Protocol

PROCLAIM – A Randomized, Double-Blind, Placebo-Controlled Trial to Assess the Efficacy and Safety of Misoprostol in the Prevention of Recurrence of Clostridium Difficile Infection in Adults

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PROCLaIM

PREVENT RECURRENCE OF *CL*OSTRIDIUM *DIFFICILE* INFECTION WITH MISOPROSTOL

Protocol

Version 8.0: May 24, 2021

A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL TO ASSESS THE EFFICACY AND SAFETY OF MISOPROSTOL IN THE PREVENTION OF RECURRENCE OF *CLOSTRIDIUM DIFFICILE* INFECTION IN ADULTS

Short Title: PROCLAIM – Prevent Recurrence of *Clostridium difficile*[1] Infection with Misoprostol

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[1] *Clostridium difficile* was renamed to *Clostridioides difficile*, and all uses of each term in this document are equivalent

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PROTOCOL NUMBER: MISO-001

INVESTIGATIONAL MEDICINAL PRODUCT: Misoprostol Oral Tablet

PATIENT POPULATIONS: Patients 18 years of age or older with *C. difficile* Infection

SPONSOR: Vanderbilt University Medical Center

PARTICIPATING SITES: Vanderbilt University Medical Center (Nashville, TN), Washington University in St. Louis (St. Louis, MO), and The University of North Carolina at Chapel Hill (Chapel Hill, NC)

1. OBJECTIVES, INTRODUCTION AND BACKGROUND

Study Objectives

The objectives of this study are to:

1. Evaluate the efficacy of misoprostol for prevention of recurrent *Clostridium difficile* Infection (rCDI), by determining if misoprostol can modify the rate and severity of rCDI in adults ≥18 years of age during the first 8 weeks after standard of care is completed.
2. Evaluate the safety and tolerability of misoprostol dosed orally for up to 14 days in adults (≥18 years of age) with *Clostridium difficile* Infection (CDI), measured by treatment emergent adverse events and standard laboratory assessments.
3. Determine the effect of misoprostol on the gut microbiome and diarrhea occurrence/severity, in order to inform the design of future studies.

Primary Hypothesis

The treatment of patients with CDI with misoprostol, in addition to standard care, will result in a relative reduction in the rate of recurrence by 40% when compared to patients treated only with standard care.

Introduction

Clostridium difficile is a Gram-positive, spore-forming, toxin-producing bacillus that causes diarrhea and colitis often following exposure to antibiotics. It is emerging as one of the most common healthcare-associated infections. *C. difficile* infection (CDI) ranges in severity from mild diarrhea to severe colitis (sometimes resulting in septic shock and/or death). In addition, CDI has a high tendency to recur following treatment. First clinical recurrence of infection occurs in about 25% of patients initially treated with antibiotic therapy with rate of recurrence increasing with age. Once a first recurrence has occurred, the risk for future recurrences exceeds 40% with highest rates being in older adults. The problem of recurrent CDI (rCDI) is a significant one, negatively impacting quality of life. There are patients for whom the cycle of recurrences does not remit. Experimental treatments such as fecal transplant are being investigated for patients with rCDI. Misoprostol, a prostaglandin analogue, has been shown to enhance recovery from CDI

in animal models, and decreased prostaglandin pathway signaling is associated generally with GI inflammatory conditions in a phenome wide association study (PheWAS) analysis. Misoprostol is FDA-approved for chronic use in the prevention of gastric ulcers given its established mucosal protective properties and inhibitory effects on gastric acid. This trial will evaluate the rate and severity of rCDI in patients treated with oral misoprostol vs placebo subsequent to the initiation of standard treatment(s) in adults ≥18 years of age following a primary episode of CDI.

Background

Clostridium difficile

CDI is a serious problem worldwide, and in the United States, it comprises approximately 17.1% of hospital-associated infections affecting 500,000 individuals annually. It leads to worse clinical outcomes, more frequent readmissions, longer hospital stays, and costs billions of dollars each year in health care expenses.[1] In this disease there are focal areas of epithelial loss and an exudate consisting of polymorphonuclear cells, fibrin, and cellular debris. Focal inflammation might arise after exposure to high local concentrations of toxins released from *C. difficile* adherent to specific small regions of the epithelium.[2] There are two main toxins that cause damage, Toxins A and B, and these cause the symptoms of CDI.

The most well-known risk factor for the infection is antibiotic use, but other risk factors include advanced age, hospitalization within preceding 2 months, proton-pump inhibitor use, and severity of underlying illness. Inflammatory bowel disease (IBD), which includes Crohn's disease (CD), ulcerative colitis (UC), and patients after ileal pouch anal anastomosis (IPAA), is known to be an independent risk factor for CDI.[1] A history of CDI has also been associated with failure of IPAA reconstruction, which is a frequent treatment for UC.[3]

Humoral immunity is an increasing area of interest in understanding the varied clinical presentation of CDI in both IBD and non-IBD patients, and also in investigating why an IBD patient may be more susceptible to clinical disease. Hughes et al. (2016) conducted a study to determine if IBD patients have any alteration in humoral response to *C. difficile* toxins A or B when compared with a group of control patients enrolled from outpatient clinics at the same medical center. They found that patients with UC have lower IgA levels to *C. difficile* toxins compared to those with Crohn's disease and those after IPAA. Patients with IBD with prior CDI failed to demonstrate any increase in antitoxin IgG. These findings suggest that IBD patients may benefit from immunization strategies targeting *C. difficile* toxins.[1] However, If prostaglandins maintain mucosal integrity and enhance almost all mucosal defensive mechanisms, perhaps they can protect against other exogenous insults, lower in the GI system.[4]

A Prostaglandin E_2 receptor 4 (EP_4) receptor agonist, KAG-308, is in Phase II study for ulcerative colitis. Oral administration of KAG-308 suppressed onset of dextran sulfate sodium-induced colitis and promoted mucosal healing in a mouse model; it also prevented colorectal carcinogenesis by inhibiting colitis development in another mouse model (conversely, an EP4 antagonist increased mortality).[5]

PheWAS in drug discovery and drug repurposing

One source of data guiding the design of this study is BioVU, a large repository of de-identified DNA samples that Vanderbilt University Medical Center (VUMC) has catalogued for >10 years from what otherwise would have been excess, discarded patient blood samples collected during

routine clinical testing. This biobank is a centralized resource for investigating genotype-phenotype associations. Biospecimens within BioVU are linked to corresponding longitudinal clinical and demographic data derived from the Synthetic Derivative, VUMC's de-identified database of EMRs.[6–8] More recently, PheWAS has been introduced as a systematic and efficient approach to discover novel disease-variant associations and pleiotropy using BioVU.[9] It is the comprehensive and diverse nature of the diagnostic information within EMRs that enables PheWAS. PheWAS not only replicates known genetic-phenotypic associations, but also reveals new phenotypic associations with genetic variants, enhancing analyses of the genomic basis of human diseases and providing genetic support for drug discovery and drug repurposing efforts.[10–13]

Prostaglandin receptor function and misoprostol pharmacology

Prostaglandin E_2 (PGE_2) plays a pivotal role in maintaining local homeostasis in a variety of pathophysiological settings, including the colon. PGE_2 receptors (EPs) mediate the effects of this molecule and include four subtypes: EP1–4. PGE_2 participates decisively in the defense of the colonic mucosa. For example, misoprostol, a synthetic PGE_1 analog, potently protects human colonic mucosa against mucosal insults. In addition, in mice, PGE_2 and EP4-selective agonists significantly improved colitis induced by dextran sodium sulfate (DSS) treatment. Nakatsuji et al. (2015) investigated the effect of the recently identified EP4-association protein (EPRAP), which is essential for anti-inflammatory function of EP4 signaling in macrophages, on colitis and colitis-associated tumorigenesis in mice. They observed that EPRAP deficiency exacerbated DSS-induced colitis and found support for the idea that EPRAP in macrophages functions crucially in suppressing colonic inflammation.[14]

Misoprostol is utilized clinically as an anti-ulcer agent and signals through the protective PGE_2 EP2, EP3, and EP4 receptors. It increases the cytoprotective levels of PGE_2 necessary for maintaining integrity of the gastric mucosa. The risk for nonsteroidal anti-inflammatory drug (NSAID)-induced gastric or duodenal ulcer is decreased with concomitant use of misoprostol.[15] Misoprostol can affect gastric, esophageal and duodenal mucosal integrity from insults related to acids and exodenous factors like NSAIDS. NSAIDs inhibit prostaglandin synthesis, thereby reducing mucus production, bicarbonate secretion, and mucosal blood flow. Continuous blood flow through the microvessels is very crucial for the function and maintenance of structural integrity of the gastrointestinal tract. Microcirculation delivers oxygen and nutrients to all tissues and cells and removes *ad hoc* generated toxic metabolites. See **Figure 1** below for the signaling pathway of these events.

Ahluwalia et al. (2014) attempted to better understand the roles of PGEs and their receptors, signaling pathways including cAMP and CREB, and their relation to VEGF and angiogenesis in the tissue injury healing process (i.e. gastroduodenal and dermal ulcers). Using an esophageal ulcer model in rats, they demonstrated that esophageal mucosa expresses predominantly EP2 receptors and that esophageal ulceration triggers an increase in expression of the EP2 receptor, activation of CREB (the downstream target of the cAMP signaling), and enhanced *VEGF* gene expression. Treatment of rats with misoprostol, a PGE_1 analog capable of activating EP receptors, enhanced phosphorylation of CREB, stimulated VEGF expression and angiogenesis, and accelerated esophageal ulcer healing. In cultured human esophageal epithelial (HET-1A) cells, misoprostol increased intracellular cAMP levels (by 163-fold), induced phosphorylation of CREB, and stimulated VEGF expression. A cAMP analog (Sp-cAMP) mimicked, whereas an inhibitor of

cAMP-dependent protein kinase A (Rp-cAMP) blocked, these effects of misoprostol. These results indicate that the EP2/cAMP/protein kinase A pathway mediates the stimulatory effect of PGEs on angiogenesis essential for tissue injury healing via the induction of CREB activity and VEGF expression.[16]

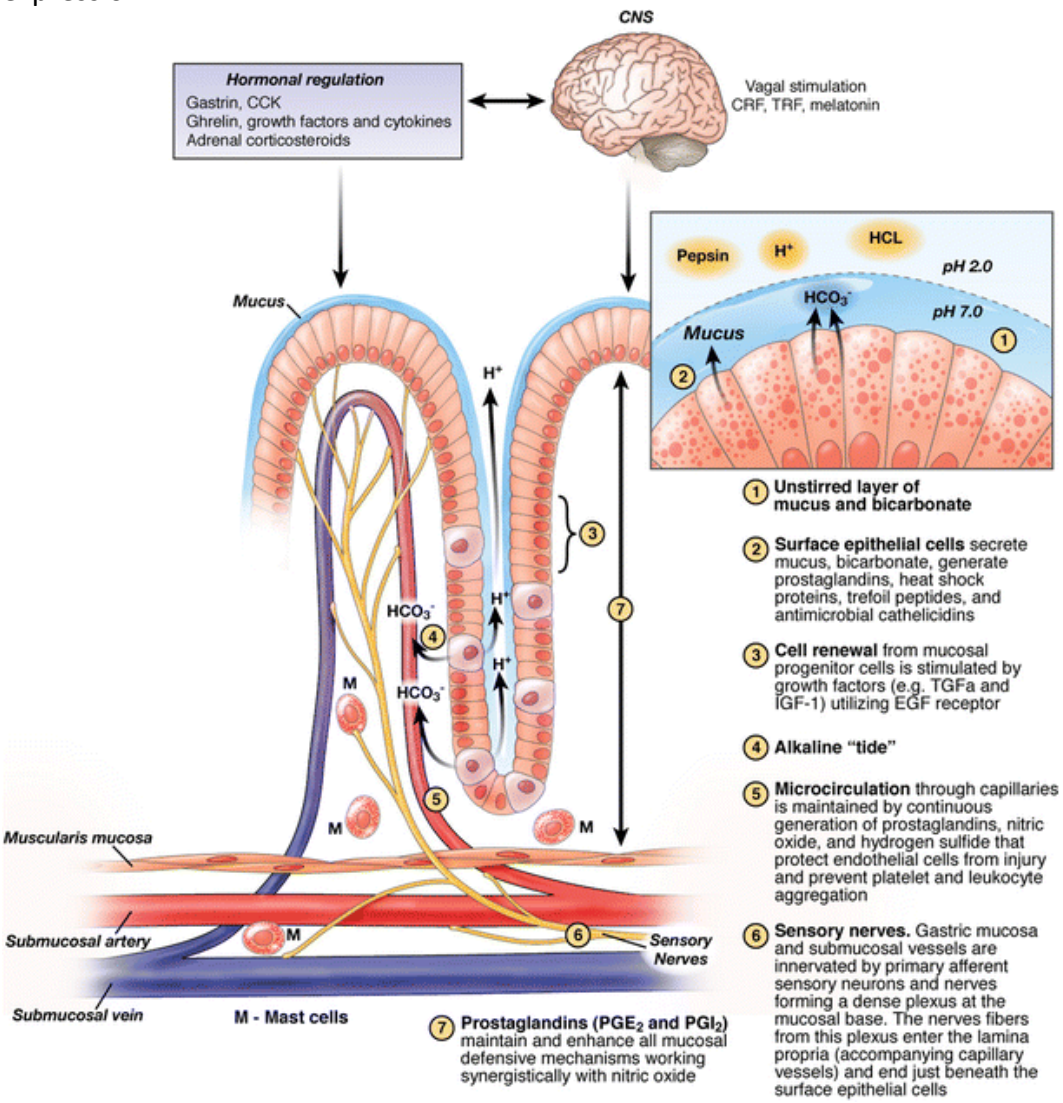

Figure 1. Protective Factors of the Gastric and Duodenal Mucosa [4]

PheWAS data supporting the use of misoprostol in CDI

The PheWAS data summarized in **Appendix A** support, and are consistent with, a role for prostaglandin biology in various GI inflammatory diseases. More specifically, these PheWAS data are consistent with evidence from a variety of sources which suggest a potential role for misoprostol in preventing recurrent CDI.

Preclinical data supporting the use of misoprostol in CDI

Data from mouse model (of CDI) experiments conducted in David Aronoff's laboratory further suggest that misoprostol may be effective in helping to prevent recurrence of CDI infection and improve outcomes of patients with CDI (Data shown in **Appendix B**). Mice first treated with cefoperazone and then challenged by inoculation with spores of *C. difficile* fared significantly

better when treated with misoprostol compared to placebo on multiple outcome variables including survival, diarrhea severity and intestinal permeability.

2. STUDY DESIGN

Experimental Design

A total of 440 patients meeting enrollment criteria (listed below) will be consented and randomized across 3 sites. All patients will receive standard care intended to treat CDI under the care of their physician. Potential participants will be contacted and consent will be obtained if the individual is interested in participating in the study. Those who consent and enroll will be randomized to receive either misoprostol (200 mcg po BID) or matching placebo for 14 days. Participants will be monitored for a total time-period of approximately 70 days with the goal of monitoring for recurrence of CDI during an 8-week follow-up period from the time that standard care (often a short course of antibiotics) is completed. Patients will have blood and stool samples, or rectal swabs if participants are unable to provide a stool sample, collected throughout the study (as described below) to assess biomarkers and to confirm recurrence of CDI (if necessary). The total study time period for study procedures followed by clinical monitoring is anticipated to be about 36 months (biomarker assays and other analyses may be completed after the 36-month time period).

Screening

Patients with a documented positive *C. difficile* lab test (lab tests outlined in inclusion criteria) will be screened with the possibility of enrollment. Outpatients meeting the inclusion criteria will be followed up with via phone call after a positive laboratory test. Inpatients may be followed up with in person during the admission to discuss enrollment in the study. Additionally, patients at other outside/community clinics who hear about the study may contact a study site to determine eligibility as well.

Inclusion Criteria

1. Episode of CDI, defined as both a and b below:
    a. A documented positive *C. difficile* toxin assay (enzyme immunoassay [EIA] or cell cytotoxicity assay) or NAAT for toxigenic *C. difficile* from a stool sample collected while the subject was symptomatic; and
    b. No other plausible explanation for diarrhea (e.g. laxatives).
2. Be ≥18 years of age.
3. Be able to provide signed and dated informed consent.
4. Must be able to read and understand English.

Exclusion Criteria

1. Pregnant, nursing, or planning to become pregnant.
2. If female:
    a. be pre-menopausal (cessation of menses less than or equal to 1 year) and not surgically/medically sterile or not following acceptable non-hormonal method of birth control such as abstinence, intrauterine device, or barrier control for at least 1 complete menstrual cycle before the enrollment visit, or
    b. not using estrogen/progestin containing products for at least 2 months before the enrollment visit through completion of the study.

3. Current or planned treatment with prostanoid therapy.
4. Known hypersensitivity to misoprostol.
5. Have any contraindication to oral/enteral therapy (e.g., severe nausea/vomiting or ileus).
6. Have any clinically significant medical or surgical condition that in the investigator's opinion could interfere with the administration of study drug, interpretation of study results, or compromise the safety or well-being of the subject.
7. Unreliable access to telephone service to allow for contact with study personnel.

3. DRUG PRODUCTS

Misoprostol

The study drug, misoprostol, will be purchased from Novel Laboratories, Inc. Misoprostol will be over-encapsulated using #00 locking capsules by each site's Investigational Drug Service/Pharmacy for the participants at the respective sites. The capsules containing either misoprostol or placebo will be prepared in an identical fashion. To use misoprostol in this study, an Investigational New Drug (IND) application was submitted to the FDA and the safe to proceed letter from the agency (see **Appendix C**).

The matching placebo compounded for this study is identical in appearance and will also be compounded at each site's Investigational Drug Service/Pharmacy. The Investigational Drug Service at the sponsor's institution will manage all shipment of study drug products to the other investigational sites following federal and local shipping regulations.

Dose and Mode of Administration

Patients randomized to receive misoprostol will take two 100 mcg capsules, by mouth, twice per day (200 mcg po BID) for 14 days. Per the drug label, patients will be instructed to take the capsules with food. Patients randomized to placebo will take two capsules, by mouth, twice per day for 14 days.

4. ENROLLMENT AND INFORMED CONSENT

Enrollment

The study will be discussed with patients with a documented positive *C. difficile* lab test (lab tests outlined in inclusion criteria). Outpatients will be scheduled to come into the study site for Visit 1 of the study to be enrolled. Alternatively, it may be arranged for a study team member, to go to the patient's home for the enrollment visit. Inpatients will be followed up with in person.

Informed Consent

Those participants meeting eligibility criteria will be informed by study staff that their participation is voluntary, and they may withdraw from the study at any time, for any reason prior to study completion. Participants will be given ample time to ask questions and will have their questions answered. Those that are interested will be consented. Study staff will give a copy of the informed consent document to the potential participant. No research procedures will be performed until after consent has been provided. Consent or refusal to participate in this study will not affect medical care. An alteration of consent will be used in this study to allow for storage of the standard of care stool sample collected prior to consent if the sample is available. No

samples will be obtained by study staff until participants have consented to participate in the study.

Due to the timing of the clinical lab testing (which, per SOC, occurs before patients will be eligible to be enrolled), when possible, all available positive samples will be held by the clinical lab until patients consent, decline to consent and/or are determined not to be eligible to participate in the study. Samples for patients who consent to participate will be frozen and kept for future study related testing, and all other samples will be discarded.

5. STUDY PROCEDURES

This is a randomized, double-blind, placebo-controlled study in patients age 18 years of age or older who have been recently diagnosed with an episode of CDI. The study will be discussed with patients presenting with an episode of CDI as defined in the inclusion criteria. Patients who meet inclusion/exclusion criteria, will be consented and a pregnancy test will be completed for women of childbearing potential. If participants are pregnant, they will be determined to be a screen failure and will not proceed with any further study procedures. Participants will then be randomized to receive misoprostol or matching placebo. For patients taking antibiotics, consenting patients will be contacted on the last regular business day before the seventh day of oral antibiotic treatment, to remind all patients to begin the treatment that they were randomized to on day 7 of their oral antibiotic treatment; patients not taking oral antibiotics will start taking misoprostol 7-10 days after a positive CDI lab test. **All randomized patients who were taking antibiotics will continue to take oral antibiotics for an additional 4-7 days after starting misoprostol treatment (such that those participants have a 4-7 day overlap of antibiotic and misoprostol treatment),** and begin taking an investigational 200 mcg oral misoprostol or placebo two times a day (200 mcg po BID) for 14 days. Participants will be randomized 1:1 between the misoprostol and placebo arms of the study. All randomized participants will be monitored for recurrence via phone call once per week for an additional 49 days (the total duration of study participation will be 62 days).

In the event that a participant thinks he/she is having a recurrence of CDI, the participant will collect a stool sample and the sample will be tested for the presence of *C. difficile*. If the participant has a confirmed recurrence (positive toxin test) participant will continue to participate in the study procedures as described in Table 1 below. The participant's care subsequent to a recurrence will be the SOC at the discretion of participant's physician (participant will stop taking misoprostol/placebo if recurrence occurs during misoprostol/placebo treatment).

Throughout the study, participants will be followed through their inpatient or outpatient care, depending on their individual clinical conditions and routine care context. Safety will be monitored through the recording of adverse events (all AEs, including SAEs, will be collected while participants are on study drug, and only SAEs will be collected after participants have finished taking the study drug through the end of the study), and clinical safety laboratory testing. A diagnostic stool sample (or rectal swab samples if stool sample cannot be produced) will be collected (self-collection) to confirm CDI infection and at periodic study visits, including (1) the study visit at day -5 to 0, (2) in the case of a possible recurrence (for toxin testing), and (3) at the end of study period at day 62 (+4 day window for scheduling). Blood draws, when possible, will

also occur at periodic study visits, including (1) the study visit at day -5 to 0 (to obtain baseline data before treatment with study drug or placebo), (2) in the case of a possible recurrence (to test for antibodies and other biomarkers), and (3) at the end of study period (to test for antibodies and other biomarkers) at day 62 (+4 day window for scheduling) if possible at an in-person visit. Participants who are unable to return for the day 62 visit will have the option to mail in a self-collected stool sample but would not contribute a blood sample.

An overview of an example study timeline is presented in **Figure 2** below, followed by more detail.

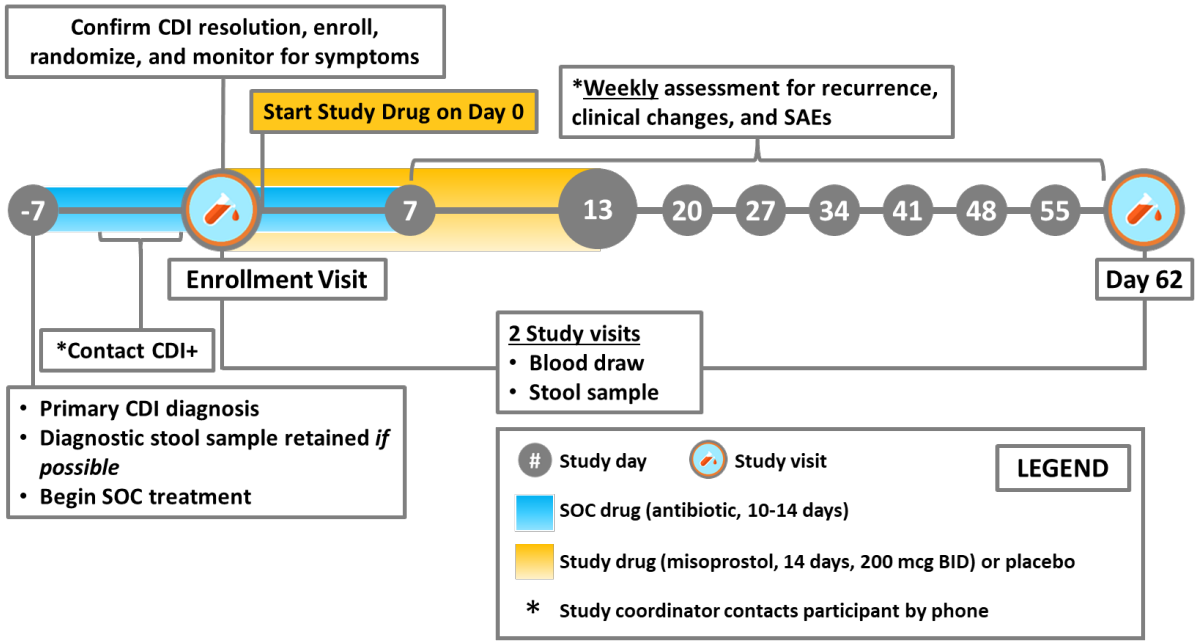

Figure 2. Study Timeline

Pre-enrollment period (Days -7 to Enrollment Study Visit): Patients with a documented positive *C. difficile* lab test (lab tests outlined in inclusion criteria) will be screened for the proposed study and the possibility of enrollment. Once a positive *C. difficile* laboratory test (Inclusion Criteria 1b) is obtained, outpatients will either be asked to schedule a time to come to the study site for an enrollment visit or study staff may travel to the patient for the enrollment visit (if distance is reasonable etc.). When possible, stool samples will be held such that stool samples from patients with confirmed CDI infection (defined in inclusion criteria) who give informed consent and enroll in the study will be available for future analyses.

Enrollment, Study Visit and Randomization (Day -5 to Day 0): Participants meeting inclusion/exclusion criteria will be consented and upon completion of a negative pregnancy test result, if needed, the patient will be randomized to misoprostol or placebo. The project statistician will implement a permuted block allocation scheme, likely based on block sizes of 2 and 4, stratified by site, sex, and severity of illness, as defined by IDSA criteria for severity (if bloodwork is not available, the patient will be assumed to be "non-severe"):

- Non-severe (or bloodwork unavailable): Leukocytosis with a WBC count of ≤15,000 cells/mL and serum creatinine <1.5 mg/dL

- Severe: Leukocytosis with a WBS count >15,000 cells/mL or serum creatinine >1.5 mg/dL
- Fulminant: Hypotension or shock, ileus, megacolon

Randomization will be accessed at each site through REDCap. The randomization module in REDCap allows the statistician to load a randomization table that will allow the sites to click a 'randomize' button. Study participants will be dispensed study drug at this visit and will be instructed on the use of the medication. If study participants are on a course of antibiotics, they will begin taking investigational therapy on Day 0 (Day 7 of antibiotics) in addition to oral antibiotics. If study participants are not on a course of antibiotics, they start taking the study drug 7-10 days after their positive CDI lab test. Participants will be given the study medication and stool diary and instructed on how to complete the diary. Blood samples (if possible) and stool samples (or rectal swabs) will be collected at this visit. A stool sample collection kit to collect a stool sample at home for future visits will be provided.

Study Drug Administration Period (Days 0-13): Participants will be called (or visited in person if inpatient or present at clinic) on Day 0 (or on the last regular business day before day 0 occurs) to be reminded to begin study drug. Participants will continue taking oral antibiotics, if applicable, such that there is a 4-7 day overlap during at which time the participant will be taking both oral antibiotics and investigational therapy. They will be instructed to record date and time of each study drug administration (approx. 12 hours apart), all adverse events, date and time of any unformed (loose or watery) stools, and any other medications taken.

Follow-up phone calls 1 & 2 (Day 3 +/- 1 day, Day 7 +/- 1 day): All participants will be contacted on Day 3 and Day 7 of taking the study medication to review symptoms, discuss adherence to study drug, review stool and drug diary, and complete the CDI QOL Patient Questionnaire and the symptom questionnaire.

Follow-up phone calls 3-9 (Days 13-55 +/- 2 days): Study personnel will contact participants at least once weekly through Day 62 (via telephone contacts) to inquire about any occurrence of diarrhea and/or loose/watery stools, completion of a symptoms questionnaire, and the CDI QOL Patient Questionnaire.

Follow-up visit (Day 62-66): If possible, participants will return on day 62 for a study visit to collect blood and stool samples; a four day window is permitted in case of scheduling difficulties. Participants will complete the CDI QOL Patient Questionnaire, the symptom questionnaire, blood samples will be collected (if possible) and patients will be asked to bring in a stool sample (if a stool sample cannot be provided, participants will be asked to provide a rectal swab). If patients cannot attend this visit on site, a home visit can be arranged for the study team to come to their home or they will be given the option to provide a self-collected stool sample that will be picked up from their home. A blood sample will not be obtained if an in-person visit is not completed.

Recurrence visit (Day 7 - Day 62): If possible, participants exhibiting symptoms of a recurrence of their CDI after completion of antibiotic treatment will come to the study site for a follow-up visit. At this study visit participants will complete the CDI QOL Patient Questionnaire, the symptom questionnaire, blood samples will be collected (if possible) and patients will be asked to bring in a stool sample to this visit. A home visit can also be arranged if the participant would prefer a site study team member to come to their home. If an in-person visit is not able to be

scheduled within 24 hours of the recurrence of symptoms, the participant will be asked to follow the instructions provided so that a stool sample/rectal swab can be picked up at their home.

A schedule of the study events is detailed below in **Table 1**.

Procedures	**Clinical Care** Day -7 to -3	**Pre-Enrollment Period** Day -7 to enrollment	**Enrollment Visit** Day -5 to Day 0	**Start Study Drug** Day 0	**Follow-up Phone Calls 1&2** Day 3 & Day 7 (+/- 1 day)	**Follow-up Phone Call 3** Day 13 (+/-2 days)	**Follow-up Phone Call 4** Day 20 (+/-2 days)	**Follow-up Phone Call 5** Day 27 (+/-2 days)	**Follow-up Phone Call 6** Day 37 (+/-2 days)	**Follow-up Phone Call 7** Day 41 (+/-2 days)	**Follow-up Phone Call 8** Day 48 (+/-2 days)	**Follow-up Phone Call 9** Day 55 (+/-2 days)	**Recurrence Visit**[1] Day 14 - Day 62	**Final Study Visit** Day 62 (+4 days)
Start Standard of Care Treatment	X													
Contact CDI+ potential participants		X												
Informed consent			X	X[8]										
Demographics			X	X[8]										
Medical history			X	X[8]										
Pregnancy test			X	X[8]										
Concomitant medication review			X	X	X	X	X	X	X	X	X	X	X	X
Physical Exam			X	X[8]										
Vital signs			X										X[1]	X
Height			X											
Weight			X										X[1]	X
CDI toxin test[2]	X[2]												X[1, 2]	
Participants begin taking study drug				X										
Confirm CDI symptom resolution			X	X										
Symptom questionnaire			X	X	X	X	X	X	X	X	X	X	X[1]	X
CDI Patient Quality of Life Questionnaire			X	X	X	X	X	X	X	X	X	X	X[1]	X
Participant Medication & Stool Diary				X	X	X								
Blood draw			X										X[1]	X
CBC w/ diff[2]			X[2]										X[1, 2]	X[2]
Complete Metabolic Panel[2]			X[2]										X[1, 2]	X[2]
Stool Sample or Rectal Swab Collection	X[4]		X										X[1]	X
Serum anti-toxin IgG to TcdA & TcdB[6]			X[6]										X[1, 6]	X[6]
Phone call to participants		X		X	X	X	X	X	X	X	X	X		
Randomization			X											

Procedures	**Clinical Care** Day -7 to -3	**Pre-Enrollment Period** Day -7 to enrollment	**Enrollment Visit** Day -5 to Day 0	**Start Study Drug** Day 0	**Follow-up Phone Calls 1&2** Day 3 & Day 7 (+/- 1 day)	**Follow-up Phone Call 3** Day 13 (+/-2 days)	**Follow-up Phone Call 4** Day 20 (+/-2 days)	**Follow-up Phone Call 5** Day 27 (+/-2 days)	**Follow-up Phone Call 6** Day 37 (+/-2 days)	**Follow-up Phone Call 7** Day 41 (+/-2 days)	**Follow-up Phone Call 8** Day 48 (+/-2 days)	**Follow-up Phone Call 9** Day 55 (+/-2 days)	**Recurrence Visit**[1] Day 14 - Day 62	**Final Study Visit** Day 62 (+4 days)
Dispense study drug			X	X[8]										
Complete Case Report Forms (CRFs)	X	X	X	X	X	X	X	X	X	X	X	X	X[1]	X
Collection of adverse events				X	X									
Collection of serious adverse events			X	X	X	X	X	X	X	X	X	X	X	X

Table 1: Schedule of events

Procedures	**Clinical Care** Day -7 to -3	**Pre-Enrollment Period** Day -7 to enrollment	**Enrollment Visit** Day -5 to Day 0	**Start Study Drug** Day 0	**Follow-up Phone Calls 1&2** Day 3 & Day 7 (+/- 1 day)	**Follow-up Phone Call 3** Day 13 (+/-2 days)	**Follow-up Phone Call 4** Day 20 (+/-2 days)	**Follow-up Phone Call 5** Day 27 (+/-2 days)	**Follow-up Phone Call 6** Day 34 (+/-2 days)	**Follow-up Phone Call 7** Day 41 (+/-2 days)	**Follow-up Phone Call 8** Day 48 (+/-2 days)	**Follow-up Phone Call 9** Day 55 (+/-2 days)	**Recurrence Visit**[1] Day 14 - Day 62	**Final Study Visit** Day 62 (+4 days)
Metabolomic profiling of stool sample[3, 5]	X[3,5]		X[3,5]										X[1,3,5]	X[3,5]
Ribotyping of stool sample[5]	X[3,5]		X[3,5]										X[1, 3, 5]	X[3,5]
Nutritional Immunity/Fecal metals analysis[6]	X[3,6]		X[3,6]										X[1,3, 6]	X[3,6]
Microbiome sequencing of stool samples[7]	X[3,7]		X[3,7]										X[1, 3, 7]	X[3,7]

[1] – This visit will occur if participant experiences symptoms of CDI recurrence.
[2] – Completed at individual sites' local Clinical Laboratory.
[3] – To be completed at completion of study.
[4] – Positive samples will be retained by local Clinical Laboratory until consent is obtained, all samples that are retained and consent is not obtained will be discarded by local Clinical Laboratory. Note, it may not always be possible to obtain this sample.
[5] – To be completed by Microbiology lab at Washington University in St. Louis.
[6] – To be completed by sponsor investigator's lab at Vanderbilt University Medical Center.
[7] – To be completed by Vanderbilt University Medical Center's Genomics Core Facility.
8 – To be completed if not obtained prior to Day 0.

Table 2: List of the research laboratory tests that will be completed on the stool samples collected throughout the study. Note, it may not be possible to collect all samples from all participants. The exploratory analyses will be completed on the samples it is possible to collect.

A schedule of the laboratory tests that will be completed on the stool samples collected at the following study visits appears above in **Table 2.**

Misoprostol may have gastrointestinal (GI) side effects (*e.g.*, diarrhea, abdominal cramping) during the first few days of use, which is not to be confused with recurrence of disease. Since patients do not generally experience a recurrence until after they have completed the full course of antibiotic therapy, any GI symptoms occurring during the first 4-7 days of investigational therapy (which overlaps with antibiotic treatment) will be presumed to be a side effect of misoprostol (captured as an adverse event) and not a recurrence of CDI. Depending on the

severity of symptoms (specifically GI symptoms such as diarrhea and abdominal cramping), misoprostol dose may be reduced to minimize discomfort (100 mcg po BID minimum dose) after contacting the lead center for guidance.

An independent unmasked Data Safety Monitoring Board (DSMB, more detail in **section 7** below) will review available data at predefined time points during the study.

Both the participant and everyone interacting with the participant on the study team (PI, coordinator, nurses, etc.) will be blinded throughout the duration of the study. There is no plan to ever make the treatment assignments known to the participants during or after the completion of the study. In the event of a serious adverse event requiring knowledge of the treatment allocation to protect the safety and welfare of the participant, the blind may be broken following study standard operating procedures. Study personnel will remain blinded.

Concomitant Medications
All medications that a patient is taking at Day -7 through completion of final study follow-up visit will be collected. At each study visit, all concomitant medications will be reviewed and changes will be recorded. Medications will also be reviewed over the phone at each follow up phone call and changes will be recorded.

Medication information collected will include:

- Drug
- Dose
- Unit
- Route of administration
- Frequency

Screen Failures and Study Withdrawals
Participants not meeting inclusion/exclusion criteria will not be allowed to participate in any further study procedures. Participants who are no longer allowed to participate in the study or voluntarily remove themselves from the study after consent and prior to randomization will be considered a Screen Failure.

Participants who are removed from the study, either by discretion of the PI or participant withdraws consent after randomization, will be considered a Study Withdrawal.

6. RISKS

Drug Related Risks
See package insert.

Misoprostol has been used clinically for several decades and potential risks are well documented in the medical literature. Specific to this study, Misoprostol may have gastrointestinal (GI) side effects (e.g., diarrhea, abdominal cramping) during the first few days of use. Depending on the severity of symptoms, misoprostol dose may be reduced to minimize discomfort (100 mcg po BID minimum dose).

Any clinical event of diarrhea or loose/watery stools will be recorded on a case report form. Participants who have ≥3 unformed (loose or watery) stools within a 24-hour period (or those with any diarrhea of concern to the subject or investigator) will contact study personnel as soon as possible for clinical assessment. If the clinical event of diarrhea occurs after completion of final antibiotic dose (if applicable), arrangements will be made for collection of a stool sample to determine whether the event is a recurrence of CDI. Whenever possible, the collection of the stool sample should occur at the study site or collected by a study team member at an in-home visit. However, because it is important to collect a stool sample while the subject is symptomatic, participants who are outpatients will be provided with a stool collection kit and instructions for stool sample collection at home; participants can bring these samples with them to the study site, have them available for an at-home visit, or have them picked up and delivered to the study site under direction of study personnel. Stool samples collected in association with an adverse event of diarrhea or loose/watery stools will be tested for *C. difficile* toxin to confirm if recurrence has occurred. All other aspects of management of participants with diarrhea or loose/watery stools (including additional laboratory testing and any treatment for diarrhea/CDI) will be at the discretion of the treating physician based on clinical presentation.

Other Risks

1. **Blood sampling:** There are minor risks and discomforts associated with blood sampling and venipuncture. This may cause a brief period of pain and possibly a small bruise at the site. Occasionally, a person feels faint when their blood is drawn. There is a small risk of bleeding after removal of the needle and possibly a bruise at the site, which can be prevented by tight compression on the site. Rarely, an infection develops which can be treated. Proper aseptic techniques will be used to minimize these side effects.
2. **Pregnancy Risks:** If a subject is a female who could be pregnant, a urine pregnancy test will be done to make sure she is not pregnant. If she is pregnant, she will be excluded from the study.
3. **Other Risks:** There may be risks that are not known at this time. If any new information becomes known, participants will be notified.

7. REPORTING OF ADVERSE EVENTS OR UNANTICIPATED PROBLEMS INVOLVING RISK TO PARTICIPANTS OR OTHERS

Adverse Event Reporting

Serious adverse events (SAEs) may occur in older adults in the study population, but are not generally expected otherwise. Note, serious adverse events related to the use of the study drug (misoprostol) are not expected to occur based on extensive previous human experience. Since misoprostol is a marketed drug with a known safety profile, non-serious adverse events will only be collected while participants are receiving study drug, after completion of the study drug only SAEs will be collected. Individual sites must report SAEs to the sponsor within one business day of the study team's awareness of the event. Additionally, the site will report all SAEs to their IRB per local policy.

All study personnel will be responsible for the accurate documentation, investigation and follow-up of all serious adverse events and unanticipated problems involving risks to participants and

others that are possibly related to study participation. When a serious adverse event is identified, the necessary medical personnel will immediately be notified, as well as the DSMB Chair.

Adverse Event

An AE is any unexpected medical occurrence in any study participant administered an investigational product and may or may not have a causal relationship with the treatment. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not considered related to the medicinal (investigational) product.

AEs include any of the following:

- Worsening (change in nature, severity or frequency) of conditions present at the onset of the trial
- Subject deterioration due to the primary illness
- Intercurrent illnesses
- Drug interactions
- Events related or possibly related to concomitant medications
- Abnormal laboratory values or changes of vital signs, as well as significant shifts from baseline within the range of normal, which the Investigator considers to be clinically significant

Serious Adverse Event

During clinical investigations, serious AEs may occur. If the event is suspected to be drug-related, the event may be significant enough to lead to important changes in the way the medicinal product is developed (*e.g.*, change in dose, population, needed monitoring, consent forms). This is particularly true for reactions, which, in their most severe forms, threaten life or function. A serious AE (SAE) is any untoward medical occurrence that:

- Results in death
- Is life-threatening. 'Life-threatening" refers to an event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe (ICH E6).
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/ incapacity (as per PI's opinion)
- Is a congenital anomaly/birth defect
- Is another medically important condition. Important medical conditions that may not result in death, be life-threatening or require hospitalization may be considered as SAEs when, based upon appropriate medical judgment, they may jeopardize the subject or may require intervention to prevent one of the outcomes listed in the definition above. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse [Code of Federal Regulations Title 21, Volume 5, 21CFR312.32, revised April 1, 2006].

Please note: Serious is not synonymous with severe. An event may be severe (e.g., severe headache) but still be of minor medical significance. Serious refers to an event that poses a threat to the subject's life or functioning.

Assigning Severity to an Adverse Event

Severity of SAEs will be categorized based on Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0.

Assigning Relationship of Adverse Event to Study Drug (Causality)

The PI will determine the relationship of each SAE to study drug (i.e., causality) by using the classification criteria 'not related', 'possibly related', or 'probably related'. Descriptions of the three classification categories are as follows:

Not Related

Exposure to study drug has not occurred; administration of study drug and the adverse event are not reasonably related in time; or the AE/SAE is considered by the Investigator to be due to a pre-existing condition, a known manifestation of the target disease, a recurrent condition, or is likely explained by environmental or diagnostic therapeutic factors or was pre-existing and did not deteriorate.

Possibly Related

The AE/SAE occurred during or within a reasonable period of time after administration of the study drug, or a pre-existing event worsened within an appropriate period of time after administration of study drug, but the AE/SAE could be explained equally well by factors or causes other than exposure to the study drug. This category will also be used if there is a lack of information, or insufficient or conflicting evidence exists for classifying the causality of the AE/SAE.

Probably Related

The AE/SAE occurred during or within a reasonable period of time after administration of the study drug or a pre-existing event worsened within an appropriate period of time after administration of study drug, and at least one of the following criteria is applicable:

- the event could not be explained by the clinical condition or history of the subject, environmental or toxic factors, or other diagnostic or therapeutic measures;
- the event was an expected ADR associated with study treatment or a class-labeled drug effect;
- the AE/SAE subsided or disappeared after withdrawal or dose reduction of study treatment; or
- the AE/SAE recurred after re-exposure to study treatment.

Adverse and Serious Event Recording

During study drug administration all AEs and SAEs will be recorded. Each event occurring to a subject, either spontaneously revealed by the subject or observed by the Investigator, whether believed by the sponsor to be related or unrelated to the study drug, must be recorded on the AE/SAE Case Report Form within the EDC.

After a participant has completed taking the study drug only SAEs (not all AEs) will be recorded.

Site investigators will record all appropriate information into the CRF including time of onset, duration, and the precautions carried out.

Laboratory results will be recorded on the case report form and the investigator will indicate whether abnormal results (high or low) are clinically or are not clinically significant. Clinically significant laboratory abnormalities will be entered on the 'AE/SAE Event Form' of the CRF. Clinically significant changes in vital signs (*e.g.*, tachycardia) or other clinically significant changes observed by the physician will be entered in the appropriate CRF.

Serious Adverse Event Reporting

All SAEs must be reported to the sponsor within 24 hours of knowledge of the event to the lead site/sponsor and will be reported to the IRB, DSMB Chair and FDA in accordance with applicable regulations and policies.

Data Safety Monitoring Board (DSMB)

An independent, unmasked Data Safety Monitoring Board (DSMB) will review available data at predefined time points during the study according to a monitoring plan. Briefly, all SAEs will be reviewed by one member of the DSMB (DSMB Chair) and this individual will make a determination if it is clear that the SAE was not (or was) related to the use of study drug. If it is unclear whether or not the SAE was not (or was) related to the use of study drug, then the case will be discussed by the entire DSMB. All SAEs will be reported promptly to the sponsor and will be reported to the IRB and FDA in accordance with applicable regulations and policies.

8. STUDY WITHDRAWAL/DISCONTINUATION

Participants will be advised that they have the right to withdraw from the study at any point in time for any reason. If during the course of the study they no longer want to participate, they will be withdrawn from the study. Subjects who wish to withdraw from the study will be asked permission to allow the study team to review their medical records through the Day 62 visit window to determine if recurrence occurred.

Participants who begin to exhibit symptoms of recurrence between the enrollment visit and randomization will be considered a screen fail for the study.

9. STATISTICAL CONSIDERATIONS

The text below is a summary of the statistical analysis plan (SAP), for full details see the SAP.

Primary Hypothesis

The treatment of patients with CDI with misoprostol, in addition to standard care, will result in a relative reduction in the rate of recurrence by 40% when compared to patients treated only with standard care.

Planned Interim Analyses

We will perform an interim safety analysis after 44 patients (10% of accrual) have completed study treatment. It is expected that of the first 44 patients, although there might be dose adjustments, there will be no greater than 5 withdrawals due to diarrheal events reasonably attributed to misoprostol use. If more than 5 diarrheal events reasonably attributed to

misoprostol use occur among the first 44 patients which require study withdrawal, enrollment will be interrupted for safety concerns. All currently enrolled participants will be followed to completion and the data will be analyzed as planned to estimate effect sizes; no statistical testing will be performed. The data will be presented to the DSMB to determine whether the study should proceed or not. If no more than 5 diarrheal events (which can be attributed to treatment with misoprostol) occur among the first 44 patients which require study withdrawal occur, the study will continue without interruption of enrollment.

A planned interim futility analysis at 176 participants will be conducted. This will allow the study to be terminated early in the event there is a less than acceptable chance of detecting the desired effect, therefore not wasting funds or exposing patients to unnecessary risk with no commensurate benefit. We propose the interim analysis for efficacy will be completed using the Lan-DeMets procedure with an O'Brien-Fleming stopping boundary to account for the 2 interim analyses and the final analysis. The significance boundary for final analysis will be $P \leq .048$. The interim analysis for futility will be based on the stochastic curtailment method. The interim analyses for futility will be performed by calculation of conditional power, an estimate of the probability that the study shows a statistically significant effect on the primary endpoint, i.e. a reduction in the rate of recurrence, given the results to date and assumptions regarding outcome through the end of the study. A recommendation to stop the trial for futility will require a conditional power below 30%, under the observed efficacy trend at the time of interim analysis, with two-sided type I error less than 5%. The proposed sample size of 196 per arm is adjusted for the interim analyses, i.e., types I and II errors.

Endpoints

To evaluate the exploratory objectives of the study assessing oral misoprostol compared to placebo in patients aged 18 years or older with an episode of CDI, the following endpoints will be measured:

- Primary endpoint: Rate of clinical recurrence of CDI in patients aged 18 years or older during the first 42 days after standard of care therapy is stopped. Rate of recurrence among misoprostol treated participants will be compared with that of placebo treated participants to determine if misoprostol has efficacy in preventing rCDI. rCDI will be defined as people who meet criteria for CDI in the 42 day follow-up period.
- Safety endpoint: Safety and tolerability of misoprostol in patients aged 18 years or older with rCDI compared to placebo as measured by treatment emergent adverse events and standard laboratory assessments.
- Secondary endpoint: Number of recurrences during the follow-up period (for those who have a first recurrence during the follow-up time period)
- Secondary endpoint: Time to resolution of diarrhea (TTROD; for those with recurrence).
- Exploratory endpoint: Serum biomarkers/toxin A and B antibodies (from blood draws).
- Exploratory endpoint: Recovery of bowel microbiota (from microbiome collection).
- Exploratory endpoint: Titer of toxins A and B in stool (for those with recurrence).
- Exploratory endpoint: Severity of disease (for those with recurrence), determined using a combination of severity criteria used in Hospital specific guidelines, SHEA/IDSA guidelines, Zar criteria, or other sources:
    - WBC count

- Serum creatinine
- Temperature
- Albumin level
- ICU or step down unit admission
- Endoscopically or histologically confirmed pseudomembranous colitis
- Toxic megacolon, perforation, colectomy, or septic shock requiring ICU admission and pressors
- Hospital readmission
- Validated quality of life instrument

Assessments by interview, recording of adverse events, clinical laboratory tests, and other evaluations. Patients will be asked to record self-administration of study drugs and symptoms.

Safety analyses will include displays of adverse events and laboratory values and will include descriptive statistics for the study population where applicable. Adverse events will be summarized by treatment, intensity, and relationship to study drug.

Statistical methods

For data summary, frequency tables will be generated for categorical variables, and continuous variables will be expressed as means ± SD or medians and interquartile ranges as appropriate. We will then compare the outcomes of interest between the patient groups who receive treatment vs placebo. The primary outcome of this study is the clinical recurrence rate of CDI in patients aged 18 years or older during the first 8 weeks after SOC therapy is completed. Secondary outcomes will include treatment emergent adverse events (AE), standard laboratory assessment for AE, recovery of bowel microflora, time to recurrence, time to resolution of diarrhea, severity of diarrhea, titer of toxin in stool. Serum biomarkers/toxin antibodies from blood draws will be recorded for each patient. Comparisons between control and treatment patients will use *t*-test or Kruskal-Wallis test for continuous variables and Fisher exact tests for categorical variables. Secondarily, we will model outcomes using generalized linear mixed-models with linear or logit link functions as appropriate. A two-tailed $p < 0.05$ will be considered significant. Data analyses will be performed using standard statistical software, such as the current version of R (http://www.r-project.org/).

The sample size calculation is based on relative reduction in the proportion of participants with recurrent events between the placebo and misoprostol treatment group. We assume the patient population may be heterogeneous and has variable baseline recurrences rate due to their baseline variables before applying misoprostol, such as disease severity (as determined by IDSA guidelines), sex differences, or study site differences (stratification variables). However, recurrence rates are, in general, well established, with age being a key variable influencing recurrence rates. For patients experiencing their first ever episode of CDI, a conservative estimate of recurrence rate is 20% and a recurrence rate more consistent with previous data for older patients is closer to 30-40%. There are demographics of patients with even higher recurrence rates. We performed power calculations using a range of combinations of control group recurrence rate (p0) and % reduction in recurrence rate by the treatment (effect size).

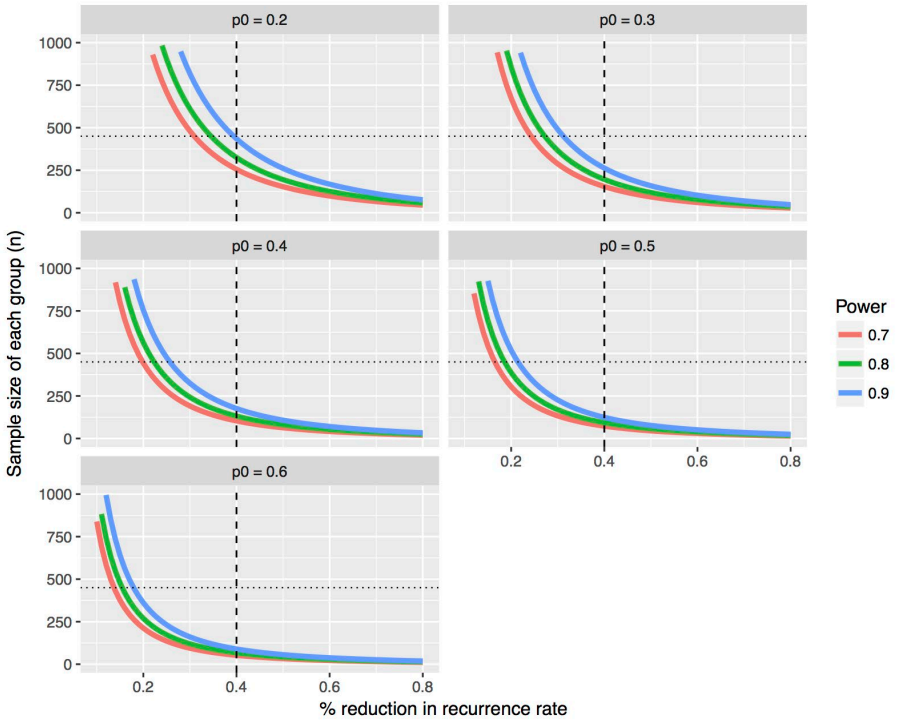

Figure 2. Power Curves for control recurrence rates from 20% to 60%

As illustrated in **Figure 2**, for a set of control infection recurrence rates (p0) varying from 0.2 – 0.6 (corresponding subpanels), the power curves show the sample size required (assumed same for the control and the treatment groups) as a function of % reduction in recurrence rate (x-coordinate), in order to achieve the anticipated power in the 70% - 90% range (red, green, and blue lines). The vertical dashed line indicates the anticipated 40% reduction in recurrence rate. For example, to gain 80% power for a 50% reduction in recurrence rate, we would require 119 or 80 patients in each group when control recurrence rate is at 0.3 or 0.4 respectively. To gain 80% power for a 40% reduction in recurrence rate, we would require 196 or 132, patients in each group when control recurrence rate is at 0.3 or 0.4 respectively. **We aim to collect data on 196 participants in each group (392 total participants)** as this will afford us 80% power to detect a 40% reduction in recurrence when the control recurrence rate is 30%. **We aim to enroll 440 total participants** which would allow for a 10% overall attrition rate.

10. PRIVACY/CONFIDENTIALITY ISSUES

Data will be maintained in paper and computer files that will be locked and password-protected. Only the study team will have access to identifying information. Information will be maintained indefinitely by the PI.

Each site will be required to have a local coordinator(s) who will be responsible for entering the study information into the web-based database and uploading the identifiable source documents/medical records associated with the study information. For Vanderbilt participants, access to the electronic health record (EHR) will be provided to study data monitors. A web-based database housing the electronic case report forms (eCRFs) has been designed for the study, which will improve efficiency, lower cost of the study, and speed up publication of the results. Use of drop-down selection lists, radio buttons, checkboxes, and validation checks will be incorporated

to aid the speed, accuracy and consistency of data entry. The database will be backed up regularly.

This project will utilize the Research Electronic Data Capture (REDCap) platform for data collection and management. Project team members listed as Key Study Personnel with existing electronic health record (EHR) system access rights may also be granted use of REDCap Clinical Data Pull (CDP) tools. These tools are designed to enable transfer of relevant study-related data directly from the EHR into REDCap. The latest version of each eCRF and any applicable source document worksheets will be available as a PDF file on the REDCap website for use by study personnel.

The web-based Randomization Module will be used by authorized site personnel for the purpose of randomizing eligible patients. The Study Coordinator (or other appropriate study team member) will log onto the REDCap system via the PROCLaIM Portal (Proclaimstudy.org) using a unique username and confidential password. When a subject is deemed eligible, a unique subject ID and record will be created. Once the Study Coordinator has entered the required subject information and clicked "Randomize", the computer program will display a message that the treatment assignment has been sent to the pharmacy for the subject. The subject is considered randomized at the time the REDCap system generates the treatment assignment.

VUMC employs several layers of data protection to ensure data security. The first part of security is physical protection of the hardware systems, access to which is limited to authorized personnel. By limiting access, ensuring only authorized personnel have access, and tracking all entry, we can ensure this risk is minimal. Additionally, REDCap has a built-in audit trail that tracks all user activity and changes made to the data entry fields with a date-time stamp.

11. FOLLOW-UP AND RECORD RETENTION

The research material obtained in this study will be recorded data available through the EMR and volunteered by participants, and recorded during study procedures, obtained specifically for research purposes and kept indefinitely. Study teams (except VUMC) will be expected to upload non-redacted source into the EDC for source verification. For more detail regarding data monitoring, please refer to the trial's Data Safety Monitoring Plan. Blood and stool samples will be obtained for screening purposes and for analysis of the various parameters under study. Initial, available diagnostic stool specimens for patients who test positive for CDI, but do not enroll in the study, will follow local lab/hospital policy for discarding sample. A separate secure database of identified patient information will be maintained in order to store and analyze collected study data. Blood and stool samples will be stored at VUMC indefinitely. Samples sent to Washington University in St. Louis will also be stored indefinitely. All study data will be recorded in REDCap through the Portal will be stored indefinitely on secure servers. For more detail regarding sample collection and storage, please refer to the trial's Lab Manual. Any research records that are kept on paper or in other electronic formats will be stored according to applicable laws and regulations. All research records will be accessible for inspection by authorized representatives of the IRB, federal regulatory agency representatives, and the department or agency supporting the research.

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APPENDIX A PHEWAS DATA TABLES

The tables below show information about the variants for the relevant prostaglandin receptors covered in our genotyping platform, the Illumina Infinium Exomchip. **Table 1** shows basic information on the variants covered on our platform.

***Table 1**. Relevant SNPs for PTGER2-4*

SNP	rsID	Mutation	SIFT	PP2	Exome MAF*	Populations with highest MAF[17]
Gln379Arg	rs141688168 *(PTGER3)*	Missense	0.63	0.0	0.2%	Punjabi in Pakistan 1% Bengali in Bangladesh 0.6%
Tyr30Cys; Tyr285Cys	rs139552094 *(PTGER2)*	Missense	0.0	0.566	0.3%	Toscani in Italy 0.9% Mexican ancestry in LA 0.8%
Val294Ile	rs111866313 *(PTGER4)*	Missense	0.4	0.002	2.7%	Finnish in Finland 3.5% Utah residents with Northern/Western European ancestry 3% Puerto Ricans in Puerto Rico 2.4%
Cys83Gly	rs111965614 *(PTGER2)*	Missense	0.0	0.999	0.6%	Colombians in Colombia 3.2% Iberians in Spain 2.8% Toscani in Italy 2.8%
-	rs11209710 *(PTGER3)*	Intronic	-	-	32%	East Asians 62% South Asians 57%

* Minor allele frequency reflects the MAF in the Exomechip European ancestry population genotyped for this analysis.

Sorting Intolerant from Tolerant (SIFT) scores at or below 0.05 are considered to be deleterious; those above 0.05 are considered to be tolerated.[18] Polyphen2 (PP2) scores below 0.447 are considered benign; those higher than 0.908 are considered probably damaging; and those in between possibly damaging.[19] The scores above are color coded to indicate benign or tolerated (green), possibly damaging (orange) and probably damaging (red).

Table 2 below summarizes existing literature on the functional effects of SNPs with PheWAS associations that will be displayed in subsequent tables below.

***Table 2**. Literature Describing SNP Functional Effects*

SNP	Additional SNP details	Predicted effects	Studies of SNP effects
rs141688168 *(PTGER3)*	Gln379Arg (missense)	Predicted to be benign/tolerated by SIFT and PolyPhen2 algorithms	None identified to date
rs139552094 *(PTGER2)*	Tyr30Cys; Tyr285Cys (missense)	Predicted to be deleterious/possibly damaging by SIFT and PolyPhen2 algorithms, respectively	None identified to date
rs111866313 *(PTGER4)*	Val294Ile (missense)	Predicted to be benign/tolerated by SIFT and PolyPhen2 algorithms	Known IBD gene [20]; Protective in IBD [21]
rs111965614 *(PTGER2)*	Cys83Gly (missense)	Predicted to be deleterious/probably damaging by SIFT and Poly-Phen2 algorithms, respectively	None identified to date
rs11209710 *(PTGER3)*	Intronic	(no prediction data for intronic SNPs)	No significant effect on cancer risk [22]; no significant effect on ACE-inhibitor associated cough; patent application, Biomarkers for assessing peripheral neuropathy response to treatment with a proteasome inhibitor[23]

Based on directionality of the ORs displayed in **Table 3**, the below SNPs would all function like receptor antagonists (i.e. opposite of misoprostol's effects). If this is true, any other diseases associated with those SNPs with a >1 OR (risk) would be potential new therapeutic indications for misoprostol.

***Table 3**. PheWAS Results Validating Current Use*

Condition	PheWAS Code	rsID (gene)	p Value	Odds Ratio	Case Carriers	Total Cases
Gastritis and duodenitis	535	rs111965614 *(PTGER2)*	0.00476	1.88	22	968
Ulcer of esophagus	530.12	rs139552094 *(PTGER2)*	0.01113	6.44	2	72

Table 4 below shows known and potential new GI inflammatory conditions that could be plausible to treat with misoprostol.

Table 4*. Gastrointestinal PheWAS Results*

Condition	PheWAS Code	rsID (gene)	p Value	Odds Ratio	Case Carriers	Total Cases
Ulcerative colitis	555.2	rs139552094 *(PTGER2)*	0.02431	3.19	4	238
Ulceration of intestine	556.1	rs141688168 *(PTGER3)*	0.02549	5.01	2	76
Other specified gastritis	535.8	rs11209710 *(PTGER3)*	0.01728	1.32	106	161

APPENDIX B MOUSE MODEL OF CDI DATA

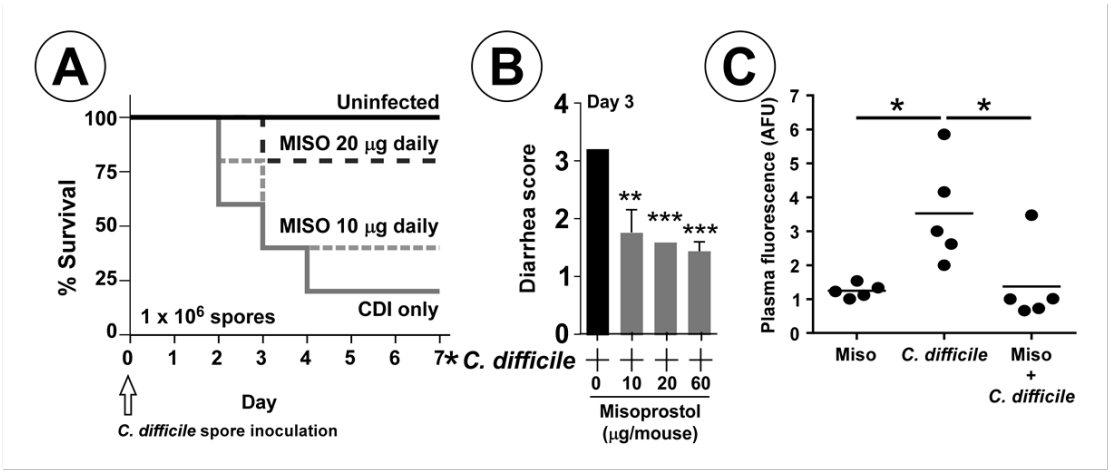

Figure 5. Misoprostol improves CDI in mice. Female C57BL/6 mice were treated with cefoperazone for 5 days followed by 2 days of recovery in regular drinking water and then challenged with 1 x 10^6 spores of strain M7404. In (**A**) mice received misoprostol by intraperitoneal (i.p.) injection daily starting on the day of inoculation. 60 μg i.p. daily showed the same results as 20 μg (not shown). N = 5 mice per group. * *P* <0.05 compared to uninfected control by Log-rank (Mantel-Cox) test. In (**B**) mice were treated with cefoperazone for 5 days followed by 2 days of recovery in regular drinking water and then challenged with 1 x 10^4 spores of strain M7404. Mice received misoprostol by i.p. injection (or vehicle) daily and stools were scored for severity of diarrhea on a 4 point scale (1 -- normal, 2 -- soft stool/discolored, 3 -- wet stained tail/mucous, 4 -- liquid/no stool). N = 5 mice per group. ***P* <0.01, ****P* < 0.001 by ANOVA followed by Tukey's multiple comparisons test. In (**C**) to assess intestinal permeability mice were infected with 1x10^4 spores of M7404 and given misoprostol 20 μg/mouse by IP injection 30 min before *C. difficile* inoculation, 24 h later and at the time of FITC-dextran treatment. 2 d post infection mice were gavaged with FITC-dextran or vehicle control, then euthanized 4 h later and concentrations of FITC-dextran in plasma were determined. N = 5 mice per group. **P* < 0.05 by ANOVA followed by Tukey's multiple comparisons test.

Appendices C & D are separate documents

Appendix C IND safe to proceed letter from FDA

Appendix D Misoprostol Package Insert