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Alanyl-glutamine Supplementation for *Clostridioides difficile* Infection Treatment (ACT): A double-blind randomized controlled trial

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ABSTRACT

Introduction: *Clostridioides difficile* is the leading cause of healthcare-associated infections in the US, with an estimated 1 billion dollars in excess cost to the healthcare system annually. *C. difficile* infection (CDI) has high recurrence rate, up to 25% after first episode and up to 60% for succeeding episodes. Preliminary in vitro and in vivo studies indicate that alanylglutamine (AQ) may be beneficial in treating CDI by its effect on restoring intestinal integrity in the epithelial barrier, ameliorating inflammation, and decreasing relapse.

Methods and analysis: This study is a randomized, placebo-controlled, double-blind, phase II clinical trial. The trial is designed to determine optimal dose and safety of oral AQ at 4, 24, and 44g doses administered daily for ten days concurrent with standard treatment of non-severe or severe uncomplicated CDI in persons age 18 and older. The primary outcome of interest is CDI recurrence during 60 days post-treatment follow-up, with the secondary outcome of mortality during 60 days post-treatment follow-up. Exploratory analysis will be done to determine the impact of AQ supplementation on intestinal and systemic inflammation, as well as intestinal microbial and metabolic profiles.

Ethics and dissemination: The study has received University of Virginia Institutional Review Board approval (HSR200046, Protocol v9, April 2023). Findings will be disseminated via conference presentations, lectures and peer-reviewed publications.

Trial registration: NCT04305769

Key words: *C. difficile* infection, treatment, diarrhea, antibiotic-associated, alanyl-glutamine, glutamine

Word count: 4008

Strengths and Limitations

- **A randomized, placebo controlled, double-blinded study to test efficacy and safety of alanyl-glutamine supplementation to standard of care for *C. difficile* infection in adult patients.**
- **The clinical trial is supported by previously published data showing benefit of alanyl-glutamine in preventing toxin- and infection- induced intestinal injury in cell culture and animal models.**
- **This study will determine the effect of alanyl-glutamine supplementation on inflammation, and microbial and metabolic profiles.**
- **The clinical trial is limited to non-severe and severe uncomplicated *C. difficile* infection.**

INTRODUCTION

Diarrhea is a major cause of mortality from infectious diseases in the US. From 1980 to 2014, deaths from diarrheal diseases increased from 0.4 to 2.4 per 100,000 persons while the overall mortality due to all infections dropped from about 43 to 34 deaths per 100,000 persons [1]. The likely cause of the observed increase in death is *Clostridioides difficile* infection (CDI), the most common cause of antibiotic-associated diarrhea and healthcare-associated infections [2]. In 2017, the estimated number of CDI cases in the U.S. was greater than 450,000, first recurrences was approximately 70,000 and number of deaths around 20,000 [3]. Up to $4.9 billion in excess health care costs was attributed to CDI in 2008 in US acute care facilities alone [4]. Unfortunately, antibiotic treatment is still the recommended approach to this antibiotic-associated disease. Vancomycin and fidaxomicin are currently the drugs of choice for initial treatment [5]. Although fidaxomicin is reported to be equivalent to vancomycin in treating acute CDI but superior in preventing relapses, its advantage over vancomycin is lost in infections caused by the prevalent epidemic strain, BI/NAP1/027 [6]. We have shown that like vancomycin, fidaxomicin increases susceptibility to initial infection and is as likely to promote recurrent disease in mice [7]. In humans, vancomycin or metronidazole treatment of asymptomatic infection has only led to recurrent and prolonged clostridial shedding [8]. Consistent with these findings is the observation that the risk of recurrence in humans increases from 24% in individuals with one episode of CDI to up to 64.7% in those with prior recurrences [9] (and therefore, consequent CDI treatments). Strategies that target the microbiome—probiotics [10] or fecal transplant (FMT) [11], or the toxins (not the bacteria)—tolevamer [12] or monoclonal antibodies [13] appear to be better than antibiotics in preventing recurrences. Strategies to prevent recurrence is critical to stop the vicious cycle of more antibiotic use in this antibiotic-induced disease. None of the current strategies address repair of toxin-mediated epithelial damage or prevention of the unregulated host inflammatory response.

Alanyl-glutamine (AQ) is a dipeptide with a glutamine amino group joined to an alanyl residue. It has the chemical structure: C8H15N3O4. Glutamine is an amino acid that serves as an important energy source in the body, particularly for enterocytes. It is a non-essential amino acid in healthy people but is considered “conditionally essential” during critical illness, injury, and other stressful states [14]. Our preliminary data indicate that AQ may specifically be beneficial for CDI. We found that glutamine and AQ reduced *C. difficile* toxin A, Tcd-A, induced apoptosis and that this was associated with inhibition of caspase 8 activation in intestinal cell line [15,16]. Migration of intestinal epithelial cells after injury is also inhibited by both TcdA [17] and TcdB [18], an effect that is prevented in the presence of either glutamine or AQ. Glucosylation of Rho by *C. difficile* toxins causes cytoskeletal disruption. We found that supplementation of the media with glutamine or AQ partially reversed the altered F-actin distribution and increased RhoA expression [19]. *In vivo* studies confirmed the benefit of AQ in *C. difficile* associated diarrhea. In rabbit ileal loops, TcdA caused intestinal inflammation and secretion. In the presence of glutamine or AQ, ileal histopathology is improved and secretion is decreased [17]. As previously observed *in vitro*, TcdA-induced intestinal cell apoptosis was decreased by the dipeptide in rabbit ileal tissues. In *C. difficile* infected mice, treatment with vancomycin plus AQ reduced post-antibiotic associated relapse, diarrhea and mortality [18]. Furthermore, histopathology, intestinal inflammation and apoptosis were all improved with dipeptide supplementation. In a limited single-arm preliminary study of AQ supplementation of antibiotic treatment for CDI to test safety and efficacy of AQ at a dose of 44 g given orally with standard treatment in 7 hospitalized patients, 2 recurrences occurred within 6 months after

treatment and both were from subjects who had < 1 dose of the study agent [NCT02053350]. The rest of the subjects who had 2 to 10 doses of AQ did not develop recurrent disease.

Objectives

Given our preliminary data showing the beneficial effects of AQ and published benefits and safety of glutamine supplementation in persons with diarrhea and other conditions, we now conduct this double-blind, placebo-controlled randomized controlled trial to determine the benefit of AQ supplementation of standard of care in patients with CDI.

METHODS AND ANALYSIS

Study design

This is a phase II, randomized, double-blinded, placebo controlled clinical trial in adult patients with non-severe or severe uncomplicated CDI. It is designed to test the hypothesis that compared to standard of care, daily AQ supplementation will reduce recurrence (primary outcome) and mortality (secondary outcome) during 60 days post-treatment follow-up. Furthermore, we hypothesize that alanyl-glutamine supplementation will be associated with decreased intestinal and systemic inflammation and improvement of intestinal microbial and metabolic profiles. Both the treatment and control groups will receive antibiotics for treatment of CDI as outlined in consensus guidelines for management of CDI10].

Study Setting

The primary sites of enrollment are the University of Virginia Health, a tertiary academic center located in Charlottesville VA and Carilion clinic in Roanoke VA.

Sample size justification

Sample size estimations are based on the presence of four study groups (placebo, 4 mg dose, 24 mg dose and 44 mg dose), a 40% recurrence rate of CDI in the standard treatment control arm (placebo) with a 15% difference between best intervention and the standard control treatment, alpha level of 0.05 and power of 90%. With these specifications, 59 participants per group (total n=236) are required using a single stage approach for randomized Phase II trial designs with multiple groups. Fifty-nine persons per group will also achieve 80% power for a minimum difference of 12%. Assuming a 17% mortality during 60 days post-treatment follow-up in this population, the proposed sample size of 236 provides 90% power for a minimum detectable difference of 10% in mortality between the active treatment groups and control group. Assuming a loss of 60-days follow-up rate not more than 10%, 260 participants (65 persons per group) will be required to meet our primary objectives.

Eligibility, Recruitment and enrollment

Potential participants will be identified through the microbiology reports and limited review of the EMR for the enrollment criteria (Figure). Once a potential candidate is identified, the clinical research coordinator shall contact the primary healthcare team to inform them of the study. The potential candidate is then approached to discuss the trial including the purpose of the study, the study intervention and other study procedures including follow-up visits, specimen collection,

time commitment, and compensation. Signed informed consent will be obtained from all participants. Within 120 hours of screening, participants will be consented and randomized in the trial. The inclusion and exclusion criteria for subject enrollment are summarized in **Table** 1. Schedule of activities are presented in **Table 2**.

Randomization

This study will employ a randomized, double-blind design which will be maintained throughout the conduct of the trial. A study statistician who will not be involved in determining participant eligibility, will create the randomization scheme within each of the study sites using a SAS randomization algorithm and a block randomization approach. Because we are enrolling subjects over a longer period of time within each of the study sites, we will utilize block randomization to ensure that relative temporal balance is maintained throughout the trial. Random block sizes of 8 will be employed (last block of 4), and accruing block size information will not be shared with study personnel. After consent, participants will be randomly assigned to one of the four study groups, either one of the three experimental (AQ) or control (placebo) group in 1:1:1:1 allocation from a list containing the randomized and blinded treatment assignments. Study subjects will be assigned a unique consecutive three digit study identification number regardless of experimental or control allocation. Study personnel responsible for collecting, recording, and interpreting clinical and safety follow-up information will remain blinded to treatment assignment.

If a subject has an adverse event and the investigator or the participant's physician of record feels it is necessary to break the blind for that participant, they will contact the unblinded pharmacist and study statistician. In this case, the code will be broken only for the participant in question. Health authorities, the DSMB or appropriate auditors may request code-breaking. Intentional or unintentional breaking of the blinding will be reported to the IRB, NIH and other bodies as appropriate.

Intervention

The intervention is the administration of AQ at 4, 24, and 44g, or placebo (water) supplementation in combination with standard antibiotic treatment. The subject takes the oral AQ supplement or placebo every day for 10 days. After 10 days, the subjects cease taking the supplement or placebo. All subjects will receive the standard antibiotic treatment for CDI, as directed by the treating physician. AQ is tasteless, odorless and highly soluble in water and thus, water was chosen as the placebo.

Outcomes

Primary efficacy endpoint is the recurrence rate of CDI within 60 days after completion of treatment. After completion of study treatment, the research team will call the participant weekly (until 60 days after completion of treatment, Study visits 12-18) to check for recurrence of diarrhea, occurrence of other adverse events, new medications or procedures, clinic/urgent care/ER visits or other developments. Diarrhea is defined as unformed or liquid stool taking the shape of the receptacle and bowel movement > 3 within 24 hours. If with recurrent diarrhea and if not yet evaluated by a clinician, the participant will be advised to come to the ID clinic where the research clinician will evaluate the participant and collect stool specimen for evaluation of recurrent CDI

Secondary endpoint is mortality within 60 days after expected completion of treatment (Day 70 post-enrollment). For an individual, this will be considered as death for any reason, determined during the follow-up visits or phone calls and by reviewing the EMR. CDI-associated mortality will be defined as mortality with CDI listed as a cause of death in the medical or vital record. Mortality with concomitant diagnosis of CDI will be noted.

Sample collection and laboratory evaluation

As part of the screening process, the potential subject's stool that tested positive by *tcdB* PCR from the Microbiology laboratory will be retrieved. Subjects with stool positive for tcdB will be enrolled. Blood, stool and urine will be collected at days 0 and 10 and 70. Laboratory methods are described in the **Appendix**.

Statistical plans

All randomized participants will be included in the analysis based on the intent-to-treat principle. Standard descriptive statistics will be used to summarize participants' baseline demographic and clinical characteristics by four treatment groups. Percentages and counts will be used for categorical variables, while mean with standard deviation and interquartile range will be used for continuous variables. Inferential tests will be treated conservatively as two-sided with an alpha-level of 0.05, including calculation of confidence intervals. Covariates such as age, gender, comorbidities, CDI-related risks and other factors will be addressed in greater detail in a statistical analysis plan and will be addressed in comparisons between randomized groups in the descriptive and early stage inferential analyses. Evaluation for skewness, kurtosis and scedasticity will be conducted as appropriate to the variable types and consideration for non-parametric analyses will be made when necessary.

Primary efficacy endpoint is the recurrence rate of CDI within 60 days after completion of treatment. For an individual, this will be considered as the persistence or redevelopment of symptoms requiring repeat or further standard treatment after the treatment index date, which is the first day following a positive test result in which standard treatment (plus intervention or placebo) is provided – this is a single dichotomous measure (yes/no) at the individual level. On the treatment group level (N = 4), the group rate is an interval measure representing the prevalence of recurrence at 60 days post-treatment where the numerator is individuals in the group with recurrence and the denominator is total individuals in the group. **Secondary endpoint** is mortality within 60 days after expected completion of treatment (Day 70 post-enrollment). This is not dependent upon the primary endpoint. For an individual, this will be considered as death for any reason – this is a single dichotomous measure (yes/no) at the individual level. On the treatment group level (N = 4), the group rate is an interval measure representing the mortality rate at 70 days post-enrollment, where the numerator is the number who have died and the denominator is total individuals in the group. Analysis will use ANOVA unless statistically significant differences in the distribution of baseline characteristics or features of non-normality are detected and relevant, at which point contingency utilization of ANCOVA, logistic regression, or other approaches as appropriate will be implemented. Treatment group level rates will be presented as period prevalence risk ratios relative to the control (placebo) group with 95% confidence intervals. As noted above, we will emphasize an intention to treat analysis of the Modified Intention to Treat Analysis Data Set comprised of all participants who took at least one dose of study intervention (placebo or treatment), regardless of completeness of follow-up outcome data. Individuals lost to follow up or otherwise with

missing outcome data will be censored, noting oversampling for attrition and a conservative power of 90% for sample size. There will be no planned interim futility analysis, or stopping rules for achieved efficacy but we will follow stopping rules based on safety review.

Exploratory analysis using either parametric or nonparametric regression as appropriate will be used to assess relationships between AQ supplementation and intestinal and systemic inflammation, as well as intestinal microbial and metabolic profiles.

Discontinuation of study agent or participant withdrawal

Participants are free to withdraw from participation in the study at any time upon request. An investigator may discontinue or withdraw intervention or participant from the study for the following reasons: if any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant; disease progression which requires discontinuation of the study intervention; and, if the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation.

Safety monitoring

The research team will monitor the participant daily while on the study agent to check for compliance to treatment and adverse event monitoring. If the participant is discharged before end of treatment, the patient will bring home a detailed instruction about storage and administration of the study agent at home and contact information. A Study Diary will be provided for documentation purposes. The research team will call the participant daily to monitor for adherence and adverse events. After completion of study treatment, the research team will call the participant weekly until 60 days after completion of treatment, to check for recurrence of diarrhea, occurrence of other adverse events, new medications or procedures, clinic/urgent care/ER visits or other developments.

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor. All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate electronic case report form. Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution or end of study.

Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the study participant's condition deteriorates at any time during the study, it will be recorded as an AE. Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode. The research team will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. 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.

Safety oversight will be under the direction of a Data and Safety Monitoring Board (DSMB) composed of individuals with the appropriate expertise, including a doctor of Pharmacy, a Statistician, an ID specialist/Safety Officer, a hospital epidemiologist and a colorectal surgeon. Members of the DSMB are independent from the study conduct and free of conflict of interest.

Protocol reporting

For this manuscript, SPIRIT reporting guidelines was used [37].

Patient and public involvement

It was not possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

ETHICS AND DISSEMINATION

Research ethics

All study procedures and informed consent documents have been approved by the University of Virginia Health Sciences Research Institutional Review Board (IRB). Consent documents are available on request from the communicating author. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. Consent forms describing in detail the study intervention, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study intervention. All changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study, or the data will be released to any unauthorized third party without prior written approval of the sponsor.

Data collection and management

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into Red Cap, a 21 CFR Part 11-compliant data capture system provided by the Analytics and Reporting Team of the UVA HS. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

Dissemination policy

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial is registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to

publish results in peer-reviewed journals. Data from this study may be requested from other researchers 5 years after the completion of the primary endpoint by contacting the principal investigator.

DISCUSSION

Vancomycin and fidaxomicin are the only 2 FDA-approved antibiotics for CDI. During acute infection, vancomycin is comparable to fidaxomicin, in terms of clinical cure. Vancomycin is the drug of choice for severe complicated disease and fulminant CDI per multiple professional society guidelines [5, 20, 21]. However, collateral damage to the microbiota leads to recurrence rates of up to 30% after the first episode of treatment with vancomycin, and expected to be higher in persons > 65 years [12]. The risk of recurrent disease after treatment in humans increases to up to 65% in those with prior CDI recurrences [9]. Unfortunately, most antibiotics in the pipeline are also associated with significant recurrence rates [23]. Given the limited antibiotic armamentarium for CDI, strategies to prevent recurrence is critical to stop the vicious cycle of more antibiotic use in this antibiotic-induced disease. Adjunctive treatment with agents that facilitate repair of the intestinal mucosa, enhancement of immune response and recovery of the microbiota will likely offset untoward effects of anti-*C. difficile* agents and limit the use of more antibiotics in the long run. Our hypothesis is AQ as an adjunctive immunonutrient to repair intestinal epithelial function and restore microbial homeostasis can decrease disease severity and recurrence in CDI.

Given the observed benefit of AQ in intestinal cell lines and mouse models, we anticipate improved outcomes of infection with supplementation of standard of care for CDI in human disease in this clinical trial. The primary endpoint of interest is recurrence as in the mouse model of infection, AQ prevented relapse of infection after discontinuation of treatment with vancomycin [18]. It is unclear whether restoration of microbiota is as underlying mechanism involved in prevention or relapse but there have been few animal and human studies that have directly examined the effect of glutamine on microbiome. Glutamine supplementation enhanced intestinal secretory IgA secretion through its effects on the microbiota in mice [22], modified nitrogen and gut microbiota metabolism in rats [23], and mitigated intestinal dysbiosis by increasing beneficial bacteria and reducing pathogenic organisms in various animal models [24-26]. In a small study in obese and overweight adults, those randomized to receive oral L-glutamine (compared to alanine) had significant decreases in Firmicutes to Bacteroidetes ratio, a biomarker of obesity. [27] Interestingly, elevated Firmicutes to Bacteroidetes has also been associated with increased susceptibility to CDI in other studies.[28] In two small studies in HIV-infected subjects with untreated viremia, a glutamine-rich prebiotic (scGOS/lcFOS/glutamine) increased the abundance of Bifidobacteriaceae- a predominant bacteria in healthy hosts [29], Faecalibacterium and Lachnospira [30]. Noteworthy, both Faecalibacterium (specifically, F. prausnitzii) and Lachnospira spp. are also reported to provide protection against CDI and are increased after successful fecal microbiota transplantation for recurrent CDI [31-33]. Together, these published results of both animal and human studies suggest glutamine has beneficial effects in the intestinal microbiota.

The safety and effectiveness of AQ for treatment of diarrhea was reported previously in patients with HIV infection. In a randomized cohort of HIV+ patients with persistent diarrhea on highly active anti-retroviral agents, orally-administered AQ has been shown to be safe and effective in

reducing diarrhea and improving absorption of antiretroviral drugs [34]. In this study 8 of 9 (89%) subjects treated with high-dose (44 g) AQ had improved clinical symptoms (frequency of bowel movement, consistency of stool, and other intestinal complaints) compared with 3 of 8 (38%) control subjects ($p<0.05$). Twenty-six of 30 (87%) subjects who received any study drug (AQ at 4 and 44 g and glutamine at 30 g) and 19 of 20 (95%) subjects who received either glutamine (30 g) or high-dose AQ (44g) improved compared to placebo treated control subjects ($p<0.01$ and $p<0.003$, respectively). Moreover, antiretroviral drug levels significantly increased in patients given AQ or glutamine. In another double-blinded RCT in 46 HIV+ participants, supplementation with AQ at 24 g was noted to significantly decrease urinary mannitol excretion among those that had diarrhea within 14 days of enrollment [35]. These findings suggest that AQ is able to restore intestinal integrity and function. A recent systematic review of 53 clinical trials with random or quasi-random allocation (N = 4671 patients) examined the effects of glutamine or AQ in adults with critical illness or undergoing elective major surgery. Supplementation with glutamine or AQ was found to reduce infection rate and days on mechanical ventilation (moderate evidence) and length of hospital stay (low quality or evidence) but no significant impact on mortality in subjects included in the analyses [36]. Thus, overall, glutamine or AQ supplementation has been shown to be safe and beneficial in various conditions and relevant to our study, is found to be effective in restoring intestinal integrity and diarrhea.

In conclusion, this clinical trial will determine the potential efficacy and safety of AQ supplementation to standard of care for CDI. Adjunctive treatment with AQ is an affordable, accessible, safe and simple strategy which can potentially impact quality of life, infection control, and healthcare economics indices in the management of CDI.

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AUTHOR'S CONTRIBUTIONS

CW and LAP developed and provided oversight for study design and drafted manuscript. JS edited the manuscript and participated in study design. PT and XW designed the statistical plans and edited the manuscript. MW, JS, DC, BW participated in study design and critically edited the manuscript. JS is from the University of Southamton while the rest of the authors are from the University of Virginia. All authors have seen the final version of the manuscript and are accountable for all aspects of the study.

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COMPETING INTERESTS STATEMENT

CW is a medical advisor for Seres-109 of Aimmune and Seres Therapeutics. The rest of the authors have no conflict of interest to disclose.

APPENDICES

Informed consent

Laboratory methods

Study Flow Diagram

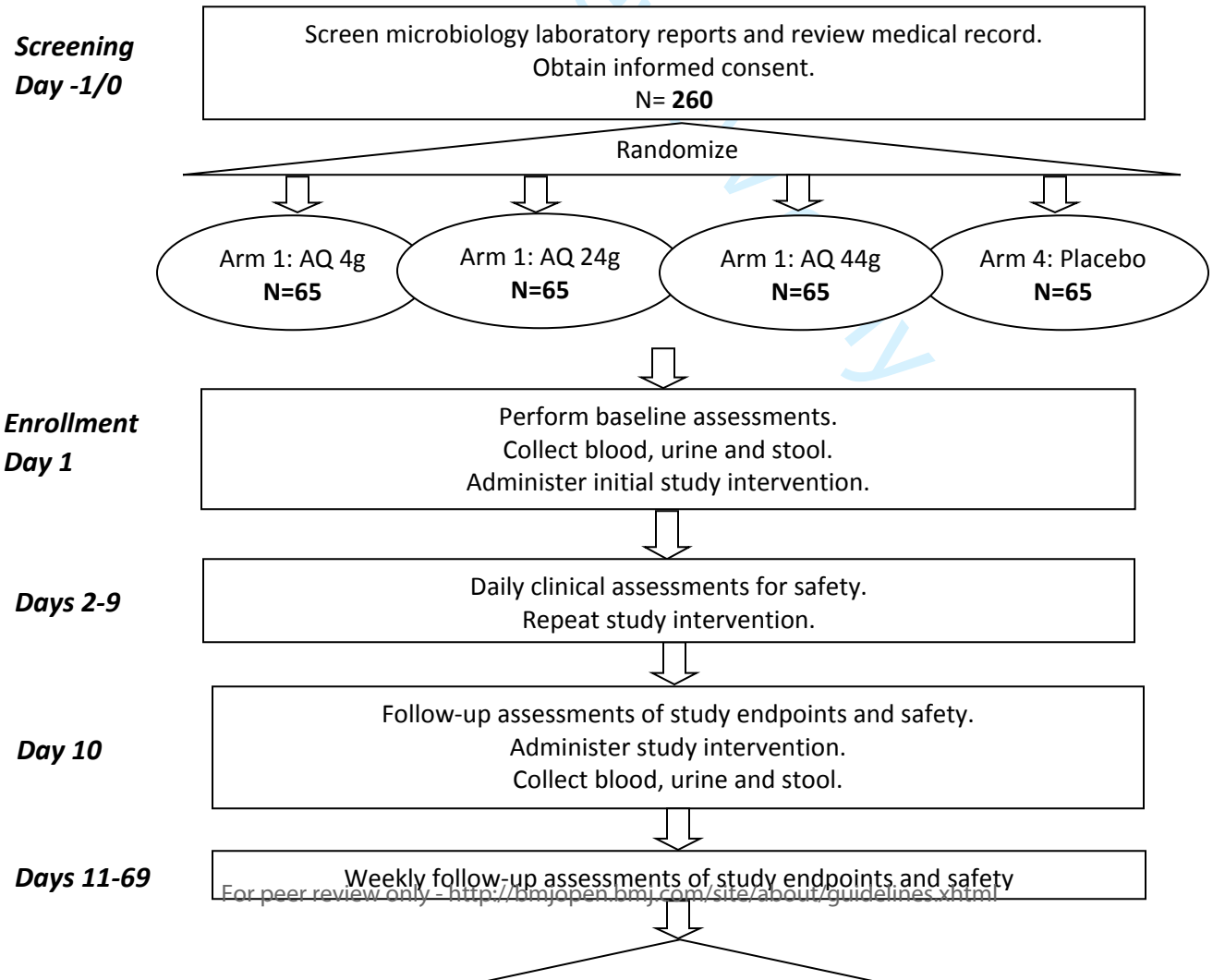

Table 1. Enrollment eligibility

Inclusion criteria	• Age 18 years and above, all gender • Diarrhea* • Stool positive for *tcdB* • Non-severe or severe uncomplicated CDI • Within 120 hours of receiving standard therapy • Ability to comply with study procedures for the length of the study

Exclusion criteria	• At enrollment, presence of any of the following:
	  ○ Hypotension or shock
	  ○ Megacolon or moderate to severe ileus
	  ○ Acute abdomen
	  ○ Admission to intensive care unit
	• Inability to tolerate oral or enteral medication
	• Presence of other known infectious etiology of diarrhea
	• COVID-19 co-infection at the time of CDI diagnosis.
	• Absolute neutrophil count <500 mcl
	• Within 100 days of hematologic or solid organ transplant
	• Uncontrolled inflammatory bowel disease (e.g. Crohn's disease, ulcerative colitis) or other etiology of non-infectious diarrhea
	• Enrollment in another investigational drug trial

	• Current use of alternative treatment for CDI (e.g. antibiotics other than metronidazole, vancomycin or fidaxomicin; IVIg; fecal transplant). • On probiotics and not willing to discontinue. • Cirrhosis or in participants with ALT > 3X normal • End stage renal disease, not on dialysis, or creatinine clearance or estimated GFR of <30mL/min even after adequate hydration • Life expectancy of < 6 months.

**Diarrhea is defined as liquid stool or stool that takes the shape of the receptacle, with bowel movements occurring > 3x within a 24 hour period.*

Table 2. Schedule of Activities

Procedures	Screening Day -1 to 0	Enrollment/Baseline Visit 1, Day 1	Study Visit 2-9[a]	Study Visit 10[b] Day 10+/- 1 day	Study Visit 11-1[c] Days 11-69 +/-2 days	Final Study Visit 18 Day 70+/-7 days
Informed consent	X					
Demographics	X					
Medical history	X					
Randomization	X					
Administer study intervention		X	X	X		
Concomitant medication review	X	X				
Physical exam	X	X		X		
Hematology	X			X		X
Complete metabolic panel	X			X		X
CRP, ESR, serum cytokines	X			X		X

Procedures	Screening Day -1 to 0	Enrollment/Baseline Visit 1, Day 1	Study Visit 2-9[a]	Study Visit 10[b] Day 10+/- 1 day	Study Visit 11-1[c] Days 11-69 +/-2 days	Final Study Visit 18 Day 70+/-7 days
Adverse event review and evaluation	X	X				
Fecal TcdB, *tcdB,* lactoferrin, cytokines	X			X		X
Collect stool for microbiome and urine for metabolomics	X			X		X
Complete Case Report Forms (CRFs)	X	X	X	X	X	X

[a]Daily visits while in the hospital or phone calls, if discharged.
[b]Participant comes to the clinic for assessment and specimen collection, if discharged.
[c]Weekly calls, if discharged; participant may be seen any day of the week (M-F) for episodes of diarrhea, if not yet seen by other healthcare providers; stools will be collected for *C. difficile* testing, if not yet done.

Biological specimen collection and laboratory evaluation

As part of screening process, the potential subject's stool that tested positive by *tcdB* PCR from the Microbiology laboratory will be retrieved. Blood, stool, and urine will be collected at days 0, 10 and 70.

For patients who consented to the study, the following will be evaluated:

Clinical I Laboratory:

1. Complete blood count (CBC): white blood cells (WBC) with differential counts, hemoglobin, hematocrit, platelet count
2. Complete metabolic panel or basic metabolic panel plus liver function test: Sodium, potassium, chloride, bicarbonate, creatinine, BUN, glucose, alkaline phosphate, AST, ALT, direct bilirubin, total bilirubin, albumin
3. Erythrocyte sedimentation rate (ESR)
4. C-reactive protein (CRP)

Note: Blood results (as part of medical care) from the previous 24 hours can be used for this purpose. If any of the tests are not available, the test/s will be added on to previously collected blood, if available. If not, a phlebotomist will draw approximately 10 mL of blood for the tests below. These blood tests will be performed at days 0 and 10 (before and after treatment) and 70. Results of diagnostic tests performed as part of regular medical care will be used in accordance with Health Insurance Portability and Accountability Act (HIPAA) and IRB rules.

Research Laboratory:

- Fecal lactoferrin
- Anaerobic culture for *C. difficile* and ribotyping of *C. difficile* isolates
- Antimicrobial susceptibility testing
- Quantification of TcdA and TcdB
- DNA extraction and microbiome assay
- Metabolomics assay
- 38-plex cytokine profiling (*Luminex*) at the UVA Flow Cytometry Core facility

Research Laboratory Assays

Isolation of C. difficile from human fecal samples

C. difficile spores will be recovered using alcohol shock followed by inoculation into Chromid (BioMérieux) or TCCFA agar plates: *C. difficile* agar base (Oxoid) supplemented with 1% taurocholate (Sigma-Aldrich), 7% defibrinated horse blood (Remel) and cycloserine/cefoxitin (Oxoid, 2297109). Briefly, approximately 1 mL of fecal samples will be incubated with equal volumes of ethanol 200 proof (Decon laboratories) at room temperature for 1h and centrifuged (3500 rpm, 10 min). Then, the pellet will be inoculated in Chromid or TCCFA agar plates using a swab and incubated at anaerobic chamber (Bactrom) for 1 and 2-5 days, respectively, at 37°C. One single colony with morphology similar to *C.difficile* will be cultured in Brucella blood agar plates for 48h. One single colony will be grown and stocked in Chopped Meat Broth (Remel). After 24h, 10 µL of culture supernatant will be spread in BHIS agar plates for aerotolarance test and 1mL will be used for PCR analysis (for identifying *C. difficile* triose phosphate isomerase, *tpi)*.

Measurement of TcdA, TcdB and CDT in stools

Levels of TcdA and TcdB will be measured by ELISA. Briefly, a high binding 96 well plates will be coated overnight with a polyclonal *C. difficile* toxin A antibody (Novus biological, NB100-62473) or polyclonal *C. difficile* toxin B antibody (Thermo Fisher, ACDTB). Plates will be washed three times with wash buffer and incubated with samples and a standard curve (TcdA and TcdB from Labtech) overnight at 4°C. After washing the plates three times with wash buffer, each well will be incubated with HRP *C. difficile* toxin A antibody (Novus biological, NBP3-08858H) or HRP *C. difficile* toxin B antibody (R&D system, AF6246) for 2h at room temperature. Plates will be washed and incubated with substrate reagent (R&D system) for 20 min and the reaction will be stopped by adding stop solution (R&D system). Absorbance of the reaction will be detected at 450 nm in an ELISA reader. Optimal dilution of each antibody will be experimentally determined.

PCR to identify C. difficile genotyping

C. difficile genomic DNA will be extracted using a DNA extraction kit (Qiagen), according to the manufacturer's recommendations. PCR amplification of *tcdA, tcdB, cdtB*, and *tpi* will be performed in a CFX Connect system (Bio-Rad) with the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 s and 55°C for 30 s. All PCRs will be performed with iTaq Universal SYBR Green Supermix (Bio-Rad). The primer sets are listed in supplementary Table 1.

Antimicrobial susceptibility test

To determine minimum inhibitory concentrations (MICs) of clindamycin (CLI), fidaxomicin (FDX), metronidazole (MTZ), moxifloxacin (MXF), tigecycline (TGC), and vancomycin (VAN), spores of *C. difficile* isolates will be inoculated onto BHIS agar plates supplemented with taurocholate followed by 20h growing in BHIS broth. MIC will be determined using broth microdilution and Etest strips (Biomerix) according to the manufacturer's instructions. Agar dilution with specific antibiotic concentration in Brucella agar plates containing haemin (Sigma, 5 mg/l), vitamin K1 (Sigma, 1 mg/l), and 5% defibrinated sheep red blood cells (Remel) will be used to confirm the resistant

strains. E-test strips will be used to test the susceptibility to TGC (ranging from 0.016 µg/mL to 256 µg/mL), MXF (0.002 µg/mL to 32 µg/mL), VAN (0.016 µg/mL to 256 µg/mL), CLI (0.016 µg/mL to 256 µg/mL) and MTZ (0.016 µg/mL to 256 µg/mL) according to the manufacturer's instructions. Microbroth dilution will be used to test susceptibility to FDX. The interpretation of minimum inhibitory concentration (MIC) will be done according to the recommendations of CLSI M11-A7 and EUCAST. *C. difficile* ATCC 700057 will be used for quality control.

Ribotyping

Isolates will be ribotyped using an internationally standardized, high-resolution capillary gel-based electrophoresis PCR ribotyping protocol for *C. difficile*. The 16S and 23S rRNA genes will be amplified using 1 µL of DNA, 12.5 µL of HotStaq (Qiagen, 203443), 9.5 µL of nuclease-free water and 1 µL of each primer (16S and 23S) at 95°C for 15 min. PCR products will be analyzed on a 2100 Agilent bioanalyzer using a DNA HS kit (Agilent) performed by Genome analysis and Technology Core (RR:SCR_018883). Samples containing 1 µL of amplified DNA, 0.5 µL of 1200 LIZ standard, and 8.5 µL of Hi-Di formamide (Life Technologies, Carlsbad, CA) will be injected at 5 kV for 5 s and resolved using a separation voltage of 6.5 kV for 103 min. Major peaks in fluorescent signal will be imported into BioNumerics v.5.1 software (Applied Maths, Austin, TX) for analysis. Fragments will be initially sized using GeneMapper v.4.0 software (Life Technologies) before being imported into BioNumerics. All signals with a height <10% of the highest peak in the individual profile will be excluded (as these were considered background rather than evidence of a major DNA fragment). Ribotyping will be identified based on Leeds-Leiden *C. difficile* reference strain library.

Measurement of inflammation biomarkers in fecal samples

Protein will be extracted from stools using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS,

adjusted to pH 7.5) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (Sigma-Aldrich). Samples will be centrifuged at 13000 rpm for 15 min and the supernatant will be used to perform the protein assay using the bicinchoninic acid assay (Thermo Fisher Scientific). Levels of inflammatory mediators (such as MPO, calprotectin, lactoferrin an others) will be measured using a commercial 38-plex cytokine profiling kit (R&D Systemsor ELISA kit (BD bioscience) according to the manufacturer's instructions. For the single ELISA assay, the absorbance (450 nm) will be determined using an Epoch plate reader (BioTek). For the Bio-plex assay, the samples will be run on a Luminex machine.

Microbiota analysis

Fecal DNA will be extracted using Qiagen stool extraction kit (Qiagen). The V1-V3 hypervariable regions of *16S rRNA* gene from fecal DNA samples will be amplified by PCR with broad range primers 8F and 534R *16S rRNA* libraries from up to 100 samples will be pooled and sequenced using MiSeq Reagent Kit v3. From the *16S rRNA* sequences, bacteria present in each sample will be identified and relative abundance quantified using the QIIME package [1] for sample demultiplexing, quality filtering, chimeric sequence removal, identification of operational taxonomic units (OTUs), and taxonomic classification. Changes in the bacterial composition will be analyzed using multivariate technique Principal Coordinate Analysis (PCoA) as previously described [2]. Signature genera will be identified by Random Forrest machine-learning classification. Model accuracy will be calculated using the 10-fold cross validation error estimate, which is an approximation of how frequently a sample is misclassified. The discriminatory power of each genus is assessed by comparing the classification accuracy with and without including the genus in the model. Genera that led to more loss of classification accuracy will be considered to be more discriminatory.

Metabolomics

Metabolic profiles of blood, urine and fecal samples will be comprehensively measured using a dual platform approach incorporating 1H nuclear magnetic resonance (NMR) spectroscopy and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) [3-6]. For the NMR analysis, an untargeted approach will be adopted to measure H-containing metabolites in the study samples using standard one-dimensional 1H NMR experiments. These will be performed using standard methods on a 700 MHz Bruker NMR spectrometer equipped with a cryoprobe for enhanced sensitivity. These will be optimized for quality, sensitivity and solvent suppression. Quality control samples will be created from pooled samples and analyzed intermittently for analytical validation. This untargeted approach allows the simultaneous unbiased assessment of a broad range of metabolite classes including several metabolites previously associated with CDI, such as p-cresyl-sulfate, 4-hydrophenylacetate, tyrosine, glycine, short-chain fatty acids (acetate, propionate, butyrate), and caproate. For the UPLC-MS analysis, a triple-quadrupole platform will be used for the targeted profiling of bile acids in the blood and fecal samples. These microbial-host co-metabolites have been previously implicated with CDI. This approach provides enhanced sensitivity and includes conjugated, and unconjugated bile acids as well as a range of primary, secondary and tertiary bile acids..

Standard multivariate statistical approaches will be applied to elucidate metabolic perturbations in the individuals associated with infection, antibiotic intake and alanyl-glutamine supplementation. This will include, but will not be limited to, principal components analysis (PCA), projection to latent structures-discriminant analysis (PLS-DA), self-organizing maps (SOMs), random forest-based methods, and linear regression techniques. Biochemical variation related to the additional clinical and phenotypic data will also be investigated. In addition, data fusion strategies will be used to couple the metabolic phenotypes with high resolution microbial profiles. This will allow pathogen-host, pathogen-microbiome and microbiome-host interactions to be studied as well as the impact of alanyl-glutamine treatment on these biochemical

relationships. For the NMR datasets, once candidate spectral components have been identified as discriminatory features, metabolite identification will be performed. This will involve the use spectral compound libraries (e.g. Human metabolome database [HMDB], KEGG, the Biological Magnetic Resonance Data Bank, published literature and in-house databases). The structural identity will be investigated if necessary using two dimensional NMR experiments (e.g. 1H-1H COrrelation SpectroscopY [COSY] and 1H-1H TOtal Correlation SpectroscopY [TOCSY]) and statistical approaches (Statistical TOtal Correlation SpectroscopY [STOCSY]).

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IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

Consent of an Adult to Be in a Research Study

In this form "you" means a person 18 years of age or older who is being asked to volunteer to participate in this study.

Participant's Name_____ Medical Record Number_____

What is the purpose of this form?

This form will provide you with information about this research study. You do not have to be in the study if you do not want to. You should have all your questions answered before you agree to be in this study. Please read this form carefully. If you want to be in the study, you will need to sign this form. You will be given a copy of this form.

Who is funding this study?

This study is being funded through a grant provided by the National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases.

Key Information About This Research Study

Principal Investigator:	Cirle Warren, MD University of Virginia Division of Infectious Diseases and International Health, MR6 Bldg 345 Crispell Drive, Charlottesville, 22908 Telephone: 434-924-9676 Email: Ca6t@virginia.edu

You are being asked to take part in a research study. You do not have to take part in this study.
You should only agree to take part in this study after reading this consent form and discussing it with the study team. You may also discuss this with your family, friends, health care providers or others before you make a decision.

What problem is this study trying to solve?

A C. difficile infection, or "C. diff", is a serious intestinal disease caused by a bacteria called C. difficile. C. diff can return even after treatment with antibiotics. New and affordable treatments are needed to both prevent the C. difficile infection from returning, and reduce the chance of death caused by this infection. Alanyl glutamine is a man-made form of a naturally occurring amino acid (amino acids make proteins), called glutamine. Research indicates that this product may help protect and restore your intestines that could be damaged by a C. diff infection.'

Research has shown that alanyl-glutamine (AQ) may be beneficial to patients who have C. difficile infections.

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

This study is being done to see which dose of AQ is the safest and most beneficial to patients when combined with antibiotic treatment. Three different doses are being provided in this study: 0, 4, 24, and 44g. This study will compare patients who receive standard antibiotic treatment alone for C. difficile infections (0g) with patients who receive standard treatment with the addition of one of the three different AQ doses (4, 24, and 44g). The study agent is to be taken once a day only.

Why would you want to take part in this study?

You might like to take part in this study because you may see an improvement of your diarrhea symptoms and a decrease in abdominal symptoms. Receiving the AQ supplement may better prevent the C. difficile infection from returning than the current drugs without AQ supplementation. This study may lead to a better understanding of how to treat C. difficile infections.

Why would you NOT want to take part in this study?

You might not want to take part in this study because you do not want to have any additional blood drawn, answer questions, provide stool samples, and have follow up phone calls. If you do not wish to take the study medication at home, you may not want to take part in this study.

After you have completed taking the study medication, you will be called weekly by a member of the study team to ask you about diarrhea symptoms, side effects, new medications, and if you have visited a doctor or clinic. If you are unable or unwilling to talk to a member of the study team weekly, you should not participate in this study.

You might not want to participate in this study because you cannot choose whether you receive AQ or the dose of AQ you receive.

What will I have to do if I take part in this study?

Full details of all the procedures are found later in this form. If you take part in this study, you will:

- Receive study medication each day for 10 days.
- Receive a physical exam (including height and weight) at three study visits.
- Have blood drawn at screening, at the last day of receiving the study drug and at 2 months after end of treatment.

What is the difference between being in this study and getting usual care?

If you take part in this study, the following things will be done differently than if you do not take part in this study.

- You will receive AQ supplements at one of four doses.
- You will have additional blood tests performed, and more blood drawn, if needed.
- You will provide urine and stool specimens that will be tested for research purposes.

What other treatments may I receive if I decide to not take part in this study?
The following alternative treatments are available to you if you decide not take part in this study:

- Standard antibiotic treatment for C. difficile infection

You are being asked to be in this study because you have a C. difficile infection and are 18 years of age or older. Up to 260 people will be in this study at UVA.

How long will this study take?

Your participation in this study will require 18 study visits or telephone calls over about 2 months. You will have daily study visits if you are in the hospital. Study visits or telephone calls will last about 30 minutes. If you are an outpatient, a referral to the UVA C. Diff clinic, or have been discharged by the emergency department, you will meet with a member of the study team at Fontaine Research Park according to the below schedule (study visit day 1, day 10 and day 18).

What will happen if you are in the study?

SCREENING (visit will last about 1 hour)

If you agree to participate, you will sign this consent form before any study related procedures take place. Before you can start in the study, there will be a screening period. You will have tests and procedures during this time to make sure you are eligible, and it is safe for you to participate. These include the following:

- Review of your medical history and demographic information
- Review of medications you are currently taking
- Physical exam including height and weight and vital signs (pulse, breathing, temperature and blood pressure)
- Blood testing
- Stool and urine samples collected

If these tests show you are eligible, you will begin study treatment.

RANDOMIZATION and STUDY TREATMENT

You will be randomly assigned (like the flip of a coin) to 1 of 4 study treatment groups. You have an equal chance of being assigned to any one of the groups. Neither you nor your doctor can choose which treatment you are assigned. Neither you nor your doctor will know which study treatment you will get until the study is done. But if your doctor needs to know, the people doing this study can find out.

GROUP 1: AQ supplement 0g (placebo)
GROUP 2: AQ supplement 4g
GROUP 3: AQ supplement 24g
GROUP 4: AQ supplement 44g

Your nurse will give you the study agent (water or AQ supplement) daily. AQ will be dissolved in water, and you will not be able to taste it or tell if you have been given AQ or not. If you have a feeding tube, the nurse will give you the medication in the feeding tube. You will take this medication once every day for 10 days. If you are an outpatient, UVA C. Diff Clinic referral, or have been discharged by the emergency department, you will be required to take the study agent (water or AQ supplement) daily.

Visits 1-10

(Each visit will last about 1 hour**)**

Each day (Visit 1 and 10), you will be given the study drug (water with or without AQ) and we will review your medical record for any changes in your health and to collect a list of your current medications.

You will be provided with a symptom diary to keep track of how you are feeling. A member of the study team will go over this document with you during each visit. If you develop unexpected symptoms, you may be asked to have an additional physical exam or blood testing.

During Visit 1 and Visit 10, you will have a physical exam and your height, weight, and vital signs measured.

During Visit 10, we will take (or "draw") up to 2 tablespoons of blood, if you do not have enough leftover blood available from the medical laboratory**.** The blood we take will be tested to measure your blood cell count, markers of inflammation, kidney function, liver function and electrolytes. When these tests are done any left-over sample will be thrown away or they will be de-identified. This means there is no information that could be used by anyone to determine who the sample came from.

You will have a stool and urine sample collected at Visit 10 to use for research testing. When these tests are done any left-over sample will be thrown away or they will be de-identified. This means there is no information that could be used by anyone to determine who the sample came from.

You will not be told the results of the tests performed for research purposes. The results of the research tests will NOT be put in your medical record

If you are discharged from the hospital before you complete Visit 10, you will be given the study medication to take home. A member of the study team will call you daily (for 30 minutes) to review any new medications or symptoms you are having. A study diary will be given to you to help you remember. If you are an outpatient, a UVA C, Diff Clinic referral, or were in emergency department or discharged early, you will meet with a member of the team at Fontaine Research Park to receive your study medication and to collect stool, urine and blood samples.

FOLLOW UP:

Visits 11-18

(Each visit will last about 30 minutes)

After you have completed Visit 10, the study team will continue to monitor you weekly. They will ask you about any new medications you are taking. Your symptom diary will be reviewed at each visit. They will ask about your diarrhea symptoms, and if you have had to visit the doctor or hospital.

About 60 days after you have stopped taking the study medication, you will return to the clinic to meet with the study doctor. They will review your medications, any new symptoms that you have, and will collect another set of blood, stool and urine samples for research.

Study Schedule

	Visits				
	1	2 to 9	10	11 to 17	18
Study Day	0/1	1-9	10	11-69***	70
Informed Consent, review of study eligibility, medical history	x				
Alanyl-glutamine study treatment		X	X		
Clinical monitoring, medicine review and follow-up	X	X	X	X	
Blood collection*	X		X		X
Stool collection**	X		X		X
Urine collection**	X		X		X

*Blood collection will be performed for both routine clinical care (as requested by your primary team) and research purposes (if blood test was already performed for routine clinical care, no additional blood will be drawn). All labs at visit 18 are done as part of research.
**Stool and urine collections are for research purposes only.
***During this period, you will be called weekly.

What are your responsibilities in the study?

You have certain responsibilities to help ensure your safety. These responsibilities are listed below:

- You must attend each study visit.
- You must be completely truthful about your health history.

- Follow all instructions given.
- You should tell the study doctor or study staff about any changes in your health or the way you feel.
- Ensure that the study medication is taken as instructed, keep the study drug in a safe place away from children, return any unused study medication at each visit, and report any lost or missed doses.
- Ensure that the study medication is taken only by you, the person for whom it has been prescribed.
- Answer all of the study-related questions completely.
- Inform the study doctor or study staff as soon as possible if you have to take any new medications, including anything prescribed by a doctor or those that you can buy without a prescription (over the counter), including herbal supplements and vitamins. The study doctor will let you know if you can take these medications.

If you want to know about the results before the study is done:

During the study you are having an investigational test done. The purpose of the test is NOT to diagnose any disease or abnormality you may have. Because the test is investigational there is no way for the study leader to understand if the results are "normal" or "abnormal". However, if any test results are concerning, your study leader will let you know.

In addition, as the research moves forward, your study leader will keep you informed of any new findings about the research itself that may be important for your health or may help you decide if you want to continue in the study. The final results of the research will not be known until all the information from everyone is combined and reviewed. At that time, you may ask for more information about the study results.

Collection of Samples and Health Information for Specimen Banking

What Sort of Research Will Be Done On Your Samples?

You are being asked to provide samples of your blood, stool and urine to be used for research. Along with specimens, researchers may need to collect some health information about you. Combining information from the specimen with information from your health records may be useful for this research. For this research, the following types of information could be included:

- Demographic information
- Other diagnoses
- Signs and symptoms
- Results of laboratory tests, radiologic imaging and procedures done in the hospital
- List of medications
- Presence of C. difficile infection

In addition, if you agree, specimens collected for research will be added to a research specimen bank. The purpose of a specimen bank is to process, and store samples until researchers need them for future research. The long-term goals of the samples collected in this bank will be mainly used for research on causes of diarrhea and to identify different types of bacteria in stool and urine. It is not possible to list every research project that will include the samples because we cannot predict all of the research questions that will be

important over the coming years. As we learn more, new research questions and new types of research may be done.

What will you have to do to give samples for research?
Your doctor will obtain blood, stool and urine samples from you for testing. This will be collected at the Screening visit, Visit 10, and Visit 18.

After the tests for your medical care are completed, there may be samples left over. Normally, these leftover samples would be thrown away. We are asking you to allow us to collect this leftover material for specimen banking.

How Will Your Sample(s) Be Labeled?
Dr. Cirle Warren will be responsible for storing your sample and for protecting your privacy.

Your samples will not be labeled with your name or other information that would identify you directly. Instead, it will have a unique code that allows for it to be linked to some of your health information. This link means that your specimen can be identified but only indirectly. We can find out if we need to know which sample is yours in the event you wish the sample to be removed from the bank later.

Which researchers can use your samples and what information about you can they have?
Your samples may be shared with researchers at the University of Virginia and at other institutions. Dr. Warren will not give your name to other researchers who want to use your sample, but will only give them information like your age and what disease/condition you have. Those who would see the information would include researchers and the others listed under "Who will see your private information?" section of this consent document.

Some of the people who receive your information may not have to follow the privacy laws and may share or release your information because they do not have to follow the privacy laws.

What Are the Benefits To Donating Your Samples Specimen Banking?
The research and/or specimen banking that is done with your sample is not meant to help you. But, doctors hope that in the future it will help people who have other diseases or conditions.

What Are The Risks of Donating Your Sample(s) For This Study?
Risks to Privacy from Specimen Banking:
The main risk of allowing us to store and use your samples and certain limited health information for research is a potential loss of privacy. One of the risks to you is the release of information from your health records. The University of Virginia will do its best to protect your records so that facts about you and your health will be kept private. The chance that information identifying you will be given to someone else is very small. However, we cannot *guarantee* it will be safe. To further safeguard your privacy, information obtained from future research will not be placed in your medical record.

Will You Find Out the Results of the Research on Your Samples for Specimen Banking?

Neither you, your health care provider**,** nor anyone in your family, will receive the results of any research done on your samples. The results will not be put in your health records. Therefore, results from any research done on your samples will not affect your medical care. This helps protect you and other members of your family from harm that might be caused by this information.

What If You Change Your Mind About Donating Your Samples for Specimen Banking?

If you decide now that your samples can be kept for specimen banking, and later change your mind, you can simply withdraw the samples at that time. To withdraw you will need to write to the Principal Investigator listed on the first page of this form. We will then destroy any of your tissue that has not already been used. Unless you withdraw from the study, permission for researchers to use your samples and to use and share your private health information for this study will never end.

Will You Be Paid For Donating Your Sample(s) for Specimen Banking?

You will not be paid to donate your samples for specimen banking.

Will Donating Your Sample(s) Cost You Any Money?

There is no cost to you to have your samples collected or used for genetic research and/or specimen banking.

Specimen Banking Options:

You do not have to participate and agree for specimens to be collected for specimen banking in order to be in the main part of this study. No matter what you decide to do, your decision will not affect your medical care. You can tell us your choice by placing your initials in one of the options below:

SPECIMEN BANKING:

Please indicate your choice by placing your initials below:

___ YES **Your sample(s) may be saved for future research and stored in a specimen bank.**

___ NO **Your sample(s) may not be saved for future research and stored in a specimen bank.**

What are the risks of being in this study?

Risks and side effects related to the AQ Supplement include:

Less Likely

- Elevation of liver enzymes which could harm the function of your liver
- Increase in your blood ammonia levels which could harm your liver, kidneys and brain.
- Nausea and vomiting

Rare but serious

- Increase in the chance of death for up to 6 months

Risks of Sharing the Drug

Do not share the study drug with anyone. It is prescribed only for you and could hurt someone else. Keep it out of reach of children and people not able to read or understand the label.

Risks of having your blood drawn:

Having blood drawn may cause:

- ✓ pain (common),
- ✓ a bruise (sometimes),
- ✓ fainting or passing out (not very often), and
- ✓ infection (rare).

If the people doing the study are exposed to your blood or body fluids in a way that could give them a disease, your blood may be tested. The tests might check for:

- ✓ hepatitis,
- ✓ HIV (Human Immunodeficiency Virus), or
- ✓ other infections.

You and the person exposed would be told the test results. However, your name would be kept private. If your test is positive for hepatitis or HIV or any other infection that may affect your clinical care, we will tell you the results and help you understand what the results mean for you.

Other unexpected risks:

You may have side effects that we do not expect or know to watch for now. Call the study leader if you have any symptoms or problems.

Could you be helped by being in this study?

You may or may not benefit from being in this study. Those who are assigned to the placebo are not expected to benefit from this study. For those assigned to receive AQ, this study may result in an improvement in diarrhea and abdominal symptoms**.** Another benefit is that AQ supplements may reduce the risk that you contract another C. difficile infection. In addition, information researchers get from this study may help others in the future.

What are your other choices if you do not join this study?

You do not have to be in this study to be treated for your illness or condition. You can get the usual treatment even if you choose not to be in this study. The usual treatment would include antibiotic treatment.
If you are an employee of UVA, your job will not be affected if you decide not to participate in this study. If you are a student at UVA, your grades will not be affected if you decide not to participate in this study.

Will you be paid for being in this study?

You will be paid $50 for completing the first visit and providing specimens, $100 for completing the study treatment (visit 10) and providing specimens, and $150 for providing blood, urine, and stool samples for visit 18. There will be a $25 gas voucher given, per visit for, subjects traveling from 30 miles away.

You should get your payments about 6 weeks after the end of the study. The income may be reported to the IRS as income.

If you do not finish the study, you will still be paid as detailed above. If the study leader says you cannot continue, you will be paid the full amount for the study.

By agreeing to be in this study, you are donating your bodily fluids, and giving up any property rights you may have in them. The results of this research using your donated materials may have commercial value. However, you will not receive any payments.

Will being in this study cost you any money?

The following procedures/tests, which are being done for research purposes, will be provided at no cost to you or your health insurance:

- AQ supplements or placebo
- Follow-up visits
- Blood draws and testing of the blood
- Testing of urine and stool

You and/or your insurance company must pay for any tests or care given beyond what is required in this study. In addition, you and/or your health insurance may also have to pay for other drugs or treatments that are given to help you control any side effects. You will have to pay for any costs not covered by your health plan. You may be responsible for any co-payments or deductibles. You may wish to ask your insurance company for an estimate of what these costs might be or if pre-approval is required.

You will be responsible for the cost of travel to come to any study visit and for any parking costs.

What if you are hurt in this study?

You do not give up any legal rights, such as seeking compensation for injury, by signing this form. If you feel you have been injured as a result of this study, you may contact the Principal Investigator or the IRB (phone numbers are located near the end of this form). If you are hurt as a result of being in this study, there are no plans to pay you for medical expenses, lost wages, disability, or discomfort. The charges for any medical treatment you receive will be billed to your insurance. You will be responsible for any amount your insurance does not cover.

What happens if you leave the study early?

You can change your mind about being in the study any time. You can agree to be in the study now and change your mind later. If you decide to stop, please tell us right away. You do not have to be in this study to get services you can normally get at the University of Virginia.

Even if you do not change your mind, the study leader can take you out of the study. Some of the reasons for doing so may include

a) Your study physician is concerned about your health
b) Your condition gets worse
c) The side effects of the treatment are too dangerous for you
d) New information shows the treatment will not work or is not safe for you
e) You do not follow your doctor's instructions
f) The study doctor closes the study for safety, administrative or other reasons

If you decide to stop being in the study, we will ask you to let us know as soon as possible. If you are taking the study drug at home, you will be asked to discontinue using the drug and return any unused study drug to the study team.

Any data collected about you up until the time you leave the study must be kept in order to determine the results of the study.

How will your personal information be shared?

The UVA researchers are asking for your permission to gather, use and share information about you for this study. If you decide not to give your permission, you cannot be in this study, but you can continue to receive regular medical care at UVA.

If you sign this form, we may collect any or all of the following information about you:

- Personal information such as name, address and date of birth
- Social Security number ONLY IF you are being paid to be in this study
- Your health information if required for this study. This may include a review of your medical records and test results from before, during and after the study from any of your doctors or health care providers. This may include mental health care records, substance abuse records, and/or HIV/AIDS records.
- Tissue samples if you agree to provide them for specimen banking

Who will see your private information?

- The researchers to make sure they can conduct the study the right way, observe the effects of the study and understand its results
- People or groups that oversee the study to make sure it is done correctly
- Insurance companies or other organizations that may need the information in order to pay your medical bills or other costs of your participation in the study
- Tax reporting offices (if you are paid for being in the study)

- People who evaluate study results, which can include sponsors and other companies that make the drug or device being studied, researchers at other sites conducting the same study, and government agencies that provide oversight such as the Food and Drug Administration (FDA) if the study is regulated by the FDA.
- If you tell us that someone is hurting you, or that you might hurt yourself or someone else, the law may require us to let people in authority know so they can protect you and others.

Information about you and/or samples from you may be given to other researchers outside of the University of Virginia after all identifiers such as name, address, phone number have been removed. Some of the people outside of UVA who will see your information may not have to follow the same privacy laws that we follow. They may release your information to others, and it may no longer be protected by those laws.

The information collected from you might be published in a medical journal. This would be done in a way that protects your privacy. No one will be able to find out from the article that you were in the study.

Information and samples obtained from you during this study may be used in future research. Your information and samples may be shared with other researchers inside or outside of the University of Virginia. They will not be sent with information that could identify you such as name, address or phone number.

A description of this clinical trial will be available on *www.ClinicalTrials.gov*, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

What if you sign the form but then decide you don't want your private information shared?

You can change your mind at any time. Your permission does not end unless you cancel it. To cancel it, please send a letter to the researchers listed on this form or complete the "Leaving the Study Early" part of this form and return it to the researchers. Then you will no longer be in the study. The researchers will still use information about you that was collected before you ended your participation.

Please contact the Principal Investigator listed earlier in this form to:

- Obtain more information about the study
- Ask a question about the study procedures or treatments
- Report an illness, injury, or other problem (you may also need to tell your regular doctors)
- Leave the study before it is finished
- Express a concern about the study

What if you have a concern about this study?

You may also report a concern about this study or ask questions about your rights as a research subject by contacting the Institutional Review Board listed below.

University of Virginia Institutional Review Board for Health Sciences Research
PO Box 800483
Charlottesville, Virginia 22908 Telephone: 434-924-2620

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

When you call or write about a concern, please give as much information as you can. Include the name of the study leader, the IRB-HSR Number (at the top of this form), and details about the problem. This will help officials look into your concern. When reporting a concern, you do not have to give your name.

Signatures

What does your signature mean?

Before you sign this form, please ask questions about any part of this study that is not clear to you. Your signature below means that you have received this information and all your questions have been answered. If you sign the form, it means that you agree to join the study. You will receive a copy of this signed document.

Consent From Adult

_____	_____	_____
PARTICIPANT (SIGNATURE)	PARTICIPANT (PRINT)	DATE

To be completed by participant if 18 years of age or older.

If an interpreter is involved in the consent process because the potential subject does not speak English well or at all, the participant should NOT sign on the line above – leave this line blank. Instead, the participant should sign the Short Form or full consent written in the language they can understand.

Person Obtaining Consent

By signing below, you confirm that you have fully explained this study to the potential subject, allowed them time to read the consent or have the consent read to them, and have answered all their questions.

_____	_____	_____
PERSON OBTAINING CONSENT (SIGNATURE)	PERSON OBTAINING CONSENT (PRINT)	DATE

Interpreter

By signing below, you confirm that the study has been fully explained to the potential subject in a language they understand and have answered all their questions.

_____	_____	_____
INTERPRETER (SIGNATURE)	INTERPRETER (PRINT)	DATE

If an interpreter was used to explain this study to a potential subject, the interpreter must sign and date the line above.

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

Surrogate Consent for Adult Participant

In the event the adult participant is unable to give informed consent for participation in this study:

_____/_____ _____

PERSON GIVING CONSENT FOR PARTICIPANT (Signature/ Printed) DATE

RELATIONSHIP TO PARTICIPANT: *Check one of the options below*

_____Agent under an Advance Directive that authorizes participation in research
_____Court-appointed Guardian
_____Spouse (unless divorce action has been filed)
_____Next of kin

If an interpreter is involved in the consent process because the surrogate does not speak English well or at all, the surrogate should NOT sign on the line above – leave this line blank. Instead, the surrogate should sign the Short Form written in the language they can understand.

Person Obtaining Consent of the Surrogate

By signing below, you confirm that you have fully explained this study to the potential subject's surrogate, allowed them time to read the consent or have the consent read to them, and have answered all their questions.

_____	_____	_____
PERSON OBTAINING CONSENT (SIGNATURE)	PERSON OBTAINING CONSENT (PRINT)	DATE

Impartial Witness for Consent from Surrogate

If this consent form is read to the subject's surrogate because the subject's surrogate is blind or illiterate, an impartial witness not affiliated with the research or study doctor must be present for the consenting process and sign the following statement. The subject's surrogate may place an X on the "Person giving consent for participant" signature line above.

I agree the information in this informed consent form was presented orally in my presence to the subject's surrogate and the subject's surrogate had the opportunity to ask any questions he/she had about the study. I also agree that the subject's surrogate freely gave their informed consent for the subject to participate in this trial.

_____	_____	_____
IMPARTIAL WITNESS (SIGNATURE)	IMPARTIAL WITNESS (PRINT)	DATE

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

Person Obtaining Assent of the Adult Subject

The subject is unable to give assent due to the following reason: _____

By signing below you confirm that the study has been explained to the adult subject, all questions have been answered and the adult subject has not demonstrated resistance or dissent by word or gesture to enroll in the study. You also confirm that if the subject demonstrates resistance or dissent at any point in the study that they will not be subjected to any additional study interventions.

_____	_____	_____
PERSON OBTAINING ASSENT (SIGNATURE)	PERSON OBTAINING ASSENT (PRINT)	DATE

Interpreter

By signing below you confirm that the study has been fully explained to the potential subject's surrogate in a language they understand and have answered all their questions.

_____	_____	_____
INTERPRETER (SIGNATURE)	INTERPRETER (PRINT)	DATE

If an interpreter was used to explain this study the interpreter must sign and date the line above.

Consent of the Participant to Continue to Be in the Study

Your legal representative gave his/her permission for you to be in this research study. This is because you were not able to make your own decision due to your illness. Your condition is now better. You are being asked to decide whether to continue to be in this study. The decision is up to you. Before you sign this form, please ask questions about any part of this study that is not clear to you. When you sign below, you are saying you understand the information we gave you about the study and in this form.

If you sign this form it means that you agree to continue being in the study.

_____	_____	_____
PARTICIPANT (SIGNATURE)	PARTICIPANT (PRINT)	DATE

If an interpreter is involved in the consent process because the subject does not speak English well or at all, the subject should NOT sign on the line above – leave this line blank. Instead, the subject should sign the Short Form written in the language they can understand.

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

Person Obtaining Consent of the Subject

By signing below you confirm that you have fully explained this study to the subject, allowed them time to read the consent or have the consent read to them, and have answered all their questions.

_____	_____	_____
PERSON OBTAINING CONSENT (SIGNATURE)	PERSON OBTAINING CONSENT (PRINT)	DATE

Interpreter

By signing below you confirm that the study has been fully explained to the potential subject in a language they understand and have answered all their questions.

_____	_____	_____
INTERPRETER (SIGNATURE)	INTERPRETER (PRINT)	DATE

If an interpreter was used to explain this study to a potential subject, the interpreter must sign and date the line above.

Consent from Impartial Witness

If this consent form is read to the subject because the subject is blind or illiterate, an impartial witness not affiliated with the research or study doctor must be present for the consenting process and sign the following statement. The subject may place an X on the Participant Signature line above.

I agree the information in this informed consent form was presented orally in my presence to the subject and the subject had the opportunity to ask any questions he/she had about the study. I also agree that the subject freely gave their informed consent to participate in this trial.

_____	_____	_____
IMPARTIAL WITNESS (SIGNATURE)	IMPARTIAL WITNESS (PRINT)	DATE

Notification of My Health Care Provider

Your health care provider will be notified of your participation in this study.

IRB-HSR# 200046: Alanyl-glutamine supplementation of standard treatment for C. difficile infection: A randomized, double-blind, placebo-controlled trial

Leaving the Study Early

Signatures should be obtained in this section if the subject decides to leave the study early.

If you leave the study early the study leader will keep the data collected about you up until the time you leave the study to help determine the results of the study.

Check one option below:

____ I am withdrawing my consent from the intervention or treatment part of this study but agree to continue to have follow up information about me collected by the study team.
The follow up information will be collected by:

- Obtaining information from my medical records
- Phone call weekly

____ I am withdrawing my consent for this study. No additional information may be collected about me including follow up information from my medical records.

Consent From Adult

_____	_____	_____
PARTICIPANT (SIGNATURE)	PARTICIPANT (PRINT)	DATE

To be completed by participant if 18 years of age or older.

Person Obtaining Consent

By signing below you confirm that you have fully explained the implications of withdrawing from the study to the subject and have answered all their questions.

_____	_____	_____
PERSON OBTAINING CONSENT (SIGNATURE)	PERSON OBTAINING CONSENT (PRINT)	DATE

Interpreter

By signing below, you confirm that the study withdrawal section has been fully explained to the subject in a language they understand and have answered all their questions.

_____	_____	_____
INTERPRETER (SIGNATURE)	INTERPRETER (PRINT)	DATE

If an interpreter was used to explain this withdrawal section to the subject the interpreter must sign and date the line above.

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. BMJ. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1

Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	na
Protocol version	#3	Date and version identifier	2
Funding	#4	Sources and types of financial, material, and other support	14
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1, 14
Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	14
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and	9

		other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	6
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5

Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Table 1
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	8
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	8
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Table 1
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	7

Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 2
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	5
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque,	6

		sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	6
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	6
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	6
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9

Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	6, 8
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	7
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	7
Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	7
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and	9

		competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	8
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	8
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	8
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	9
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	9

Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	5
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	5
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	9
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	14
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	9
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	na
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	9

Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	na
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	9
Appendices			
Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Appendix

BMJ Open

Alanyl-glutamine Supplementation for Clostridioides difficile Infection Treatment (ACT): A double-blind randomized controlled trial study protocol

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Alanyl-glutamine Supplementation for *Clostridioides difficile* Infection Treatment (ACT): A double-blind randomized controlled trial study protocol

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ABSTRACT

Introduction: *Clostridioides difficile* is the leading cause of healthcare-associated infections in the US, with an estimated 1 billion dollars in excess cost to the healthcare system annually. *C. difficile* infection (CDI) has high recurrence rate, up to 25% after first episode and up to 60% for succeeding episodes. Preliminary in vitro and in vivo studies indicate that alanylglutamine (AQ) may be beneficial in treating CDI by its effect on restoring intestinal integrity in the epithelial barrier, ameliorating inflammation, and decreasing relapse.

Methods and analysis: This study is a randomized, placebo-controlled, double-blind, phase II clinical trial. The trial is designed to determine optimal dose and safety of oral AQ at 4, 24, and 44g doses administered daily for ten days concurrent with standard treatment of non-severe or severe uncomplicated CDI in persons age 18 and older. The primary outcome of interest is CDI recurrence during 60 days post-treatment follow-up, with the secondary outcome of mortality during 60 days post-treatment follow-up. Exploratory analysis will be done to determine the impact of AQ supplementation on intestinal and systemic inflammation, as well as intestinal microbial and metabolic profiles.

Ethics and dissemination: The study has received University of Virginia Institutional Review Board approval (HSR200046, Protocol v9, April 2023). Findings will be disseminated via conference presentations, lectures and peer-reviewed publications.

Trial registration: NCT04305769

Key words: *C. difficile* infection, treatment, diarrhea, antibiotic-associated, alanyl-glutamine, glutamine

Word count: 3397

Strengths and Limitations

- **A randomized, placebo controlled, double-blinded study to test efficacy and safety of alanyl-glutamine supplementation to standard of care for *C. difficile* infection in adult patients.**
- **The clinical trial is supported by previously published data showing benefit of alanyl-glutamine in preventing toxin- and infection- induced intestinal injury in cell culture and animal models.**
- **This study will determine the effect of alanyl-glutamine supplementation on inflammation, and microbial and metabolic profiles.**
- **The clinical trial is limited to non-severe and severe uncomplicated *C. difficile* infection.**

INTRODUCTION

Diarrhea is a major cause of mortality from infectious diseases in the US. From 1980 to 2014, deaths from diarrheal diseases increased from 0.4 to 2.4 per 100,000 persons while the overall mortality due to all infections dropped from about 43 to 34 deaths per 100,000 persons [1]. The likely cause of the observed increase in death is *Clostridioides difficile* infection (CDI), the most common cause of antibiotic-associated diarrhea and healthcare-associated infections [2]. In 2017, the estimated number of CDI cases in the U.S. was greater than 450,000, first recurrences was approximately 70,000 and number of deaths around 20,000 [3]. Up to $4.9 billion in excess health care costs was attributed to CDI in 2008 in US acute care facilities alone [4]. Unfortunately, antibiotic treatment is still the recommended approach to this antibiotic-associated disease. Vancomycin and fidaxomicin are currently the drugs of choice for initial treatment [5]. Although fidaxomicin is reported to be equivalent to vancomycin in treating acute CDI but superior in preventing relapses, its advantage over vancomycin is lost in infections caused by the prevalent epidemic strain, BI/NAP1/027 [6]. We have shown that like vancomycin, fidaxomicin increases susceptibility to initial infection and is as likely to promote recurrent disease in mice [7]. In humans, vancomycin or metronidazole treatment of asymptomatic infection has only led to recurrent and prolonged clostridial shedding [8]. Consistent with these findings is the observation that the risk of recurrence in humans increases from 24% in individuals with one episode of CDI to up to 64.7% in those with prior recurrences [9] (and therefore, consequent CDI treatments). Strategies that target the microbiome—probiotics [10] or fecal transplant (FMT) [11], or the toxins (not the bacteria)—tolevamer [12] or monoclonal antibodies [13] appear to be better than antibiotics in preventing recurrences. Strategies to prevent recurrence is critical to stop the vicious cycle of more antibiotic use in this antibiotic-induced disease. None of the current strategies address repair of toxin-mediated epithelial damage or prevention of the unregulated host inflammatory response.

Alanyl-glutamine (AQ) is a dipeptide with a glutamine amino group joined to an alanyl residue. It has the chemical structure: C8H15N3O4. Glutamine is an amino acid that serves as an important energy source in the body, particularly for enterocytes. It is a non-essential amino acid in healthy people but is considered “conditionally essential” during critical illness, injury, and other stressful states [14]. Our preliminary data indicate that AQ may specifically be beneficial for CDI. We found that glutamine and AQ reduced *C. difficile* toxin A, Tcd-A, induced apoptosis and that this was associated with inhibition of caspase 8 activation in intestinal cell line [15,16]. Migration of intestinal epithelial cells after injury is also inhibited by both TcdA [17] and TcdB [18], an effect that is prevented in the presence of either glutamine or AQ. Glucosylation of Rho by *C. difficile* toxins causes cytoskeletal disruption. We found that supplementation of the media with glutamine or AQ partially reversed the altered F-actin distribution and increased RhoA expression [19]. *In vivo* studies confirmed the benefit of AQ in *C. difficile* associated diarrhea. In rabbit ileal loops, TcdA caused intestinal inflammation and secretion. In the presence of glutamine or AQ, ileal histopathology is improved and secretion is decreased [17]. As previously observed *in vitro*, TcdA-induced intestinal cell apoptosis was decreased by the dipeptide in rabbit ileal tissues. In *C. difficile* infected mice, treatment with vancomycin plus AQ reduced post-antibiotic associated relapse, diarrhea and mortality [18]. Furthermore, histopathology, intestinal inflammation and apoptosis were all improved with dipeptide supplementation. In a limited single-arm preliminary study of AQ supplementation of antibiotic treatment for CDI to test safety and efficacy of AQ at a dose of 44 g given orally with standard treatment in 7 hospitalized patients, 2 recurrences occurred within 6 months after

treatment and both were from participants who had < 1 dose of the study agent [NCT02053350]. The rest of the participants who had 2 to 10 doses of AQ did not develop recurrent disease.

Objectives

Given our preliminary data showing the beneficial effects of AQ and published benefits and safety of glutamine supplementation in persons with diarrhea and other conditions, we now conduct this double-blind, placebo-controlled randomized controlled trial to determine the benefit of AQ supplementation of standard of care in patients with CDI.

METHODS AND ANALYSIS

Study design

This is a phase II, randomized, double-blinded, placebo controlled clinical trial in adult patients with non-severe or severe uncomplicated CDI. It is designed to test the hypothesis that compared to standard of care, daily AQ supplementation will reduce recurrence (primary outcome) and mortality (secondary outcome) during 60 days post-treatment follow-up. Furthermore, we hypothesize that alanyl-glutamine supplementation will be associated with decreased intestinal and systemic inflammation and improvement of intestinal microbial and metabolic profiles. Both the treatment and control groups will receive antibiotics for treatment of CDI as outlined in consensus guidelines for management of CDI10].

Study Setting

There are two sites of enrollment: the University of Virginia Health, a tertiary academic center located in Charlottesville VA and Carilion clinic in Roanoke VA.

Sample size justification

Sample size estimations are based on the presence of four study groups (placebo, 4 mg dose, 24 mg dose and 44 mg dose), a 40% recurrence rate of CDI in the standard treatment control arm (placebo) with a 15% difference between best intervention and the standard control treatment, alpha level of 0.05 and power of 90%. With these specifications, 59 participants per group (total n=236) are required using a single stage approach for randomized Phase II trial designs with multiple groups. Fifty-nine persons per group will also achieve 80% power for a minimum difference of 12%. Assuming a 17% mortality during 60 days post-treatment follow-up in this population, the proposed sample size of 236 provides 90% power for a minimum detectable difference of 10% in mortality between the active treatment groups and control group. Assuming a loss of 60-days follow-up rate not more than 10%, 260 participants (65 persons per group) will be required to meet our primary objectives.

Study timeline

The UVA site opened for enrollment on January 2021 and the Carilion Clinic on June 2023. The planned end date of the study is June 2025.

Eligibility, Recruitment and enrollment

Potential participants will be identified through the microbiology reports and limited review of the EMR for the enrollment criteria (Figure). Once a potential candidate is identified, the clinical research coordinator shall contact the primary healthcare team to inform them of the study. The potential candidate is then approached to discuss the trial including the purpose of the study, the study intervention and other study procedures including follow-up visits, specimen collection, time commitment, and compensation. Signed informed consent will be obtained from all participants. Within 120 hours of screening, participants will be consented and randomized in the trial. The inclusion and exclusion criteria for participant enrollment are summarized in **Table 1**. Schedule of activities are presented in **Table 2**.

Randomization

This study will employ a randomized, double-blind design which will be maintained throughout the conduct of the trial. A study statistician who will not be involved in determining participant eligibility, will create the randomization scheme within each of the study sites using a SAS randomization algorithm and a block randomization approach. Because we are enrolling participants over a longer period of time within each of the study sites, we will utilize block randomization to ensure that relative temporal balance is maintained throughout the trial. Random block sizes of 8 will be employed (last block of 4), and accruing block size information will not be shared with study personnel. After consent, participants will be randomly assigned to one of the four study groups, either one of the three experimental (AQ) or control (placebo) group in 1:1:1:1 allocation from a list containing the randomized and blinded treatment assignments. Study participants will be assigned a unique consecutive three digit study identification number regardless of experimental or control allocation. Study personnel responsible for collecting, recording, and interpreting clinical and safety follow-up information will remain blinded to treatment assignment.

If a participant has an adverse event and the investigator or the participant's physician of record feels it is necessary to break the blind for that participant, they will contact the unblinded pharmacist and study statistician. In this case, the code will be broken only for the participant in question. Health authorities, the DSMB or appropriate auditors may request code-breaking. Intentional or unintentional breaking of the blinding will be reported to the IRB, NIH and other bodies as appropriate.

Intervention

The intervention is the administration of AQ at 4, 24, and 44g, or placebo (water) supplementation in combination with standard antibiotic treatment. The participant takes the oral AQ supplement or placebo every day for 10 days. After 10 days, the participants cease taking the supplement or placebo. All participants will receive the standard antibiotic treatment for CDI, as directed by the treating physician. AQ is tasteless, odorless and highly soluble in water and thus, water was chosen as the placebo.

Outcomes

Primary efficacy endpoint is the recurrence rate of CDI within 60 days after completion of treatment. After completion of study treatment, the research team will call the participant weekly (until 60 days after completion of treatment, Study visits 12-18) to check for recurrence of diarrhea, occurrence of other adverse events, new medications or procedures, clinic/urgent care/ER visits or other developments. Diarrhea is defined as unformed or liquid stool taking the

shape of the receptacle and bowel movement > 3 within 24 hours. If with recurrent diarrhea and if not yet evaluated by a clinician, the participant will be advised to come to the ID clinic where the research clinician will evaluate the participant and collect stool specimen for evaluation of recurrent CDI

Secondary endpoint is mortality within 60 days after expected completion of treatment (Day 70 post-enrollment). For an individual, this will be considered as death for any reason, determined during the follow-up visits or phone calls and by reviewing the EMR. CDI-associated mortality will be defined as mortality with CDI listed as a cause of death in the medical or vital record. Mortality with concomitant diagnosis of CDI will be noted.

Sample collection and laboratory evaluation

As part of the screening process, the potential participant's stool that tested positive by *tcdB* PCR from the Microbiology laboratory will be retrieved. Participants with stool positive for tcdB will be enrolled. Blood, stool and urine will be collected at days 0 and 10 and 70. Laboratory methods are described in the **Appendix**.

Statistical plans

All randomized participants will be included in the analysis based on the intent-to-treat principle. Standard descriptive statistics will be used to summarize participants' baseline demographic and clinical characteristics by four treatment groups. Percentages and counts will be used for categorical variables, while mean with standard deviation and interquartile range will be used for continuous variables. Inferential tests will be treated conservatively as two-sided with an alpha-level of 0.05, including calculation of confidence intervals. Covariates such as age, gender, comorbidities, CDI-related risks and other factors will be addressed in greater detail in a statistical analysis plan and will be addressed in comparisons between randomized groups in the descriptive and early stage inferential analyses. Evaluation for skewness, kurtosis and scedasticity will be conducted as appropriate to the variable types and consideration for non-parametric analyses will be made when necessary.

Primary efficacy endpoint is the recurrence rate of CDI within 60 days after completion of treatment. For an individual, this will be considered as the persistence or redevelopment of symptoms requiring repeat or further standard treatment after the treatment index date, which is the first day following a positive test result in which standard treatment (plus intervention or placebo) is provided – this is a single dichotomous measure (yes/no) at the individual level. On the treatment group level (N = 4), the group rate is an interval measure representing the prevalence of recurrence at 60 days post-treatment where the numerator is individuals in the group with recurrence and the denominator is total individuals in the group. **Secondary endpoint** is mortality within 60 days after expected completion of treatment (Day 70 post-enrollment). This is not dependent upon the primary endpoint. For an individual, this will be considered as death for any reason – this is a single dichotomous measure (yes/no) at the individual level. On the treatment group level (N = 4), the group rate is an interval measure representing the mortality rate at 70 days post-enrollment, where the numerator is the number who have died and the denominator is total individuals in the group. Analysis will use ANOVA unless statistically significant differences in the distribution of baseline characteristics or features of non-normality are detected and relevant, at which point contingency utilization of ANCOVA, logistic regression, or other approaches as appropriate will be implemented. Treatment group level rates will be presented as period prevalence risk ratios relative to the

control (placebo) group with 95% confidence intervals. As noted above, we will emphasize an intention to treat analysis of the Modified Intention to Treat Analysis Data Set comprised of all participants who took at least one dose of study intervention (placebo or treatment), regardless of completeness of follow-up outcome data. Individuals lost to follow up or otherwise with missing outcome data will be censored, noting oversampling for attrition and a conservative power of 90% for sample size. There will be no planned interim futility analysis, or stopping rules for achieved efficacy but we will follow stopping rules based on safety review.

Exploratory analysis using either parametric or nonparametric regression as appropriate will be used to assess relationships between AQ supplementation and intestinal and systemic inflammation, as well as intestinal microbial and metabolic profiles.

Discontinuation of study agent or participant withdrawal

Participants are free to withdraw from participation in the study at any time upon request. An investigator may discontinue or withdraw intervention or participant from the study for the following reasons: if any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant; disease progression which requires discontinuation of the study intervention; and, if the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation.

Safety monitoring

The research team will monitor the participant daily while on the study agent to check for compliance to treatment and adverse event monitoring. If the participant is discharged before end of treatment, the patient will bring home a detailed instruction about storage and administration of the study agent at home and contact information. A Study Diary will be provided for documentation purposes. The research team will call the participant daily to monitor for adherence and adverse events. After completion of study treatment, the research team will call the participant weekly until 60 days after completion of treatment, to check for recurrence of diarrhea, occurrence of other adverse events, new medications or procedures, clinic/urgent care/ER visits or other developments.

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor. All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate electronic case report form. Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution or end of study.

Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the study participant's condition deteriorates at any time during the study, it will be recorded as an AE. Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode. The research team will record all reportable events with start dates

occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. 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.

Safety oversight will be under the direction of a Data and Safety Monitoring Board (DSMB) composed of individuals with the appropriate expertise, including a doctor of Pharmacy, a Statistician, an ID specialist/Safety Officer, a hospital epidemiologist and a colorectal surgeon. Members of the DSMB are independent from the study conduct and free of conflict of interest.

Protocol reporting

For this manuscript, SPIRIT reporting guidelines was used [37].

Patient and public involvement

None.

ETHICS AND DISSEMINATION

Research ethics

All study procedures and informed consent documents have been approved by the University of Virginia Health and Carilion Medical Center Institutional Review Boards (IRB). Consent documents are available on request from the communicating author. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. Consent forms describing in detail the study intervention, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study intervention. All changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study, or the data will be released to any unauthorized third party without prior written approval of the sponsor.

Data collection and management

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into Red Cap, a 21 CFR Part 11-compliant data capture system provided by the Analytics and Reporting Team of the UVA HS. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

Dissemination policy

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial is registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 5 years after the completion of the primary endpoint by contacting the principal investigator.

AUTHOR'S CONTRIBUTIONS

CW and LAP developed and provided oversight for study design and drafted manuscript. JS edited the manuscript and participated in study design. PT and XW designed the statistical plans and edited the manuscript. MW, JHS, DC, BW, EB participated in study design and critically edited the manuscript. JRS is from the University of Southamton while the rest of the authors are from the University of Virginia. All authors have seen the final version of the manuscript and are accountable for all aspects of the study.

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COMPETING INTERESTS STATEMENT

CW is a medical advisor for Seres-109 of Aimmune and Seres Therapeutics and site PI for Rebyota Prospective Registry. The rest of the authors have no conflict of interest to disclose.

APPENDICES

Laboratory methods

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Table 1. Enrollment eligibility	
Inclusion criteria	• Age 18 years and above, all gender • Diarrhea* • Stool positive for *tcdB* • Non-severe or severe uncomplicated CDI • Within 120 hours of receiving standard therapy • Ability to comply with study procedures for the length of the study

Exclusion criteria	• At enrollment, presence of any of the following:
	  ○ Hypotension or shock
	  ○ Megacolon or moderate to severe ileus
	  ○ Acute abdomen
	  ○ Admission to intensive care unit
	• Inability to tolerate oral or enteral medication
	• Presence of other known infectious etiology of diarrhea
	• COVID-19 co-infection at the time of CDI diagnosis.
	• Absolute neutrophil count <500 mcl
	• Within 100 days of hematologic or solid organ transplant
	• Uncontrolled inflammatory bowel disease (e.g. Crohn's disease, ulcerative colitis) or other etiology of non-infectious diarrhea
	• Enrollment in another investigational drug trial
	• Current use of alternative treatment for CDI (e.g. antibiotics other than metronidazole, vancomycin or fidaxomicin; IVIg; fecal transplant).
	• On probiotics and not willing to discontinue.
	• Cirrhosis or in participants with ALT > 3X normal
	• End stage renal disease, not on dialysis, or creatinine clearance or estimated GFR of <30mL/min even after adequate hydration
	• Life expectancy of < 6 months.

**Diarrhea is defined as liquid stool or stool that takes the shape of the receptacle, with bowel movements occurring > 3x within a 24 hour period.*

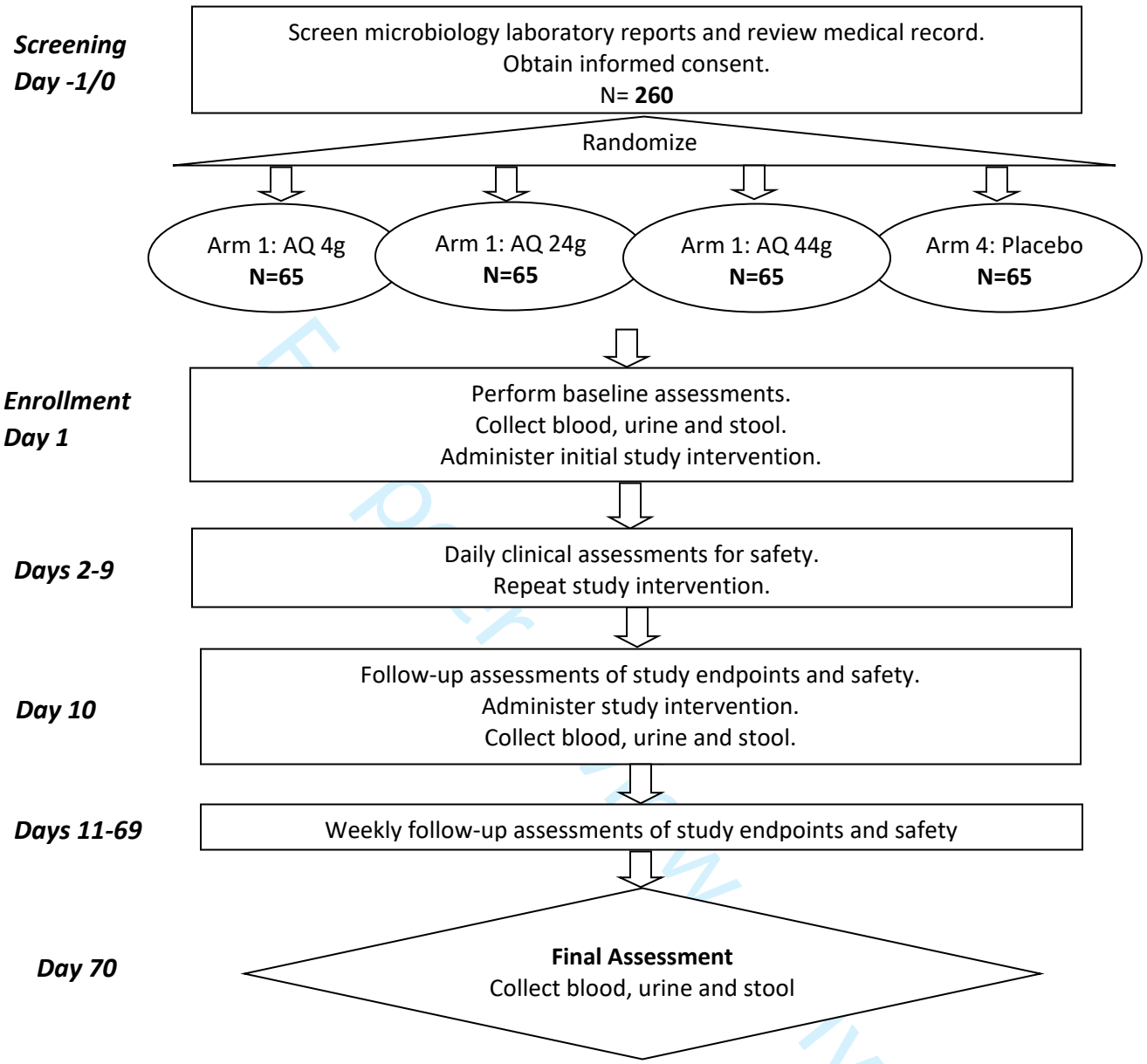
Table 2. Schedule of Activities

Procedures	Screening Day -1 to 0	Enrollment/Baseline Visit 1, Day 1	Study Visit 2-9[a]	Study Visit 10[b] Day 10+/- 1 day	Study Visit 11-1[c] Days 11-69 +/-2 days	Final Study Visit 18 Day 70+/-7 days
Informed consent	X					
Demographics	X					
Medical history	X					
Randomization	X					
Administer study intervention		X	X	X		
Concomitant medication review	X	X				
Physical exam	X	X		X		
Hematology	X			X		X
Complete metabolic panel	X			X		X
CRP, ESR, serum cytokines	X			X		X
Adverse event review and evaluation	X	X				
Fecal TcdB, *tcdB,* lactoferrin, cytokines	X			X		X
Collect stool for microbiome and urine for metabolomics	X			X		X
Complete Case Report Forms (CRFs)	X	X	X	X	X	X

[a]Daily visits while in the hospital or phone calls, if discharged.
[b]Participant comes to the clinic for assessment and specimen collection, if discharged.
[c]Weekly calls, if discharged; participant may be seen any day of the week (M-F) for episodes of diarrhea, if not yet seen by other healthcare providers; stools will be collected for *C. difficile* testing, if not yet done.

Figure Legend

Study Flow Chart from Screening to Final Assessment



Biological specimen collection and laboratory evaluation

As part of screening process, the potential subject's stool that tested positive by *tcdB* PCR from the Microbiology laboratory will be retrieved. Blood, stool, and urine will be collected at days 0, 10 and 70.

For patients who consented to the study, the following will be evaluated:

Clinical l Laboratory:

1. Complete blood count (CBC): white blood cells (WBC) with differential counts, hemoglobin, hematocrit, platelet count
2. Complete metabolic panel or basic metabolic panel plus liver function test: Sodium, potassium, chloride, bicarbonate, creatinine, BUN, glucose, alkaline phosphate, AST, ALT, direct bilirubin, total bilirubin, albumin
3. Erythrocyte sedimentation rate (ESR)
4. C-reactive protein (CRP)

Note: Blood results (as part of medical care) from the previous 24 hours can be used for this purpose. If any of the tests are not available, the test/s will be added on to previously collected blood, if available. If not, a phlebotomist will draw approximately 10 mL of blood for the tests below. These blood tests will be performed at days 0 and 10 (before and after treatment) and 70. Results of diagnostic tests performed as part of regular medical care will be used in accordance with Health Insurance Portability and Accountability Act (HIPAA) and IRB rules.

Research Laboratory:

- Fecal lactoferrin
- Anaerobic culture for *C. difficile* and ribotyping of *C. difficile* isolates
- Antimicrobial susceptibility testing
- Quantification of TcdA and TcdB
- DNA extraction and microbiome assay
- Metabolomics assay
- 38-plex cytokine profiling (*Luminex*) at the UVA Flow Cytometry Core facility

Research Laboratory Assays

Isolation of C. difficile from human fecal samples

C. difficile spores will be recovered using alcohol shock followed by inoculation into Chromid (BioMérieux) or TCCFA agar plates: *C. difficile* agar base (Oxoid) supplemented with 1% taurocholate (Sigma-Aldrich), 7% defibrinated horse blood (Remel) and cycloserine/cefoxitin (Oxoid, 2297109). Briefly, approximately 1 mL of fecal samples will be incubated with equal volumes of ethanol 200 proof (Decon laboratories) at room temperature for 1h and centrifuged (3500 rpm, 10 min). Then, the pellet will be inoculated in Chromid or TCCFA agar plates using a swab and incubated at anaerobic chamber (Bactrom) for 1 and 2-5 days, respectively, at 37°C. One single colony with morphology similar to *C.difficile* will be cultured in Brucella blood agar plates for 48h. One single colony will be grown and stocked in Chopped Meat Broth (Remel). After 24h, 10 µL of culture supernatant will be spread in BHIS agar plates for aerotolarance test and 1mL will be used for PCR analysis (for identifying *C. difficile* triose phosphate isomerase, *tpi)*.

Measurement of TcdA, TcdB and CDT in stools

Levels of TcdA and TcdB will be measured by ELISA. Briefly, a high binding 96 well plates will be coated overnight with a polyclonal *C. difficile* toxin A antibody (Novus biological, NB100-62473) or polyclonal *C. difficile* toxin B antibody (Thermo Fisher, ACDTB). Plates will be washed three times with wash buffer and incubated with samples and a standard curve (TcdA and TcdB from Labtech) overnight at 4°C. After washing the plates three times with wash buffer, each well will be incubated with HRP *C. difficile* toxin A antibody (Novus biological, NBP3-08858H) or HRP *C. difficile* toxin B antibody (R&D system, AF6246) for 2h at room temperature. Plates will be washed and incubated with substrate reagent (R&D system) for 20 min and the reaction will be stopped by adding stop solution (R&D system). Absorbance of the reaction will be detected at 450 nm in an ELISA reader. Optimal dilution of each antibody will be experimentally determined.

PCR to identify C. difficile genotyping

C. difficile genomic DNA will be extracted using a DNA extraction kit (Qiagen), according to the manufacturer's recommendations. PCR amplification of *tcdA, tcdB, cdtB*, and *tpi* will be performed in a CFX Connect system (Bio-Rad) with the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 s and 55°C for 30 s. All PCRs will be performed with iTaq Universal SYBR Green Supermix (Bio-Rad). The primer sets are listed in supplementary Table 1.

Antimicrobial susceptibility test

To determine minimum inhibitory concentrations (MICs) of clindamycin (CLI), fidaxomicin (FDX), metronidazole (MTZ), moxifloxacin (MXF), tigecycline (TGC), and vancomycin (VAN), spores of *C. difficile* isolates will be inoculated onto BHIS agar plates supplemented with taurocholate followed by 20h growing in BHIS broth. MIC will be determined using broth microdilution and Etest strips (Biomerix) according to the manufacturer's instructions. Agar dilution with specific antibiotic concentration in Brucella agar plates containing haemin (Sigma, 5 mg/l), vitamin K1 (Sigma, 1 mg/l), and 5% defibrinated sheep red blood cells (Remel) will be used to confirm the resistant

strains. E-test strips will be used to test the susceptibility to TGC (ranging from 0.016 µg/mL to 256 µg/mL), MXF (0.002 µg/mL to 32 µg/mL), VAN (0.016 µg/mL to 256 µg/mL), CLI (0.016 µg/mL to 256 µg/mL) and MTZ (0.016 µg/mL to 256 µg/mL) according to the manufacturer's instructions. Microbroth dilution will be used to test susceptibility to FDX. The interpretation of minimum inhibitory concentration (MIC) will be done according to the recommendations of CLSI M11-A7 and EUCAST. *C. difficile* ATCC 700057 will be used for quality control.

Ribotyping

Isolates will be ribotyped using an internationally standardized, high-resolution capillary gel-based electrophoresis PCR ribotyping protocol for *C. difficile*. The 16S and 23S rRNA genes will be amplified using 1 µL of DNA, 12.5 µL of HotStaq (Qiagen, 203443), 9.5 µL of nuclease-free water and 1 µL of each primer (16S and 23S) at 95°C for 15 min. PCR products will be analyzed on a 2100 Agilent bioanalyzer using a DNA HS kit (Agilent) performed by Genome analysis and Technology Core (RR:SCR_018883). Samples containing 1 µL of amplified DNA, 0.5 µL of 1200 LIZ standard, and 8.5 µL of Hi-Di formamide (Life Technologies, Carlsbad, CA) will be injected at 5 kV for 5 s and resolved using a separation voltage of 6.5 kV for 103 min. Major peaks in fluorescent signal will be imported into BioNumerics v.5.1 software (Applied Maths, Austin, TX) for analysis. Fragments will be initially sized using GeneMapper v.4.0 software (Life Technologies) before being imported into BioNumerics. All signals with a height <10% of the highest peak in the individual profile will be excluded (as these were considered background rather than evidence of a major DNA fragment). Ribotyping will be identified based on Leeds-Leiden *C. difficile* reference strain library.

Measurement of inflammation biomarkers in fecal samples

Protein will be extracted from stools using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS,

adjusted to pH 7.5) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (Sigma-Aldrich). Samples will be centrifuged at 13000 rpm for 15 min and the supernatant will be used to perform the protein assay using the bicinchoninic acid assay (Thermo Fisher Scientific). Levels of inflammatory mediators (such as MPO, calprotectin, lactoferrin an others) will be measured using a commercial 38-plex cytokine profiling kit (R&D Systemsor ELISA kit (BD bioscience) according to the manufacturer's instructions. For the single ELISA assay, the absorbance (450 nm) will be determined using an Epoch plate reader (BioTek). For the Bio-plex assay, the samples will be run on a Luminex machine.

Microbiota analysis

Fecal DNA will be extracted using Qiagen stool extraction kit (Qiagen). The V1-V3 hypervariable regions of *16S rRNA* gene from fecal DNA samples will be amplified by PCR with broad range primers 8F and 534R *16S rRNA* libraries from up to 100 samples will be pooled and sequenced using MiSeq Reagent Kit v3. From the *16S rRNA* sequences, bacteria present in each sample will be identified and relative abundance quantified using the QIIME package [1] for sample demultiplexing, quality filtering, chimeric sequence removal, identification of operational taxonomic units (OTUs), and taxonomic classification. Changes in the bacterial composition will be analyzed using multivariate technique Principal Coordinate Analysis (PCoA) as previously described [2]. Signature genera will be identified by Random Forrest machine-learning classification. Model accuracy will be calculated using the 10-fold cross validation error estimate, which is an approximation of how frequently a sample is misclassified. The discriminatory power of each genus is assessed by comparing the classification accuracy with and without including the genus in the model. Genera that led to more loss of classification accuracy will be considered to be more discriminatory.

Metabolomics

Metabolic profiles of blood, urine and fecal samples will be comprehensively measured using a dual platform approach incorporating 1H nuclear magnetic resonance (NMR) spectroscopy and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) [3-6]. For the NMR analysis, an untargeted approach will be adopted to measure H-containing metabolites in the study samples using standard one-dimensional 1H NMR experiments. These will be performed using standard methods on a 700 MHz Bruker NMR spectrometer equipped with a cryoprobe for enhanced sensitivity. These will be optimized for quality, sensitivity and solvent suppression. Quality control samples will be created from pooled samples and analyzed intermittently for analytical validation. This untargeted approach allows the simultaneous unbiased assessment of a broad range of metabolite classes including several metabolites previously associated with CDI, such as p-cresyl-sulfate, 4-hydrophenylacetate, tyrosine, glycine, short-chain fatty acids (acetate, propionate, butyrate), and caproate. For the UPLC-MS analysis, a triple-quadrupole platform will be used for the targeted profiling of bile acids in the blood and fecal samples. These microbial-host co-metabolites have been previously implicated with CDI. This approach provides enhanced sensitivity and includes conjugated, and unconjugated bile acids as well as a range of primary, secondary and tertiary bile acids..

Standard multivariate statistical approaches will be applied to elucidate metabolic perturbations in the individuals associated with infection, antibiotic intake and alanyl-glutamine supplementation. This will include, but will not be limited to, principal components analysis (PCA), projection to latent structures-discriminant analysis (PLS-DA), self-organizing maps (SOMs), random forest-based methods, and linear regression techniques. Biochemical variation related to the additional clinical and phenotypic data will also be investigated. In addition, data fusion strategies will be used to couple the metabolic phenotypes with high resolution microbial profiles. This will allow pathogen-host, pathogen-microbiome and microbiome-host interactions to be studied as well as the impact of alanyl-glutamine treatment on these biochemical

relationships. For the NMR datasets, once candidate spectral components have been identified as discriminatory features, metabolite identification will be performed. This will involve the use spectral compound libraries (e.g. Human metabolome database [HMDB], KEGG, the Biological Magnetic Resonance Data Bank, published literature and in-house databases). The structural identity will be investigated if necessary using two dimensional NMR experiments (e.g. 1H-1H COrrelation SpectroscopY [COSY] and 1H-1H TOtal Correlation SpectroscopY [TOCSY]) and statistical approaches (Statistical TOtal Correlation SpectroscopY [STOCSY]).

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2. Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 2007;62:142–60. doi:10.1111/j.1574-6941.2007.00375.x
3.) Claus SP, Swann JR. Nutrimetabonomics: Applications for Nutritional Sciences, with Specific Reference to Gut Microbial Interactions. Annu Rev Food Sci Technol 2013 Jan 3.
4. Saric J, Li JV, Swann JR, et al. Integrated cytokine and metabolic analysis of pathological responses to parasite exposure in rodents. J Proteome Res 2010 May 7;9(5):2255-64.
5. Swann JR, Tuohy KM, Lindfors P, et al. Variation in antibiotic-induced microbial recolonization impacts on the host metabolic phenotypes of rats. J Proteome Res 2011 Aug 5;10(8):3590-603.
6. Swann JR, Want EJ, Geier FM, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci U S A 2011 Mar 15;108 Suppl 1:4523-30.

Reporting checklist for protocol of a clinical trial.

Based on the SPIRIT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the SPIRITreporting guidelines, and cite them as:

Chan A-W, Tetzlaff JM, Gøtzsche PC, Altman DG, Mann H, Berlin J, Dickersin K, Hróbjartsson A, Schulz KF, Parulekar WR, Krleža-Jerić K, Laupacis A, Moher D. SPIRIT 2013 Explanation and Elaboration: Guidance for protocols of clinical trials. BMJ. 2013;346:e7586

		Reporting Item	Page Number
Administrative information			
Title	#1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1

Trial registration	#2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
Trial registration: data set	#2b	All items from the World Health Organization Trial Registration Data Set	na
Protocol version	#3	Date and version identifier	2
Funding	#4	Sources and types of financial, material, and other support	14
Roles and responsibilities: contributorship	#5a	Names, affiliations, and roles of protocol contributors	1, 14
Roles and responsibilities: sponsor contact information	#5b	Name and contact information for the trial sponsor	1
Roles and responsibilities: sponsor and funder	#5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	14
Roles and responsibilities: committees	#5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and	9

		other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	
Introduction			
Background and rationale	#6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4
Background and rationale: choice of comparators	#6b	Explanation for choice of comparators	6
Objectives	#7	Specific objectives or hypotheses	5
Trial design	#8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	#9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5

Eligibility criteria	#10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Table 1
Interventions: description	#11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6
Interventions: modifications	#11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving / worsening disease)	8
Interventions: adherance	#11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return; laboratory tests)	8
Interventions: concomitant care	#11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Table 1
Outcomes	#12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	7

Participant timeline	#13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 2
Sample size	#14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	5
Recruitment	#15	Strategies for achieving adequate participant enrolment to reach target sample size	5
Methods: Assignment of interventions (for controlled trials)			
Allocation: sequence generation	#16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	#16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque,	6

		sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	
Allocation: implementation	#16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	6
Blinding (masking)	#17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	6
Blinding (masking): emergency unblinding	#17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	6
Methods: Data collection, management, and analysis			
Data collection plan	#18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	9

Data collection plan: retention	#18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	6, 8
Data management	#19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistics: outcomes	#20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	7
Statistics: additional analyses	#20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	7
Statistics: analysis population and missing data	#20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	7
Methods: Monitoring			
Data monitoring: formal committee	#21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and	9

		competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	
Data monitoring: interim analysis	#21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	8
Harms	#22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	8
Auditing	#23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	8
Ethics and dissemination			
Research ethics approval	#24	Plans for seeking research ethics committee / institutional review board (REC / IRB) approval	9
Protocol amendments	#25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC / IRBs, trial participants, trial registries, journals, regulators)	9

Consent or assent	#26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	5
Consent or assent: ancillary studies	#26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	5
Confidentiality	#27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	9
Declaration of interests	#28	Financial and other competing interests for principal investigators for the overall trial and each study site	14
Data access	#29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	9
Ancillary and post trial care	#30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	na
Dissemination policy: trial results	#31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	9

Dissemination policy: authorship	#31b	Authorship eligibility guidelines and any intended use of professional writers	na
Dissemination policy: reproducible research	#31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	9

Appendices

Informed consent materials	#32	Model consent form and other related documentation given to participants and authorised surrogates	Appendix
Biological specimens	#33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Appendix