the lung[1].

The overall goal of this study is to determine whether prebiotic fiber supplementation leads to increased circulating SCFAs and improved clinical asthma outcomes. We propose this clinical-translational study to address precision medicine via gut microbiome manipulation. **Our Specific Aim is to evaluate changes in asthma control, type-2 airway inflammation, gut and airway microbiome, and circulating short chain fatty acids after a 4-week intervention with soluble fiber in pediatric asthma patients.**
We hypothesize that 1) fiber supplementation will increase gut microbiome diversity, lead to enrichment of carbohydrate fermenting taxa in the gut resulting in increased circulating SCFA and 2) that ingestion of soluble fiber will result in improved asthma control and reduced airway inflammation.

To complete this aim, we will recruit up to 105 children, ages 6-17 years old, with asthma from the Severe Asthma Clinic or General Pulmonary Clinic at Phoenix Children's Hospital. *Sample size justification.* The sample size for the interventional study proposed here is based on statistical power, feasibility of recruitment, and resources available. Using the same scenario as the Sample Size Justification in SA1, and using alpha diversity as a primary outcome, a total of 84 participants is required to find a significant difference of 45 units of Richness at 90% power with a small effect size (Cohen's D: 0.4). An additional outcome includes asthma control, assessed via the Asthma Control Questionnaire (ACQ). Using data from a 7-day fiber intervention in adults, we determined that n=84 participants are needed to detect changes in ACQ at 95% power with a small effect size (Cohen's D: 0.4). We will recruit up to 105 to account for attrition, variation in adherence to the study protocol, and clinical heterogeneity. This sample size is feasible (approximately 2-4 participants/month) in our clinical setting.

To minimize heterogeneity, we will select patients most likely to have type-2 airway inflammation by only enrolling children who have an exhaled nitric oxide of higher than 50 parts per billion or who have environmental allergies. Participants (or guardians) will be asked to complete the ASA24®to estimate current fiber intake. Participants will be randomly assigned (1:1) using a random number generator to ingest NOVELOSE™ 3490 soluble fiber in a fruit-flavored beverage or placebo (malodextrin in a similar fruit-flavored beverage) as previously described.[4] Soluble fiber dose nearing recommended daily levels result in shifts in the gut microbiome and increase calcium absorption in pubertal females[4]. Participants will be asked to consume the prebiotic soluble fiber (or placebo) for 4 weeks alongside their normal diet. Blood will be collected pre- and post-fiber intervention to measure baseline and post-intervention circulating SCFAs. Stool samples and nasal wash will be collected for microbiome analysis and inflammatory gene expression pre- and post- fiber consumption and SCFAs will be measured by gas chromatography (PI Cope).

The primary clinical outcome will be measured by PI Rank and the clinical team using a validated asthma questionnaire, the Asthma Control Questionnaire (ACQ), which is routinely administered in the Severe Asthma Clinic. *Secondary outcome measures* are: 1) gut and nasal microbiome composition and diversity 2) nasal inflammatory response measured via qPCR, and 3) quantification of circulating short chain fatty acids.

Participants will be recruited at Visit 1 (Week 0). At week 2, midway through the intervention, participants will be asked to collect a fecal sample at home, which will be mailed to MPI Cope's laboratory. Participants will be asked to return at Visit 2 (Week 4) for specimen collection and to return used packets to evaluate compliance.

We anticipate a biological and clinical impact of soluble fiber supplementation in individuals with Type-2 airway inflammation. The effect size of the gut microbiome response to dietary changes is large[6,7] and has been measured in a crossover study of only 10 individuals, so we anticipate to observe similar changes in the gut microbiota diversity and composition here.

Background and Significance

The human body is host to trillions of microorganisms, collectively termed the human microbiome. Recent technological advances have vastly expanded our understanding of the human microbiome, and it is becoming clear that the human gut microbiome impacts diverse aspects of human health. Gastrointestinal (GI) microbiome dysfunction has been directly linked with illnesses such as obesity and inflammatory bowel disease, and recent evidence suggests its involvement in allergic inflammation of the airways[1]. Of particular interest is asthma, a chronic airway inflammatory disease that incurs a substantial healthcare burden.

Asthma is a complex inflammatory disease of the airways that is estimated to affect 300 million people worldwide. Incidence of asthma is steadily increasing in Western populations; an additional 100 million asthma diagnoses are anticipated by the year 2025. In Arizona, Asthma prevalence has exceeded the national average for the past 10 years, with the majority of asthma diagnoses being made in Maricopa County (CDC National Health Institute Survey Public Use Data Release). Asthma is a multifactorial disease affected by genetic and environmental factors. The rise of incidence of asthma parallels a shift toward a "western" diet low in fiber, especially in urban populations. High-fiber diets have multiple health benefits, and result in reduction of allergic airway disease in mouse models[1].

Preliminary Data

Gut microbial metabolism of prebiotic dietary fiber results in production of immunomodulatory metabolites, short chain fatty acids (SCFAs; propionate, butyrate, and acetate).[1] These SCFAs can act locally, in the gut, to promote expansion of CD4+FOXP3+ regulatory T cells (Treg), or peripherally to reduce activation of Th2-polarizing dendritic cell (DCs) in the lung.[1] In mice, microbiome-driven allergic lung inflammation was reduced by short chain fatty acids.[1,2] A single soluble meal challenge was shown to reduce airway inflammatory markers in a study of 17 adults with asthma.[3] Soluble fiber supplementation at 12 grams/day (about ½ of daily recommended amount of fiber) results in shifts in the gut microbiome and an increase in calcium absorption in a study of pubertal females.[4] For this study, we will use the same dose of soluble fiber. Another study demonstrated that a low dose (6 g fiber) was sufficient to generate relevant concentrations of short chain fatty acids.[7]

Methods

Design: We will conduct a randomized (1:1) controlled, double blinded, controlled trial of 105 children with asthma.

Key definitions: Probiotics are defined as live micro-organisms that, when given in sufficient quantity, have the potential to provide a health benefit to the host. Prebiotics are defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health.[8]" Soluble fiber (NOVELOSE™ 3490), the intervention in this study, is a prebiotic. The specific changes from the soluble fiber that we anticipated seeing are an increase in the circulation of short chain fatty acids (SCFAs) which include propionate, butyrate, and acetate.

Study sample: We will recruit 6-17 year old children from the PCH outpatient pulmonary service. Drs. Rank and Williams both have asthma practices at PCH that will serve as the main method of participant recruitment. In addition, the other pulmonologists at PCH will be alerted to the study recruitment and strongly encouraged to refer interested participants to the study. The inclusion criteria are: (1) clinical diagnosis of asthma as listed as a diagnosis in the PCH medical record within the past 2 years; (2) Fractional excretion of exhaled nitric oxide (FeNO) > 50 ppb OR a clinical history of environmental allergies as defined by a positive skin prick or positive specific IgE tests to aeroallergens; (3) No emergency department or hospital visits for asthma in the past 3 months; (4) No systemic corticosteroids in the past 1 month; (5) Ability to consume a liquid drink of fiber or placebo; (6) Ability to return for a 4-6 week follow-up visit; (7) No special or unique diet as determined by PI/CO-Is. Exclusion criteria are: (1) Cystic fibrosis; (2) Bronchiectasis; (3) Change in asthma medicines other than short acting bronchodilators planned over the next 4-6 weeks (4) Baseline estimated daily fiber intake ≥ 16 grams as determined by the ASA24® (5) Sibling of a participant already enrolled in this study.

Conduct: This study will be conducted in children recruited from the PCH pulmonary clinic and in partnership with the PCH research pharmacy. The PCH research pharmacist will use a prepared simple randomization

form that will randomize each participant in a 1:1 fashion and conceal allocation from the participants and clinical investigators by using identical packaging. Participants will be asked to consume the prebiotic soluble fiber (or placebo) for 4 weeks alongside their normal diet and normal asthma treatments. The amount of fiber in the treatment arm will be determined by the difference between the estimated baseline daily intake and the recommended total daily fiber intake (based on gender and age), both of which are supplied by the ASA24®. For example, if estimated baseline fiber intake is 12 grams/day and the recommended daily fiber intake is 28 grams/day, the participant would receive 16 grams/day if they were randomized to the treatment arm. Once the amount of fiber is determined, the pharmacist will prepare daily packets containing the amount of fiber calculated, or a matching amount of placebo. The treatment packets can either be given to the study participant by the research pharmacy team on the day of enrollment, can be picked up at the clinic on a later day, or can be mailed to the participant if they live in Arizona. The research pharmacy team will provide written and verbal instruction, including counseling by phone or video if necessary.

Participants will not be allowed to change their other asthma treatments while in the study. If the participants need to change medications during the 4-week study period (other than short acting bronchodilator use), the participants will leave the study and be replaced by an additional study participant. If participants do not complete the 4-6 week follow-up visit, they will leave the study and be replaced by an additional study participant. Participants will earn remuneration ($150) based on the completion of the first and second visits, as well as receipt of samples from the home collection period. To encourage retention, the participant will receive a VIP movie pass at the last visit. Each participant will be provided an asthma action plan, as per standard of care, so that safety is not compromised. Adverse events will be captured using standard trial reporting forms.

Nasal wash will be collected during a clinic visit pre- and post- fiber intervention. Fecal specimens will be collected by the participant at home using BD collection swabs. Volunteers will be provided with a pre-paid FedEx mailer with a cold pack, biohazard bag, insulated envelope, and absorbent sheet. Volunteers will swab used toilet paper after a bowel movement using the BD swab, recap the swab, and mail the specimen to PI Cope's lab. The PMI has ~7,600 square feet in the Applied Research and Development Building on the Northern Arizona University (NAU) campus. Of these lab spaces, approximately 600 square feet are Biosafety Level 2+ (BSL-2+). Fecal and nasal samples, which may contain BSL2 pathogens, are handled in a class II type A2 biosafety cabinet, located in a dedicated area within the main lab. We also have an anaerobic chamber to grow anaerobic fiber fermenters within our BSL2 space. All study personnel have taken CITI training for human research, Bloodborne pathogens training, and shipping and receiving training.

Fecal and Nasal Wash Collection:
Nasal Wash will be collected during the clinic visit pre- and post-fiber intervention. Individual saline spray bottles (44 mL, example here) will be provided along with collection bags labeled with a study ID. Children will be instructed to use the nasal spray by the clinical team. Briefly, participants will place the tip of the squeeze bottle inside one nostril. They will tilt their head and collect the effluent from the other nostril in the collection bag.
DNA will be extracted for microbiome sequencing (bacterial and fungal) using via 16S rRNA and ITS genes. RNA will be extracted for immune analysis for Th2 genes and asthma markers *serpinb2, clca1,* and *periostin*.[5,9]

Fecal Collection – After participants are explained the project and given Informed Consent, they will be given a home fecal collection kit with a pre-paid mailer. They will take the kit home and collect a fecal swab and then mail the swab to PI Cope.

Kit contents: 1 BD swab for fecal collection, 1 Biohazard Bags, 1 ziploc bags, 1 insulated envelope, 1 ice pack, 1 prepaid mailer, and 2 absorbent sheets.

Instructions for stool sample collection:

Stool sample collection.

1. Take the red-capped Q-tip from the ziplock bag provided by the researcher

2. Following bowel movement, take the cotton Q-tip out of the labeled tube, handling it only by the plastic handle.

3. Rub the Q-tip over the used portion of the toilet paper until there is visible fecal material on the swab (Appendix A).

4. Return the sample Q-tip to the labeled tube.

5. Return the swab to the Ziploc bag.

6. Wash your hands with soap and warm water for at least 30 seconds.

7. Please write the ***date of sample collection*** on the label of the sample collection tube. **Do not write any of your personal information (such as name and birthdate) on the tube.**

Instructions for shipping:

Shipping samples

1. Following sample collection, place the ***used BD swab in the Ziploc bag*** along with the ***absorbent sheet*** in the ***biosafety bag*** provided. ***Both bags must be sealed.***

2. Place the ***Biosafety bag*** and frozen ***ice pack*** into the silver ***insulated envelope.*** Seal the envelope.

3. Place the ***sealed insulated envelope*** inside the FedEx mailer. Seal it and attach the completed address label.

4. Place the pre-paid mailer in ***any FedEx*** dropbox ***as early as possible*** to ensure next day delivery to our laboratory

Study activity	Visit #1 (baseline)	At home collection (2-3 weeks)	Visit #2 (4-6 weeks later)
Screen participants using study criteria and review informed consent form	X		

Randomization and distribution of study treatments (or placebo)	X		
Complete dietary fiber intake questionnaire	X		
Blood sample	X		X
Nasal Samples	X		X
Stool samples	X	X	X
ACQ administered	X		X
ASA24® Dietary Recall administered	X		X
Return study treatments and review for adherence to study interventions			X
Complete adverse event forms			X

Analysis: The sample size for the interventional study proposed here is based on statistical power, feasibility of recruitment, and resources available. Using alpha diversity as a primary outcome, a total of 84 participants is required to find a significant difference of 45 units of Richness at 90% power with a small effect size (Cohen's D: 0.4). An additional outcome includes asthma control, assessed via the Asthma Control Questionnaire (ACQ). Using data from a 7-day fiber intervention in adults,[63] we determined that n=84 participants are needed to detect changes in ACQ at 95% power with a small effect size (Cohen's D: 0.4). We will recruit up to 105 to account for attrition, variation in adherence to the study protocol, and clinical heterogeneity. This sample size is feasible (approximately 2-4 participants/month) in our clinical setting. A secondary outcome will be a change in the short-chain fatty acid levels (propionate, butyrate, and acetate) as measured by gas chromatography in Dr. Cope's laboratory at Northern Arizona University, comparing 4-week levels in the placebo and intervention groups, and calculated using the student's t test. Additional secondary outcomes include asthma control (measured by ACQ), respiratory microbiome (as measured from nasal wash), and adverse outcomes. ACQ is a 7-question, patient-completed questionnaire validated for children ages 6 and up.[6] ACQ is scored by taking

the total score and dividing by 7, with a range of 0.0 to 6.0. An ACQ score of < 1.5 suggests that the child's asthma is in good control, and a change of > 0.5 from measurement to measurement suggests a change that is significant (i.e. the minimum important difference). ACQ will be compared pre-and post- interventions using the paired t test. Microbiome changes will be described as in previous studies from our research team.[10,11] Briefly, alpha and beta diversity will be calculated and differences before and after fiber intervention will be analyzed using nonparametric kruskal wallis (alpha diversity) and permutational ANOVA (PERMANOVA, Bbeta diversity). Gneiss and analysis of community variance (ANCOM) will be used to determine compositional differences in the airway and gut microbiota across the cohort (unpaired data). The QIIME2 plugin q2-longitudinal samples will be used to determine differences in paired samples. Dr. Cope has extensive experience with microbiome and immune analyses. She also has access to Dr. Caporaso, Director of the Microbiome Center at PMI. His group leads the development of QIIME2, microbiome analysis software. Adverse events will be described and tabulated.

Potential problems: We have successfully used shallow shotgun metagenomics to evaluate the taxonomic composition of airway and fecal samples (publication in preparation). However, human DNA contamination in specimens with a low microbial burden can lead to low microbial sequence depth, which may impact the nasal lavage microbiome. If this occurs and shallow shotgun metagenomics becomes too costly, we will perform amplicon sequencing on samples for bacterial (16S rRNA gene) and fungal (ITS2) microbiota as described in SA1 Alternative Approaches. Based on published data and our preliminary data, we expect that the nasal specimens will be sufficient surrogates for pulmonary gene expression.[101] However, if the immune signal weak in the upper airways, we will consider alternative airway collection techniques (e.g. induced sputum, upper airway swabs) in subsequent sample collections.

Surveys will be collected using ASA24® collection software. All digital data will be stored locally on a secure server at PCH. Consent forms will be stored in a locked box or cabinet within a locked office at each site until the site Project Lead (Sophia Williams, Matthew Rank) collects them. Once collected, they will be stored in an office in a locked file cabinet. ACQ will be recorded digitally and de-identified, coded data will be sent to Dr. Cope's laboratory.

All project data will be maintained in HIPAA compliant, as well as IRB compliant, data storage facilities. Each participant will be given a unique ID. Personal information will be listed separately from study data, linked only by ID numbers. Personal data linked to study ID numbers will be maintained in secure storage both physically and electronically separate from collected data. Access to secure data files will be strictly limited to key personnel involved in the study. Data and links to identifiers will be destroyed per PCH's data retention policy.

Microbiome Related Risks: Participants will be asked the questions in the screening and asthma questionnaires provided with this submission. The questions being asked may be considered sensitive and participants may feel uncomfortable answering them. Participants are not required to answer any question that they feel uncomfortable answering.

Dietary and Asthma Control Questionnaires. Participants may feel uncomfortable answering some of the questions on the questionnaires, including identifying their socioeconomic class or racial/ethnic identity. They are informed that they can skip these questions.

Blood draw risks: The side effects of inserting needles into veins may include mild pain, bleeding, and/or bruising. Very rarely, complications such as a blood clot or an infection may occur. Some subjects may feel faint when blood is drawn.

- *Time Table:*

Query FDA for IND determination	5/01/2022-6/15/22
PCH IRB review	7/01/22-8/01/22
Funding Available (if funded)	9/29/2022
Begin study enrolment	1/01/2023
Completion of study enrolment and completion of all study visits	12/31/2027
Blood SCFA assays	1/01/2028
Stool and nasal microbiome analysis	1/01/2028
Statistical analysis and manuscript submission	7/01/2028
Submission of R01 grant proposal	10/01/28

Resources required and resource availability.

Access to pulmonary asthma patients: Drs. Williams and Rank see patients with asthma in the PCH pulmonary clinic.

Study coordinator: A study coordinator from PCH will be assigned this study and assist with study procedures. The CRC will follow up weekly by phone or text with the parents or guardians of the study participants.

Research pharmacy team: The research pharmacy team will perform randomization, allocation concealment, and dispense the study treatments. The research pharmacy team will maintain the blinded code until all patients have completed the study.

Statistical support: If needed, Drs. Rank and Williams have access to statistical support for the analysis of the study findings. Dr. Cope has access to bioinformatics support (Dr. Caporaso, PMI) if needed for microbiome analysis.

Molecular Analysis: The Pathogen and Microbiome Institute (PMI) has the sequencing capacity required (multiple MiSeq sequencers). Bioinformatic analysis will be performed on our computing cluster, Monsoon. NAU hosts a high performance computing cluster ("Monsoon"), which is available for faculty and their lab members to utilize free of charge. This cluster runs Red Hat Enterprise Linux 6 with 2928 Xeon cores, 24TB memory and 16 Nvidia GPUs (K80 and P100). There are 105 individual systems that are interconnected via FDR Infiniband at a rate of 56Gbps and <0.07us latency. Cluster nodes have access to 1.3 PB shared storage and each faculty member is allotted 10TB long term storage at no cost. Monsoon has a measured peak performance of 102 teraflops. Finally, PMI has a QuantStudio 7 qPCR machine for gene expression analysis. Dr. Koppisch (Associate Professor, Chemistry Department, NAU) will collaborate for SCFA analysis at NAU using gas chromatography.

Funding: This study is funded by the NIH/NIMHD

References from the body of the text.

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Appendices (if any). [Copy of FDA determination of IND status] (see IRB attachment)