Clinical Study Protocol

FULL PROTOCOL TITLE

Xanthohumol Microbiome and Signature (XMaS) in Healthy Adults

Randomized placebo-controlled trial of xanthohumol (24 mg/day for 8 weeks) to determine safety and biological signature in healthy adults.

Study Chairman or Principal Investigator:

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Co-Principal Investigator: Fred Stevens, PhD, Director, Linus Pauling Institute, Oregon State University

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Metagenics Inc., Gig Harbor, WA

Hopsteiner – S.S. Steiner, Inc., New York, NY

Sponsor of IND (IDE):

Fred Stevens, PhD, Oregon State University, IND#140626

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Summary of Revisions Made: Clarification of acceptable laboratory values and re-screening protocols for accessing candidate eligibility; clarification of stabilization periods for dietary supplements, changes in diet and exercise, alcohol reductions and discontinuation of Cannabis products

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PRÉCIS

Study Title

Xanthohumol Microbiome and Signature (XMaS) in Healthy Adults Trial

Objectives

The overarching objectives are to: 1. Determine safety and tolerability of xanthohumol (XN, 24 mg/day for 8 weeks) and 2. Collect data on the metabolism and biological signature of XN as a baseline for possible future studies.

Design and Outcomes

This is an 8-week triple-masked, placebo controlled, randomized clinical trial of xanthohumol (XN), which is a constituent of hops (*Humulus lupulus*) in healthy volunteers aged 21 to 50 years. Participants will complete a series of bi-weekly clinical visits and provide samples of blood, urine, and stool, plus be interviewed for adverse events.

Primary outcome measures include routine clinical toxicology measures (e.g. comprehensive metabolic panel and complete blood counts). Secondary measures include: XN metabolites, bile acid metabolism, biomarkers of inflammation and gut integrity, and composition of intestinal microbiome species.

Interventions and Duration

The intervention is xanthohumol (XN, 24 mg/day plus 288 mg of rice protein) or placebo (rice protein 288 mg/day), encapsulated in gelatin capsules, and taken by mouth once daily for 8 weeks.

Sample Size and Population

The target sample will be 32 healthy adults free of acute and chronic diseases aged 21-50 years, divided equally between XN and placebo groups.

1. STUDY OBJECTIVES

1.1 Primary Objective

The primary objective is to determine the safety and tolerability of xanthohumol in healthy adults over 8 weeks in a Phase 1 trial. The primary hypothesis is that in the XN arm, the number of participants with an adverse event or abnormal lab value will not be more than 50% higher than the number in the placebo group.

1.2 Secondary Objectives

The secondary objective is to collect comparison data for a subsequent Phase 2 trial in adults with Crohn's disease (if an acceptable safety/tolerability profile is established by the Phase 1 trial). Our primary outcome measure for this secondary objective is TNF-alpha as a biomarker of systemic inflammation. Because we are enrolling healthy adults, we hypothesize there will not be a difference in changes in TNF-alpha between XN and placebo in Phase 1. Yet the sample size provides >80% power, at a threshold of alpha=0.05, for the detection of a 25% difference in TNF-alpha changes in response to XN between the healthy adults in this trial and participants in future trials.

1.3 Tertiary Objectives

The tertiary objective is to collect comparison data related to XN metabolism, gut permeability and impact on the microbiota for a subsequent Phase 2 trial in adults with Crohn's disease. Thus our outcome measures for this objective include XN metabolites, bile acid metabolism, biomarkers of gut inflammation and gut integrity, and composition of intestinal microbiome species.

2. BACKGROUND AND RATIONALE

2.1 Background on Condition, Disease, or Other Primary Study Focus

The motivation for the current Phase 1 trial of xanthohumol (XN) in health adults is to serve as comparison data for a subsequent trial in adults with Crohn's disease (CD). Mechanistic evidence supports the therapeutic potential of XN in CD, and thus Phase 1 safety data are required prior to moving on to Phase 2 trials in CD. CD is an important source of disease burden in the United States. The Centers for Disease Control and Prevention estimates 1.3% of U.S. adults (3 million) are diagnosed with Inflammatory Bowel Disease (IBD), and the prevalence of the disease is increasing.[1] CD is the most prevalent form of IBD, characterized by predominantly small bowel inflammation (although up to 50% may also have colitis), including inflammation in the ileum. CD causes significant symptoms including diarrhea, abdominal pain, bleeding, micronutrient malabsorption, iron deficiency anemia, increased risk of colon cancer, and unintended weight loss. Medical therapy for CD is problematic and no treatment has consistently delivered success in the treatment of CD due to the episodic nature and variability in abnormality. Current treatments are costly, and associated with adverse outcomes including increased risk of lymphoma and infections.[2]

2.2 Study Rationale

The pathophysiology of CD is multi-factorial including potential genetic, environmental and microbial factors that may serve as potential triggers, and perpetuators, of the disease process. One consequence of inflammation in the ileum so common in CD is reductions in the absorption of bile acids (BA).[3,4] BAs are important agonists to the nuclear farnesoid X receptor (FXR) in the intestines, as well as in the liver. The FXR receptor is known to regulate intestinal permeability, as well as innate immunity, including production of pro-inflammatory cytokines IL-1β, IL-2, IL-6, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ).[4,5] FXR agonists have been tested in animal models and early clinical trials, and show promise for altering inflammatory signaling pathways.[4,6] Definitive clinical trials in humans with CD have not been performed.

Mechanistic evidence supports the therapeutic potential of XN in CD, and thus Phase 1 safety data are required prior to moving on to Phase 2 trials in CD. XN is a flavonoid constituent of hops (*Humulus lupulus*), with antioxidant and anti-inflammatory activities. XN acts as a prebiotic for intestinal microflora, and along with its bacterial metabolites, alters the gut microbiome.[7] In silico models suggest XN and its metabolites are receptor agonists for the FXR receptor,[8] and our preliminary in vitro data suggest XN activates FXR-regulated genes

(SREBP1, G6Pase, and PEPCK) at low concentrations, suggesting XN acts as a pharmacologic agonist of FXR (unpublished data). Additional preliminary data from our collaborators demonstrates the bioavailability of XN, including increased bioavailability of XN when combined in a rice protein matrix.[9] Thus XN has the potential to be a novel therapeutic in CD, by acting as a FXR agonist, reducing inflammation, increasing BA re-absorption, and reducing gut permeability.

3. STUDY DESIGN

This study will be an 8-week randomized, triple-masked, placebo-controlled parallel groups trial, in healthy adults aged 21 to 50 years.

The intervention is xanthohumol (XN, 24 mg/day plus 288 mg of rice protein) or placebo (rice protein 288 mg/day), encapsulated in gelatin capsules, and taken by mouth once daily for 8 weeks.

The primary objective is to determine the safety and tolerability of xanthohumol in healthy adults over 8 weeks in a Phase 1 trial. The primary hypothesis is that not more than 50% more participants receiving XN will report an adverse event or experience a laboratory abnormality compared to those receiving placebo.

The secondary objective is to collect comparison data for a subsequent Phase 2 trial in adults with Crohn's disease (if an acceptable safety/tolerability profile is established by the Phase 1 trial). Our primary outcome measure for this secondary objective is TNF-alpha as a biomarker of active inflammation. Because we are enrolling healthy adults, we hypothesize there will not be a difference in changes in TNF-alpha between XN and placebo.

The tertiary objective is to collect comparison data related to XN metabolism, gut permeability and impact on the microbiota for a subsequent Phase 2 trial in adults with Crohn's disease. Thus our outcome measures for this objective include XN metabolites, bile acid metabolism, biomarkers of gut inflammation and gut integrity, and composition of intestinal microbiome species.

4. SELECTION AND ENROLLMENT OF PARTICIPANTS

4.1 Inclusion Criteria

Participants must meet all of the following inclusion criteria to participate in this study:

- Men and women aged 21-50 years
- Willing to take isolated xanthohumol as a dietary supplement for 8 weeks
- Willing to have blood drawn bi-weekly and fast for 10-12 hours before blood draws

- Willing and able to collect bi-weekly stool samples at home
- Willing and able to collect 24-hour urine specimens prior to bi-weekly visits
- Able to speak, read, and understand English
- Must be able to provide written informed consent
- Non-smokers (including tobacco and Cannabis products, combusted or vaporized)
- For women of child-bearing potential, willingness to use an IUD or two other concurrent forms of birth control (e.g., 2 of the following categories: condoms, spermicide-containing gels, films or sponges; and/or vaginal rings) to prevent pregnancy while enrolled.

4.2 Exclusion Criteria

Any candidate meeting any of the following exclusion criteria at baseline will be excluded from study participation:

- History of any chronic disease including, but not limited to: diabetes (type 1 or 2); uncontrolled hypertension; coronary artery disease resulting in angina; cardiovascular disease requiring percutaneous coronary intervention (PCI), bypass, or past myocardial infarction or stroke; blood disease including current anemia; cancer (except non-melanoma skin cancer) within the last year or still requiring chemotherapy or hormonal therapy; chronic kidney disease; liver disease including viral hepatitis, non-alcoholic fatty liver disease, or alcoholic hepatitis/cirrhosis; any immunocompromising condition including human immunodeficiency virus/acquired immunodeficiency syndrome or organ transplant requiring anti-rejection medications; chronic osteoarthritis requiring joint replacement or daily use of NSAIDs; chronic endocrine condition including but not limited to: Cushing's, Addison's, Hashimoto's thyroiditis, Grave's disease, etc.
- Body Mass Index (BMI) less than 20 (underweight) or greater than 30 (obese)
- Consumption of more than 1 beer per day. Candidates will be given the option to stabilize intake to this level for 14 days and re-contact the study team.
- Use of NSAIDs more than once per week for headaches, routine aches/pains, etc.
- Use of any prescription drugs, including oral contraceptives (due to potential interference with mechanisms under investigation)
- Use of prescription opioids for any reason within the past 3 months
- Use of prescription corticosteroids for any reason within the past 3 months
- Acute viral or bacterial infection, or recent infection within the last 14 days or still requiring prescription medication for treatment
- Recent acute trauma occurring within the last 14 days
- Currently or recently (within last 14 days) taking any dietary supplements containing xanthohumol flavonoids, or other known herbal "anti-inflammatories" including: curcumin, turmeric, fenugreek, hops, rosemary, ginger, white willow, Devil's claw, fish oil (doses>1 g/day), or quercetin. Candidates will be given the option to "wash out" for 14 days and re-contact the study team.

- Currently receiving intravenous nutrition support therapy (or within the last 30 days)
- Currently taking anti-coagulant or anti-platelet prescription medications (or they were taken within the last 30 days)
- Currently taking antibiotic, antiparasitic, or antifungal medications orally or intravenously (or they were taken within the last 30 days)
- Initiation of or changes to supplements or medications within 14 days prior to screening. Candidates will be given the option to stabilize their allowable dietary supplements and medications for 14 days and re-contact the study team.
- Initiation of or changes to an exercise regimen within 14 days prior to screening. Candidates will be given the option to stabilize their exercise for 14 days and re-contact the study team.
- Initiation of or changes to a food plan within 14 days prior to screening. Candidates will be given the option to stabilize their diet for 14 days and re-contact the study team.
- Current involvement or within 30 days prior to screening of a significant diet or weight loss program, such as NutriSystem, Jenny Craig, Atkin's or other low-carb diet programs, or very low-calorie liquid diet programs (such as optifast, medifast, and/or HMR)
- Hospitalization (for any reason other than an elective medical procedure) within 3 months prior to screening
- Gastrointestinal surgery within 3 months prior to screening
- Undergoing UV therapy (e.g. treatment for skin conditions such as psoriasis).
- Engaging in vigorous exercise (e.g. activity at or above 80% of maximum perceived rate of exertion) more than 6 hours per week.
- Women who are lactating, pregnant or planning pregnancy within the next four months
- Typical intake of more than 2 alcohol-containing beverages per day, more than 14 per week, or more than 4 in any single day within the past 28 days. Candidates will be given the option to stabilize intake to these levels for 14 days and re-contact the study team.
- Smoking tobacco or nicotine products (combusted or vaporized)
- Use of illicit drugs/substances (such as but not limited to cocaine, phencyclidine (PCP), and methamphetamine) within 30 days of screening
- Use of inhaled or ingested *Cannabis* products, including CBD. Candidates will be given the option to discontinue intake for 14 days and re-contact the study team.
- Currently participating in another interventional research study or participated in another interventional study within 30 days of screening

4.3 Study Enrollment Procedures

Participants will be recruited from the general community via posted fliers, newspaper advertisements, online recruitment outlines, and targeted social media posts, plus at the NUNM clinic via posted fliers and IRB-approved mailings to current patients. Retention of study participants will be achieved through proactive and timely communication, and through detailed attention to professionalism and safety. Additional retention strategies

include the use of stipends dispensed for clinical screening ($50) and each clinical visit completed ($100 each) following enrollment for up to a total of $550. Ineligible candidates will be notified of reasons for ineligibility, and this information will be recorded in a screening log. All participants must read, attest understanding of, and agree to all items on the informed consent form.

Randomization will occur in up to 8 blocks of 4 based on biological sex. Initial randomization series will be generated using readily available random sequence generators designed to do so such as Randomization.com, which allows for block randomization.

5. STUDY INTERVENTIONS

5.1 Interventions, Administration, and Duration

The intervention is xanthohumol (XN, 24 mg/day plus 288 mg of rice protein) or placebo (rice protein 288 mg/day), encapsulated in gelatin capsules, and taken by mouth once daily for 8 weeks.

5.2 Handling of Study Interventions

The study product and visually-identical placebo will be delivered by Metagenics, Inc. to the NUNM via two separate shipments, i.e., one intervention and one placebo. Products will be stored at room temperature in separately locked cabinets in a secured storage location in the NUNM clinical research facility. Each locker will contain a Dispensing Log which will maintain a chain-of-custody record from receipt of the product to dispensing. Dispensation will require a Study Coordinator's signature, data of dispensation, and number of bottles removed.

Masking will be accomplished by using trained staff not involved in trial operations to:

1. Produce the "code" (e.g., A=XN and B=Placebo);

2. Label XN and placebo bottles according to the assigned code;

3. Generating the blocked randomization sequence;

4. Create 32 sequentially-numbered, sealed envelopes that contain the random group assignments. Envelopes will be opaque and signed across the seal. The randomization "code" will be kept in a sealed envelope with a signature across the label and dated the day of creation. This envelope will be kept in a dedicated study folder in the office of the Research Integrity Officer at NUNM.

Upon confirmation of eligibility and interest, Study Coordinators involved with trial operations will then open the next sequential envelop in the stack at the time of randomization, the envelop number will be recorded, and allocation disclosed to both participant and coordinator at that time as simply "A" or "B", which will correspond to a separately locked cabinets within a secure storage location from which the designated bottle(s) will be dispensed.

Upon return of the unused study agent at Study Visit 4 (4-week) and Study Visit 6 (8-week) unused capsules will be counted and noted on the corresponding CRF (see CRFs and SOPs for the relevant visits). Immediately following the completion of the study visit, the bottle containing unused capsules will be placed in a locked cabinet in the secure medical records facility in Helfgott Research Institute, where it will await a site visit by Westat personnel, at which time it may be disposed of in the dumpster. The study agent is a natural product extracted from the hops plant, which is native to North America, South America, and Eurasia. As such, it is widely distributed in the environment and does not represent a health or ecological hazard.

5.3 Concomitant Interventions

5.3.1 Allowed Interventions

- Use of over-the-counter medications as indicated on the label for less than 2 days per week
- Multi-vitamin and/mineral
- Dietary supplements except as detailed below

5.3.2 Required Interventions

N/A

5.3.3 Prohibited Interventions

- Dietary supplements containing xanthohumol
- Dietary supplements containing flavonoids
- Other known herbal “anti-inflammatories” including: curcumin, turmeric, fenugreek, hops, rosemary, ginger, willow, fish oil (doses>1 g/day), or quercetin.
- Anti-coagulant or anti-platelet prescription medications
- Antibiotic, antiparasitic, or antifungal medications orally or intravenously
- Biologic therapies (e.g., infliximab, adalimumab, certolizumab pegol, natalizumab, vedolizumab, ustekinumab)
- Glucocorticoids (e.g., prednisone, budesonide) (except in emergency management of flares)

5.4 Adherence Assessment

Participant adherence to the intervention will be determined by asking participants if they have missed any capsules during regularly scheduled bi-weekly study visits, and by counting unused capsules returned at week 4 and week 8 visits. A participant will be counted as adherent to the intervention if they consume at least 80% of the scheduled daily doses.

6. STUDY PROCEDURES

6.1 Schedule of Evaluations

Assessment	Screening Visit	Baseline, Enrollment, Randomization Visit 1 (Week 0)	Visit 2 (Week 2)	Visit 3 (Week 4)	Visit 4 (Week 6)	Closeout Visit 5 (Week 8)
Informed Consent Form	X	X				
Demographics	X					
Inclusion/Exclusion Criteria	X	X				
Medical History	X	X				
Current Medications	X	X	X	X	X	X
Anthropometrics/Vitals	X	X	X	X	X	X
Blood Chemistries	X	X	X	X	X	X
Hematology	X	X	X	X	X	X
Enrollment/Randomization		X				
Inflammatory markers (blood)		X	X	X	X	X
Urine Analysis		X	X	X	X	X
Stool Analysis		X	X	X	X	X
Adverse Events		X	X	X	X	X
Tolerability: PROMIS-29		X	X	X	X	X

6.2 Description of Evaluations

All biomarkers will be collected as closely to the indicated timepoint as possible, allowing a window of +/- 3 days at each time point, e.g., 2 weeks +/- 3 days is allowable.

Measures to meet Primary Objective: Clinical Toxicology

Clinical safety will be determined based on subjective reports of AEs, as well as, objective monitoring of changes in clinical laboratory measures. PROMIS-29 will also be administered as an additional indication of tolerability. The lab measures of safety in this Phase 1 trial include measures of hematological, renal and liver safety as: mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and hematocrit (derived from a complete blood count); estimated glomerular filtration rate; blood urea nitrogen to creatinine ratio; aspartate aminotransferase (AST); alanine aminotransferase (ALT); gamma-glutamyl transferase (GGT). All parameters will be measured at Baseline/Enrollment and after 2, 4, 6 and 8 weeks of the intervention. Results for all participants will be summarized and the following reported: % abnormal, % new abnormals, and mean change from Baseline/Enrollment.

Measures to meet Secondary Objective: Biologic Signature

- Change in levels of metabolic byproducts of xanthohumol: Xanthohumol and xanthohumol metabolites in blood, urine, and stool will be measured by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry, and expressed as change over time from baseline. Metabolites will be measured at Baseline/Enrollment and after 2, 4, 6 and 8 weeks of the intervention.

- Bile acid profile: Bile acid concentrations in stool samples will be measured by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry, and expressed as mean change over time from baseline. All parameters will be measured at Baseline/Enrollment and after 2, 4, 6 and 8 weeks of the intervention.

- Gut inflammation: Fecal calprotectin, a protein associated with gut inflammation and irritable gut syndrome, will be measured by enzyme-linked immunosorbent assay, and expressed as mean change over time from baseline. All parameters will be measured at Baseline/Enrollment and after 2, 4, 6 and 8 weeks of the intervention.

6.2.1 Screening Evaluation

Consenting Procedure

Upon initial determination of eligibility over the phone, candidates will be invited to an in person Clinical Screening Visit conducted by the Study Coordinator. Administration of a written informed consent form will be the first activity of this visit. This first consent applies to drawing a blood sample for confirmation of eligibility to participate in the full study.

Informed consent materials will be written in English at an eighth-grade reading level, and study personnel will be available to answer any questions the participant may have during the process. Candidates will have adequate time to review the written informed consent document (or have it read to them, according to their preference) which clearly indicates the voluntary nature of their participation and provides recourse should they feel their safety or privacy was violated in any way. Candidates will have an opportunity to ask any questions of the Coordinator or the site PI.

Upon confirmation of eligibility to participate in the trial following review of laboratory results, a second informed consent form will be administered during the Baseline/Enrollment visit which follows the same procedures above but will apply to participation in the full trial.

The informed consent form for full trial participation also includes a statement of consent to re-use the biologic samples collected for these trials for subsequent analyses related to the Aims of the proposed trials, should they become necessary. Participants will have the autonomy to provide, or refuse, additional consent for this purpose. A small volume (<2 mL) of blood and urine will be collected and frozen at -70C for the purposes of potential future analyses.

Any revisions of the informed consent documents deemed necessary will be approved by the NUNM IRB prior to implementation.

Signed forms will be retained for the duration of the study in a secure, dedicated records room with restricted access.

Screening

The screening visit will allow for:

- Administration of a written informed consent
- Review of eligibility criteria
- Measurement of anthropometrics and vitals
- Collection of blood to rule out anemia, hepatitis, and renal disease
- Collection of urine for determination of pregnancy status (where appropriate)
- Oral swab for HIV screening

Lab results will be reviewed within 48 hours of receipt and, if eligible, the candidate will be required to return for a Baseline/Enrollment visit within 30 days of screening, or else will require re-assessment of laboratory measures to re-assess eligibility.

Upon review and confirmation of eligibility, candidate participants will be contacted to schedule a Baseline/Enrollment visit, during which they will be given stool and urine collection kits and instructed on their proper use. The day following their Baseline/Enrollment visit, participants will return the baseline stool and urine specimens, and will receive their first bottle of study product.

6.2.2 Enrollment, Baseline, and/or Randomization

Enrollment

Enrollment in the study will occur at the baseline visit, immediately prior to randomization.

Baseline Assessments

- Demographics
- Anthropometrics (BMI, waist circumference) and vitals (BP and pulse rate)
- Health-related quality of life (PROMIS-29)
- Symptoms/Adverse-event questionnaire for Baseline anchoring
- Collection of blood for:
    - Complete blood count
    - Electrolytes
    - Estimated glomerular filtration rate
    - Blood urea nitrogen to creatinine ratio
    - Aspartate aminotransferase (AST)
    - Alanine aminotransferase (ALT)
    - gamma-Glutamyl transferase (GGT)

        - Alkaline phosphatase
        - Bilirubin
    - Collection of blood for Baseline measurement of:
        - Metabolic byproducts of xanthohumol
    - Collection of a urine specimen for Baseline measurement of:
        - Metabolic byproducts of xanthohumol
- Collection of a stool specimen for Baseline measurement of:
        - Microbiota sequencing
        - Fecal calprotectin
        - Gut permeability biomarkers
        - Metabolic byproducts of xanthohumol

Randomization

Randomization will occur within 30 days of screening during the Baseline/Enrollment visit, following confirmation of eligibility and signing of the informed consent form. Study Coordinators involved with trial operations will assign the participant to a treatment arm by opening the next sequential envelop in a previously created stack of randomized, sealed envelopes (see detailed description in section 5.2). The envelope number will be recorded, and allocation disclosed to both participant and coordinator at that time as simply “A” or “B”, indicating which bottles will be dispensed. Group allocation will be recorded in the baseline visit case report form. On the day following this visit (i.e. at the time of baseline sample return), study product will be provided to the participant to take home, but the container will only indicate “A” or “B”.

6.2.3 Blinding/Masking/Allocation Concealment

Masking procedures are detailed in section 5.2 above. This intervention has a low potential of producing effects that would compromise the masking of group allocation.

Unmasking is permissible if the Data Safety Monitoring Board requires it upon review and in the case of a Serious Adverse Event (SAE). If the DSMB identifies a need to break the allocation masking, the Chair of the DSMB will consult with relevant officials at NCCIH and FDA prior to unmasking.

6.2.4 Follow-up Visits

Bi-weekly follow-up visits will be conducted for 8 weeks total. Each follow-up visit must occur within a 72 hours window of each planned visit, e.g., the 4-week follow-up visit must occur within 4 weeks +/- 3 days. Follow-up visits for the total duration of the trial will be scheduled upon Enrollment whenever possible. Each follow-up visit will end with the dispensation of urine and stool collection kits for the next suitable follow-up visit, with the necessary instructions on sample collection. The following will be included in each follow-up visit:

- Anthropometrics (BMI, waist circumference) and vitals (BP and pulse rate)
- Health-related quality of life (PROMIS-29)
- Adverse events interview/questionnaire
- Collection of blood for:
    - Complete blood count

        - Electrolytes
        - Estimated glomerular filtration rate
        - Blood urea nitrogen to creatinine ratio
        - Aspartate aminotransferase (AST)
        - Alanine aminotransferase (ALT)
        - gamma-Glutamyl transferase (GGT)
        - Alkaline phosphatase
        - Bilirubin
    - Collection of blood for Baseline measurement of:
        - Metabolic byproducts of xanthohumol
    - Collection of a urine specimen for Baseline measurement of:
        - Metabolic byproducts of xanthohumol
- Collection of a stool specimen for Baseline measurement of:
        - Microbiota sequencing
        - Fecal calprotectin
        - Gut permeability biomarkers
        - Metabolic byproducts of xanthohumol

6.2.5 Completion/Final Evaluation

- Anthropometrics (BMI, waist circumference) and vitals (BP and pulse rate)
- Health-related quality of life (PROMIS-29)
- Adverse events interview/questionnaire
- Collection of blood for:
    - Complete blood count
    - Electrolytes
    - Estimated glomerular filtration rate
    - Blood urea nitrogen to creatinine ratio
    - Aspartate aminotransferase (AST)
    - Alanine aminotransferase (ALT)
    - gamma-Glutamyl transferase (GGT)
    - Alkaline phosphatase
    - Bilirubin
- Collection of blood for Baseline measurement of:
    - Metabolic byproducts of xanthohumol
- Collection of a urine specimen for Baseline measurement of:
    - Metabolic byproducts of xanthohumol
- Collection of a stool specimen for Baseline measurement of:
    - Microbiota sequencing
    - Fecal calprotectin
    - Gut permeability biomarkers
    - Metabolic byproducts of xanthohumol

For participants who withdraw from the trial prior to completing all scheduled visits, every attempt will be made to contact them and determine their reason for their withdrawal. Common reasons include inconvenience, geographical challenges, unexpected life events, and adverse effects. If adverse effects are noted as the reason, all reported adverse effects

will be graded and reported according to specifications noted below. All withdrawn participants will be queried for any/all adverse effects, and all reported events will be graded and included in summary reports.

7. SAFETY ASSESSMENTS

Safety will be assessed at every visit through the measurement of clinical toxicology laboratory measures (blood counts, liver and renal function tests as detailed above) and multi-system self-reported adverse events (per NCI definitions).

Previous clinical trials of XN have not resulted in any acute or chronic adverse events. However, potential adverse events are anticipated as possible:

- Allergy to study product
- Blood draw discomfort/bruising
- Gastrointestinal symptoms (described below)

Risks associated with any dietary supplement include gastrointestinal symptoms such as gas, bloating, digestive upset and change in bowel movement frequency or consistency. Also there is a chance of identifying a previously unknown allergy, and therefore a risk of rash, breathing difficulty and serious allergic reaction.

7.1 Specification of Safety Parameters

Standard clinical Complete Blood Counts and toxicology panels including liver and renal function tests will be measured at each bi-weekly clinical visit. Values will be interpreted based on: new abnormal value, change since baseline, new emergency or alert values, and/or the emergence of new diagnostic concerns. All participants' labs will be reviewed by the clinical investigator.

7.2 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

In light of the benign nature of XN, historical experience with its consumption as a common food ingredient, and the availability of preliminary human studies, the FDA has approved extending the duration of the proposed intervention to 8 weeks. Based on the available human data, it is expected that the study participants will not develop adverse effects at the proposed dose during the 8-week exposure period. However, a primary purpose of this study is to provide a clearer picture of the safety and physiological effects of XN. Therefore, blood samples will be taken at baseline and at each bi-weekly visit for complete blood counts and comprehensive metabolic panels, including liver and renal function tests, accompanied by determination of weight, blood pressure, and pulse rate. Additionally, adverse events will be solicited by interview at each visit, and participants will be encouraged to report any concerns as needed between visits.

All assessments, together with reported adverse events and unanticipated problems, will be collected and reviewed regularly, and per occurrence where appropriate (see Monitoring table in section 7.6) by the PI and specified monitoring bodies. As specified in section 7.1, values will be interpreted based on: new abnormal value, change since baseline, new

emergency or alert values, and/or the emergence of new diagnostic concerns. If statistical analysis is required for interpretation, the NUNM study Biostatistician, Dr. Doug Hanes, will conduct the analysis.

The general population is primarily exposed to XN through consumption of beer. A major brand beer contains about 0.5-1 mg prenylflavonoids (mainly XN and isoXN) per liter and a bitter microbrew ale may contain as much as 4 mg of total prenylflavonoids.[15]

XN is not currently FDA approved for the treatment, cure, prevention or mitigation of disease. Based on suspected mechanisms of action, potential medical uses include: inflammatory bowel disease, mucous colitis, prostate cancer, breast cancer, ovarian cancer, menopausal symptoms, hyperlipidemia, tuberculosis, cystitis, intestinal cramps, and neuralgia. XN is likely safe when used orally in amounts commonly found in foods. Hops and hops oil have Generally Recognized as Safe (GRAS, Flavoring Extract Manufacturers' Association records 2578 and 2580 respectively) status in the US.[10] The Stevens lab at Oregon State University has completed the first human pharmacokinetics study of XN in which 48 study participants ingested a single dose of XN of 20, 60, or 180 mg (16 men and women per dose group).[11] No treatment-related adverse effects were reported in this study (ClinicalTrials.gov Identifier: NCT01367431). Risks associated with any dietary supplement include gastrointestinal symptoms such as gas, bloating, digestive upset and change in bowel movement frequency or consistency.

Xanthohumol has been extensively studied in animals. Gerhauser and colleagues studied the safety of XN administered orally to Sprague-Dawley rats for four weeks.[12] At a dose of 1000 mg XN/kg body weight, authors reported a reduction of liver weight, but found no macroscopic or histopathologic changes of the liver, kidney, lung, heart, stomach and spleen. The mammary glands of treated rats appeared less developed compared to the control animals. In a two-generation study of the teratogenic effects of XN, Gerhauser's group found no differences in development of SD rats treated lifelong with a daily dose of 100 mg/kg body weight.

In another safety study of XN, mice exposed to XN for four weeks via the drinking water at a concentration of 0.5 mM (=177 mg XN per liter) showed no changes in blood counts or in the integrity of the bone marrow, liver, exocrine pancreas, kidneys, muscles, thyroid, ovaries or the surrenal cortex.[13] We estimate that the XN exposure in this study is equivalent to a human dose of 265 mg per day, which is much higher than the highest dose we propose in our clinical trial, i.e., 24 mg/day. In Zucker fa/fa rats study[14], there was no difference in uterus weight (n=6, females) or testes weight (n=6, males) between dose groups and the zero dose control group (n=6, females/males). Animals were given daily oral doses of 1.86 mg/kg, 5.64 mg/kg, or 16.9 mg/kg body weight for six weeks. These doses correspond to 20 mg, 60 mg and 180 mg XN in a 64 kg human by allometric interspecies scaling.

Based on in vitro data, the trial measured endpoints related to protection of DNA (single cell gel electrophoresis, 8-oxo-guanosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine levels in urine); oxidative stress (15F2t-isoprostanes in urine, and malondialdehyde, oxidized low-density lipoprotein, oxygen radical absorbance capacity, and ferric reducing ability in plasma); energy metabolism (glucose, cholesterol, and lipoproteins); inflammation (C-

reactive protein and urea); hormone-related parameters (17beta-estradiol and progesterone); and osteogenesis (alkaline phosphatase activity and osteocalcin). The observed differences between XN and placebo groups were limited to protective effects against DNA damage in the XN group (indicated through single cell gel electrophoresis in blood lymphocytes, and reduced urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-guanosine).

Additional research on XN has been performed in humans which support its safety and potentially beneficial mechanisms of action. Researchers at the Institute of Cancer Research at the University of Vienna, Austria, conducted a human study with the non-alcoholic beverage, XAN Wellness (TA-XAN AG Wiesbaden, Germany).[16] The tested beverage contained 12 mg/L XN. The study was designed as a cross-over, double-masked, placebo-controlled study with 22 subjects (11 men and 11 women, ages 19-37, nonsmokers). The subjects consumed one liter of the beverage per day, for 14 days. After a washout period of 14 days, subject crossed over to the alternate treatment (placebo or XN treatment). The daily dose was 12 mg XN.

Stevens and co-workers investigated the possible protective effects of XN on oxidative DNA damage and oxidative stress in a crossover, double-blinded, placebo-controlled human intervention study at three dose levels of XN: 6, 12 and 24 mg/day for 3 weeks (ClinicalTrials.gov Identifier: NCT02432651; IND #122633). Healthy human subjects consumed a non-alcoholic beverage without (placebo control) and with XN for 3 weeks, with a washout period of 3 weeks. Urine and blood were collected at the start and at the end of both 3-week intervention periods. Oxidative DNA damage was evaluated in peripheral white blood cells using single cell gel electrophoresis assay and by the measurement of urinary 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxo-dG). 8-Oxo-7,8-dihydroguanosine (8-oxo-Guo), a marker for RNA oxidation, was also measured in urine samples. The results showed that consumption of XN at 12 mg/day for 3 weeks protected DNA in white blood cells from oxidative damage as measured by the single cell gel electrophoresis, but did not influence the urinary excretion of 8-oxo-dG and 8-oxo-Guo. The blood levels of oxidized glutathione were significantly reduced by XN taken at a dose of 6 mg/day for 3 weeks. These findings suggest that XN administered in the form of a fortified non-alcoholic beverage is protective against DNA damage and oxidative stress in healthy individuals. No treatment-related adverse effects were reported or observed in this study. Thus the dose chosen for this trial, 24mg/day, aligns with the highest dose for which we have preliminary evidence of safety, tolerability and biologic effects.

The Van Breemen group evaluated the interaction of a hop-derived botanical supplement with drug metabolism in women.[17] Low doses of an oral cocktail of caffeine, tolbutamide, dextromethorphan, and alprazolam (probes for metabolism by CYP1A2, CYP2C9, CYP2D6, and CYP3A4, respectively) were administered to 16 peri- and post-menopausal women at baseline and then again after consuming a hop dietary supplement (2 capsules, twice daily for 14 days). The capsules contained 8 mg XN, 0.8 mg isoXN, 0.25 mg 8-prenylnaringenin, and 1.3 mg 6-prenylnaringenin. The pharmacokinetics values for each probe substrate showed no clinically relevant changes due to consumption of the hop dietary supplement. They concluded that consumption of the hop dietary supplement did not interact with drug metabolism.

There are no known toxicities for human subjects taking oral supplements containing XN. The general population is primarily exposed to XN through consumption of beer. A major brand beer contains about 0.5-1 mg prenylflavonoids (mainly XN and iso-XN) per liter and a bitter microbrew ale may contain as much as 4 mg of total prenylflavonoids.[15] In Europe, a beer/beverage, containing 4 mg of XN per liter, has been on the market for several years without adverse effects having been reported by the consumers. Based on the limited human data and the results from the above cited animal studies, we expect that the study participants will not develop adverse effects at the proposed dosage regimens during the 8-week exposure period.

7.3 Adverse Events and Serious Adverse Events

An **adverse event (AE)** is generally defined as any unfavorable and unintended diagnosis, symptom, sign (including an abnormal laboratory finding), syndrome or disease which either occurs during the study, having been absent at baseline, or if present at baseline, appears to worsen. Adverse events are to be recorded regardless of their relationship to the study intervention.

A **serious adverse event (SAE)** is generally defined as any untoward medical occurrence that results in death, is life threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability/incapacity, or is a congenital anomaly.

Adverse event (AE) monitoring will occur using a standardized multi-system AE questionnaire based on the National Cancer Institute's Common Terminology Criteria for Adverse Events v4.0 (2009). Adverse events of grades 4 and 5 will be considered Serious Adverse Events (SAE).

Standard clinical Complete Blood Counts and toxicology panels including liver and renal function tests will be measured at each bi-weekly clinical visit. Values will be interpreted and summarized based on: new abnormal value, change since baseline, new emergency or alert values, and/or the emergence of new diagnostic concerns. All participants' labs will be reviewed by the Clinical Investigator.

7.4 Reporting Procedures

Serious AEs will be reported to the PI within the same business day; all other AEs within 48 hours. Serious AEs, and any AE that in the discretion of the Clinical Investigator may require a modification to medications, will be reported to primary care doctors via phone contact between the Clinical Investigator and the PCP within the same business day, if possible, or the following. All other AEs will be reported to the PCPs in a summary report from each clinical visit within 1 week of scheduled visits. Adverse events will be dealt with directly, discussed with co-investigators and advisors, and adjudicated by the DSMB as related, possibly related, or unrelated to the study procedures.

In accordance with NUNM IRB AE reporting guidelines, SAEs (grades 4-5) will be reported to the NUNM IRB within 24 hours of PI awareness. Unanticipated AEs of any grade that are both related to research, result in a change to risk/benefit, or require protocol and/or consent modifications will be reported to the NUNM IRB within 10 working days of PI awareness. All

Anticipated AEs regardless whether they are classified as severe or related to the study intervention will be reported at the time of annual continuing review.

All adverse events will be recorded in the Adverse Event Log.

The DSMB will meet quarterly to conduct continuous review of data and participant safety.

The DSMB will review monthly safety reports, and adjudicate adverse events as required.

The DSMB will recommend amendments to the protocol, changes in study procedures, changes to the data collection plan or study forms, or study termination due to safety or other issues as needed.

The PI will have final responsibility for all reporting to the NUNM IRB, DSMB and/or NCCIH PO as required.

7.5 Follow-up for Adverse Events

All adverse events, regardless of grade, will be recorded with start dates, graded severity, medical interventions as relevant, clinical monitoring plan as indicated, and date of resolution.

If participants believe they have suffered an AE related to study procedures, but outside of routinely scheduled clinical follow-ups, they are welcome to contact us at any time and AEs will be adjudicated. At each study visit, the investigator will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization for at least 30 days following discontinuation of any study-related interventions.

7.6 Safety Monitoring

The NUNM IRB, DSMB, and NCCIH Program Officials will receive copies of all study monitoring/audit or inspection reports within 14 day of PI receipt.

Safety Review Plan

Study progress and safety will be reviewed monthly (and more frequently if needed). Progress reports, including participant recruitment, retention/attrition, and AEs will be provided to the DSMB. An Annual Report will be compiled to address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The Annual Report will be sent to the DSMB and will be forwarded to the IRB and NCCIH. The IRB and other applicable recipients will review progress of this study on an annual basis.

Monitoring		
Data type	***Frequency of review***	***Reviewer***
Subject accrual (including compliance with protocol enrollment criteria)	*Weekly*	*PI*
	Monthly	*Study team*
	Quarterly	*Independent Monitors*
Status of all enrolled subjects, as of date of reporting	*Weekly*	*PI*
	Quarterly	*Independent Monitors*
Data entry quality control checks on 15% of charts	*Monthly*	*PI*
Adherence data regarding study visits and intervention	*Per visit*	*Study team*
	Monthly	*PI*
	Quarterly	*Independent Monitors*
	Annually	*NCCIH*
SAEs (unexpected and related)	*Per occurrence*	*PI, Independent Monitors NIH/NCCIH*
SAEs (expected or unrelated)	*Per occurrence*	*PI*
	Annually	*Independent Monitors, NIH/NCCIH*
Unanticipated Problems	*Weekly*	*PI*
	Per Policy	*IRB*

8. INTERVENTION DISCONTINUATION

Participants may discontinue the intervention at any time by their own choosing. However, the intervention may be discontinued by the study staff if they experience moderate or greater AEs requiring them to withdraw or new onset laboratory abnormalities (confirmed with repeat testing), including new elevation of liver function tests (AST, ALT, bilirubin or alkaline phosphatase), newly abnormal electrolytes, new onset anemia, newly abnormal creatinine or estimated glomerular filtration rate (eGFR).

Of course, the intervention may also be discontinued if, for some reason, trial operations are suspended by the Institution, sponsor, or FDA requirement.

Because this trial is a mechanistic, proof-of-concept trial focused on highly mechanistic outcome measures *that require participants to consume the intervention (or placebo)*

participants will not be maintained in the trial, or complete follow-up visits, if they discontinue the intervention (or placebo). The study staff will however follow up with them to query any/all adverse events as relevant and attempt to ascertain the reason for withdrawal.

9. STATISTICAL CONSIDERATIONS

9.1 General Design Issues

The primary hypothesis is that not more than 50% more participants receiving XN will report an adverse event or experience a laboratory abnormality compared to those receiving placebo. For our Secondary Objective, we hypothesize there will not be a difference in changes in TNF-alpha between XN and placebo.

A placebo-controlled, parallel group trial is the only design conducive to determining if xanthohumol is the cause of any measured changes in safety parameters, inflammation and gut-related biomarkers and/or changes in microbiota composition or products in a timely manner. Season-, diet-, and stress-induced changes in the microbiota necessitate timely completion of the trial objectives, plus the unknown residual effects of XN supplementation preclude crossover or n-of-1 trial designs.

9.2 Sample Size and Randomization

A sample size of 12 per group provides 80% power to detect a 50% difference in proportions of participants between groups by chi^2 test experiencing a laboratory abnormality or adverse event at a significance threshold of $\alpha=0.05$.

Randomization and Treatment Assignment Procedures

Allocation concealment and randomization procedures are described in Section 6.2.2 and 6.2.3 above.

9.3 Definition of Populations

Only per protocol analyses will be performed for this mechanistic, proof-of-concept study. The per protocol population will be those randomized to the intervention or placebo and who take 80% or more of the assigned product.

9.4 Interim Analyses and Stopping Rules

Interim analyses will only include those analyses necessary for continuing safety assessments, including the accumulation of AEs by grade, the frequency of new onset laboratory abnormalities (confirmed with repeat testing), including new elevation of liver function tests (AST, ALT, bilirubin or alkaline phosphatase), newly abnormal electrolytes, new onset anemia, newly abnormal creatinine or estimated glomerular filtration rate (eGFR). The analyses will remain masked to group and will be prepared by the NUNM biostatistician periodically to determine accumulated frequencies of events.

This study will be stopped prior to its completion if the intervention is associated with adverse effects that call into question the safety of the intervention, if any new information becomes

available during the trial that necessitates stopping the trial; or if other situations occur that might warrant stopping the trial. The ultimate decision to stop the trial may be made by the DSMB, NCCIH, and/or the FDA.

The trial will be halted if >25% of the total sample experience moderate or greater AEs requiring them to withdraw or new onset laboratory abnormalities (confirmed with repeat testing), including new elevation of liver function tests (AST, ALT, bilirubin or alkaline phosphatase), newly abnormal electrolytes, new onset anemia, newly abnormal creatinine or estimated glomerular filtration rate (eGFR).

The trial may be stopped if:

1. SAEs attributable to the study intervention occur in 25% of the intervention group.
2. New onset laboratory abnormalities (upon repeat testing) occur in 50% of the intervention group.
3. The FDA, IRB, DSMB, and/or NCCIH recommend stopping the trial completing after review of the available data after a temporary suspension of the trial.

The DSMB will provide a final recommendation to the PI to halt the study or amend the study protocol, and will develop a response plan, in consultation with the NUNM IRB and the NCCIH Program Officer.

If immediate changes are required for participant safety, enrollment and other study activities will not continue until the modifications/amendment is approved by the IRB.

9.5 Outcomes

In this study, measured outcomes will take the form of laboratory values. Consequently, we do not anticipate the need to adjudicate any of the assessments.

9.5.1 Primary Outcome

As the primary objective relates to safety and tolerability, the associated primary outcome measures relate to cumulative routine clinical toxicology measures including: complete blood count, electrolytes, estimated glomerular filtration rate, blood urea nitrogen to creatinine ratio, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase, and bilirubin, plus self-reported adverse events and health-related quality of life (PROMIS-29).

Cumulative safety will be reported as the frequency of new onset laboratory abnormalities (confirmed with repeat testing), including: new elevation of liver function tests (AST, ALT, bilirubin or alkaline phosphatase), newly abnormal electrolytes, new onset anemia, newly abnormal creatinine or estimated glomerular filtration rate (eGFR).

9.5.2 Secondary Outcomes

The outcome measure for the secondary objective of assessing changes in inflammation is TNF-alpha as a biomarker of active inflammation.

9.5.3 Tertiary Outcomes

The tertiary objective is to collect comparison data related to XN metabolism, gut permeability and impact on the microbiota for a subsequent Phase 2 trial in adults with Crohn's disease. Thus our outcome measures for this objective include XN metabolites in the blood, stool, and urine, assessment of bile acid metabolism, biomarkers of gut permeability, enterocyte and systemic inflammation; and microbiota population and expression.

Specific biomarkers include: fecal calprotectin, soluble CD14, lipopolysaccharide-binding protein (LBP), cytokines (IL-8, IL-1β, IL-6, IL-10, and IL-12p70), 16s rRNA sequencing and gene product results measured by metagenomic DNA sequencing.

9.6 Data Analyses

Analysis of laboratory toxicology measures:

Laboratory measures including red blood cell count, white blood cell count, hematocrit (%), hemoglobin, mean corpuscular volume, AST, ALT, creatinine-estimated glomerular filtration rate (eGFR), blood urea nitrogen (BUN), and creatinine will be analyzed by first assessing descriptive statistics including mean, median and standard deviation. All distributions will be tested for skew and transformed (e.g., natural log transformed) as needed to reduce the influence of skew in subsequent analyses. All parameters will be evaluated by formally comparing means from baseline to each time point by unpaired, 2-sided t-tests, as well as for trends over time by applying linear mixed ANOVA models with time point as a repeated factor.

As an element of the evaluation for safety of the intervention, the % laboratory newly abnormal values within the clinically normal range at baseline per clinical laboratory reference ranges for each lab parameter (per list above) will be reported at each 2-week time point and the % compared between XN and placebo groups by Fisher's exact test.

Assessment for changes in the distribution of each lab parameter will be performed by presenting means and confidence intervals (CI) for the change in each group, as well as the mean CI for the difference between groups; significance will be tested using 2-sided, unpaired t-tests (or, if inspection of distributions indicates strong non-normality, with a non-parametric Wilcoxon signed rank test) of the mean values for XN compared to placebo.

Assessment for overall increases or decreases in parameters of interest will be tested for linear trends of the means by linear mixed ANOVA with time point as a repeated factor, which may be suggestive of evidence for cumulative toxicity if trending in a clinical deleterious direction.

Analysis of inflammatory cytokines, gut permeability biomarkers, and endotoxemia:

Given the small sample size of these clinical trials, results related to changes in inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, and IL-12p70), gut permeability biomarkers (CD14, intestinal fatty acid binding protein) and markers of endotoxemia (LPS, LPS-binding protein) are considered preliminary, and thus our principle assessment will be measurement of effect size as Cohen's d statistic between XN- and placebo-treated groups at each 2-week interval compared to baseline, with the primary comparison being between baseline and

week 8. We will also perform formal statistical comparisons as unpaired, 2-sided t-tests (or, if inspection of distributions indicates strong non-normality, with a non-parametric Wilcoxon signed rank test) of the means at relevant time points. The following findings will be considered evidence of possible effects: 1. Clinically and statistically significant effects for changes in cytokines of interest and/or 2. Calculation of a Cohen's d value for effect size estimation greater than 0.5 (with larger d indicating a larger potential effects size).

Assessment for overall increases or decreases in parameters of interest will be tested for linear trends of the means by linear mixed ANOVA with time point as a repeated factor. Trends will be considered significant if $p<0.05$ for the model overall.

Analysis of XN, its metabolites, and BAs to determine a small molecule signature of longer-term oral treatment with XN:

We will identify and quantify XN and XN-derived metabolites in plasma, stool, and urine. Bile acid profiles will be also be determined in stool samples. The metabolite profile data will then be used for correlation with the time-resolved gut microbiome analysis. This approach will allow us to determine links between metabolite profiles and gut microbiome compositions.

Determining differential abundance between treatments: High-level exploratory analyses will be performed through metrics such as alpha and beta diversity and ordination techniques. Further, differential abundance analysis for 16S and metagenomics data will be conducted through negative binomial models, with specifications to deal with inflation of zero counts, such as those implemented in statistical R packages edgeR[18] and DESeq2[19] or through a compositional Bayesian data analysis approach, such as the approach implemented in the R package ALDEx2.[20] Exploratory data analysis will first be done to evaluate which modeling technique is appropriate for the data generated based on assumptions made by each modeling approach. For MS-based omics data, we use a suite of robust statistical approaches for: 1) processing the data to identify outliers,[21–23] and 2) identifying statistically different proteins and metabolites between comparative samples. As a first step to statistical testing, we use the Statistical Procedure for the Analysis of molecular abundance Normalization Strategies (SPANS) approach to simultaneously evaluate several MS-based *omics* data set normalization strategies to determine the most appropriate normalization factor in the context of the experimental design.[24] For individual data streams, there are several strategies for identifying molecular features that statistically classify patients, depending on the structure of the data and the phenotype data being used for classification. For the determination of statistically relevant features, if the features follow the standard normal distribution, then we utilize standard ANOVA methods; otherwise, a non-parametric version, such as Kruskal-Wallis or sum-rank test, can be utilized.[25] To evaluate the statistical relevance of missing data, for example when the data is absent in the control group and present in the treatment group, we will utilize a G-test.[23] However, with multiple comparisons comes the problem of increased likelihood of a significant result by random chance. To counter this problem, we use either the Dunn-Sidak or Bonferroni correction[26] to adjust p-values. If the data have a linear relationship, then we use partial least squares discriminant analysis (PLS-DA) [27,28] and rank the molecules by p-value in a pair-wise comparison to the control group.

Longitudinal regression modeling:

We will apply systems biology tools to quantify how an individual's inflammation state, as measured by calprotectin, changes over time in association with XN metabolite status and microbial taxa, their genes, gut metabolites, or BAs. To link the time dependence of inflammation to these various parameters, we will follow our prior work[29] and construct compound Poisson generalized linear models (CPGLMs) that allow us to test for differences in temporal trends in the relative abundance of specific OTUs, genes, metabolites, or BAs as a function of various experimental parameters. CPGLMs include a point mass at zero as well as a weighted mixture of Poisson and gamma distributions. These features enable accurate modeling of the sparse (zero-inflated) but otherwise continuous and non-normal data usually produced during microbiome and metabolome investigations.[29] CPGLMs also correctly account for multiple sources of variation[30] due to different experimental parameters (e.g., XN metabolite status, inflammation state, time, and sex). We will construct CPGLMs that model how the temporal variation of each OTU, gene, or metabolite (i.e., response variables) relates to these experimental parameters. We will adopt a stepwise approach to model construction and optimal models will be identified. Models will include interaction terms that consider co-dependencies among the experimental parameters. Random intercepts and time slopes for each individual will be included in the model to account for within-individual correlation of a response variable's abundance over time. Response variables that manifest as significantly different slopes as a function of XN metabolite status are those variables that putatively interact with XN metabolites. Related analyses of model slopes will resolve parameters that elicit dependencies with inflammation state, as well as parameters that vary in accordance with the XN metabolite-inflammation interaction. We will also test models that include a lag effect between response variables and these parameters to resolve, for example, taxa whose temporal change in abundance predicts subsequent inflammation changes. We have extensive experience using GLMs and related techniques for the study of microbiome-phenotype associations.[19,31,32] Recognizing that the proposed CPGLMs are complex and may suffer from a lack of power, we may consider an alternative approach which avoids models that attempt to accommodate the data structure through zero-inflated distributional adjustments and instead consider a compositional data analysis approach, where the data is transformed and normalized using the Aitchison simplex, normalized through e.g. centered log ratio adjustment, and followed by fitting with traditional GLMs and accounting for other covariates and covariance structures. This approach would retain higher statistical power because there no longer would be an attempt to resolve the zero-inflation problem. This approach has been shown to perform better in terms of differential expression with small sample/replicate sizes (https://doi.org/10.1186/1471-2105-14-91). An alternative, more well-powered approach to variable/feature selection would be to perform lasso or ridge regression via elastic net, which is known to operate well in small sample relative to large feature scenarios.

Time-resolved alterations of the gut microbiome by 16S rRNA gene sequencing and metagenomics analysis:

Microbiome data generation:

We will profile the structure and functional capacity of the microbiome using 16S rRNA and shotgun metagenomic DNA sequencing. Following our prior work,[24] we will extract whole

genomic DNA from each stool sample using a QIAGEN PowerFecal DNA kit. We will then PCR amplify the V4V5 region of the 16S gene[33] and sequence amplicons on an Illumina MiSeq at OSU's Center for Genome Research and Biocomputing. We will produce 300 bp, paired-end sequences and target a sequence depth of 50,000 reads per sample. We will also generate metagenomes from the same DNA extracts using NexteraXT library prepa- ration and the CGRB's Illumina HiSeq 3000 (150 bp paired-end sequences). We will target a sequence depth of 15 million reads per sample, which our prior work showed was more than sufficient for resolving associations between IBD and microbiome taxonomy and functional capacity.[29,34]

Microbiome data analysis:

We will analyze 16S sequences to profile gut microbiome structure. The DADA2[35] software pipeline will quality filter sequences, assemble contigs between paired ends, resolve unique sequence variants (i.e., OTUs), and taxonomically annotate these sequences using the RDP database. The Phyloseq[36] R package will quantify the alpha- and beta-diversity of microbiome samples. Non-parametric tests (e.g., PERMANOVA) will measure differences in α- and β-diversity and β-dispersion across patient groups and over time. We have experience developing and applying methods for the analysis of microbiome data.[24,34,37–39] Metagenomic analysis will complement 16S assessments of taxonomy and quantify the functional capacity of microbiome samples. After quality controlling metagenomes using Shotcleaner, which follows Human Micro- biome Project[40] guidelines to trim adapters, filter low-quality sequences, and informatically subtract host genomic DNA from metagenomes, we will apply several strategies for the analysis of metagenomes. First, we will assemble paired-end metagenomes using the Automatic Tool for Local Assembly Structures (ATLAS), an open-source tool for NGS data analysis developed in-house.[41] ATLAS transforms raw sequence data into functional and taxonomic data at the microbial population level and provides genome-centric resolution by integrating genome binning. The full ATLAS protocol is developed and maintained as open-source software on GitHub and is implemented in Snakemake for modular, flexible, and reproducible workflows. Individual protein sequence databases will be created based on a combined metagenome that will serve as references for paired metaproteome analysis. To complement these assemblies and produce as comprehensive an assessment of microbiome structure and functional capacity as possible, we will also conduct analyses at the level of individual, unassembled reads. Specifically, we will analyze quality controlled metagenomes using MetaPhlan2[42] to measure the abundance of specific microbial taxa in each metagenome. We will also use ShotMAP to assign reads into KEGG Orthology groups based on homology.[34] Briefly, ShotMAP predicts coding sequences from unassembled metagenomic data using Prodigal and applies RAPsearch or DIAMOND to rapidly identify reads that show evidence of homology to KEGG Orthology groups. Our prior statistical simulations surrounding this technique highlight its accuracy[34] and our prior applications of these tools resolved taxa and microbiome metabolic modules that predict immune activation in mouse models of IBD[29] and flare-ups in IBD patients.[34]

10. DATA COLLECTION AND QUALITY ASSURANCE

10.1 Data Collection Forms

All data will be collected on Case Report Forms and by direct data transmits from the clinical laboratory.

10.2 Data Management

The investigators are responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data. The investigators will maintain adequate case histories of study subjects, including accurate case report forms (CRFs), and source documentation.

Data collection and accurate documentation are the responsibility of the study staff under the supervision of the investigator. All source documents and laboratory reports must be reviewed by the study team and data entry staff, who will ensure that they are accurate and complete. Unanticipated problems and adverse events must be reviewed by the investigator or designee.

This study will use a secure customized REDCap (Research Electronic Data Capture) database. REDCap is a secure, web-based application that supports electronic data capture for research studies. REDCap features include: 1) intuitive data entry features; 2) audit trails for tracking data manipulation and export; 3) user-based privileges that support HIPAA compliance, 4) seamless data export to common statistical packages; and 5) procedures for importing data from external sources. The REDCap database is secured with user login IDs and password protection. The database incorporates an electronic audit trail to show change(s) to data after original entry including the date/time and user making the change. Data will be stored as de-identified data; participants' names will be linked to their unique study ID in a single password protected electronic file stored on a limited access server with select access limited to the Principal Investigator, NUNM Co-Investigators and Study Coordinator. Identifiers will be destroyed after completion of the study and analysis of the data. . Electronic communication with outside collaborators will involve only unidentifiable information.

All data resulting from study visits will be collected on standardized case report forms (CRFs). Data will be transferred from CRFs to a secure REDCap database for data management and will be accessible only by the study team.

All completed forms will be kept in locked files to which only project personnel have access. These files will also be in locked rooms. Data from paper forms will be manually entered into REDCap and kept as electronic files. Electronic files will be password protected with access given to study personnel only. All analyses will be conducted by study staff at the NUNM Helfgott Research Institute or the University of Washington. The study statistician will only access de-identified data exported from REDCap to Excel or SPSS. Only de-identified data will be presented in any reports, presentations or manuscripts.

All data will be managed using Research Electronic Data Capture (REDCap).

10.3 Quality Assurance

10.3.1 Training

All study investigators and staff complete Good Clinical Practices (GCPs), Human Subjects Research (HSR) and Responsible Conduct of Research (RCR) trainings from CITI, plus HIPAA and blood-borne pathogens training.

All study coordinators have been training in the administration of informed consent, participant interviews for adverse effects, and standardized training for all visit components.

10.3.2 Metrics

Quality control metrics include documentation of training requirements and automated reminders for training updates as indicated. Training logs for study-specific trainings will be maintained in the study regulator binder.

10.3.3 Protocol Deviations

Protocol deviations are captured by interview for changes in eligibility, product adherence and adverse events at each scheduled clinical research visit. Protocol deviations such as lack of follow-up within acceptable timeframes for each visit, i.e., within “windows” are captured by regular audits of the visit schedule. Protocol deviations are reported to the IRB via periodic progress reports, as well as to the sponsor and the FDA.

10.3.4 Monitoring

Site monitoring will include internal and external audits. Internal audits of individual study participant records will occur monthly during trial operations by the Principal Investigator and will include reviews of eligibility confirmation, consent forms and all CRFs. Audit documentation will be maintained in the study binder for review by external auditors.

External site monitoring will be at the discretion of the sponsor and the FDA.

11. PARTICIPANT RIGHTS AND CONFIDENTIALITY

11.1 Institutional Review Board (IRB) Review

Any/all research activities involving human subjects are be reviewed and must approved by the NUNM IRB prior to implementation. All protocol amendments, other than minor administrative changes as defined by the NCCIH Guidance on Changes in Clinical Studies in Active Awards will be submitted in a prospective manner to NCCIH except when necessary to protect the safety, rights, or welfare of subjects. Prior to submission to NCCIH, the proposed changes will be reviewed and approved by the Independent Monitoring Committee. IRB approval will not be sought until after NCCIH approval of the protocol amendment has been obtained.

11.2 Informed Consent Forms

A signed consent form will be obtained from each participant by a trained study coordinator.

The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy will be given to each participant or legal guardian and this fact will be documented in the participant's record. Non-English speakers and children will not be enrolled in this trial. Non-writing individuals will be able to sign with an "X", stamp or personal mark, or assign a proxy for signing their consent form following witnessed verbal agreement by the candidate participant.

11.3 Participant Confidentiality

Throughout the study, measures to ensure the privacy of information on study participants will be maintained. All study investigators and staff are certified in Good Clinical Practices (GCPs), Human Subjects Research (HSR) and Responsible Conduct of Research (RCR) and have received training in HIPAA regulations. Participants and staff will be informed of the confidentiality of information and assured that data will be used only for statistical purposes and group analyses in which the individual cannot be identified. No data beyond what is stated in the Informed Consent will be sought without authorization from the participant. Information on illnesses and hospitalizations will not be sought from hospitals or doctors without a signed medical release from the subject. Conversely, no information on any individual will be released to anyone other than study personnel without a signed medical release from the participant, or where appropriate, the next of kin or a physician in case of a life-threatening emergency to the subject.

Each participant will be assigned a unique alpha-numeric ID upon screening. All blood and urine aliquot tubes, stool collection vials and associated paperwork will be marked using the unique ID to protect patient confidentiality. All data resulting from study visits will be collected on standardized case report forms (CRFs). Data will be transferred from CRFs to a secure REDCap database for data management. The REDCap database will be accessible only by the study team. Data will be destroyed 3 years after completion of the study. Computer files will be deleted from the server and paper files will be destroyed using a professional document shredding service.

All completed forms will be kept in locked files to which only project personnel have access. These files will also be in locked rooms. Data from paper forms will be manually entered into REDCap and kept as electronic files. Electronic files will be password protected with access given to study personnel only.

11.4 Study Discontinuation

This study will be stopped prior to its completion if the intervention is associated with adverse effects that call into question the safety of the intervention, if any new information becomes available during the trial that necessitates stopping the trial; or if other situations occur that might warrant stopping the trial. The ultimate decision to stop the trial may be made by the DSMB, NCCIH, and/or the FDA.

The trial will be halted if >25% of the total sample experience moderate or greater AEs or laboratory abnormalities requiring them to withdraw.

The DSMB will provide a final recommendation to the PI to halt the study or amend the study protocol, and will develop a response plan, in consultation with the NUNM IRB and the NCCIH Program Officer.

If immediate changes are required for participant safety, enrollment and other study activities will not continue until the modifications/amendment is approved by the IRB.

12. COMMITTEES

N/A

13. PUBLICATION OF RESEARCH FINDINGS

Authorship decisions on all resulting manuscripts will be made by the co-PIs of this trial. The principal manuscripts will be dependent on the safety and tolerability profile of XN in healthy adults and future comparisons made to adults with Crohn's disease.

All resulting manuscripts will be made available for review by the sponsor and the NCCIH prior to journal submission.

14. REFERENCES

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15. SUPPLEMENTS/APPENDICES

Informed Consent for Screening (File: XN_Phase 1Screening_ICF_1.10.19)
Informed Consent for Trial Participation (File: XN_Phase 1 Trial_ICF_1.10.19)