BMJ Open  Alanyl-glutamine supplementation for Clostridioides difficile infection treatment (ACT): a double-blind randomised controlled trial study protocol

Cirle A Warren,1,1 Jae Hyun Shin,1 Ekta N Bansal,1 Deiziane V D S Costa,1 Xin Qun Wang,2 Martin Wu,4 Jonathan R Swann,5 Brian W Behm,6 Paul V Targonski,3,7 Laurie Archbald-Pannone7

ABSTRACT

Introduction  Clostridioides difficile is the leading cause of healthcare-associated infections in the USA, 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 alanyl-glutamine (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 randomised, 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 44 g doses administered daily for 10 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 number  NCT04305769.

INTRODUCTION

Diarrhoea is a major cause of mortality from infectious diseases in the USA. From 1980 to 2014, deaths from diarrhoeal diseases increased from 0.4 to 2.4 per 100000 persons, while the overall mortality due to all infections dropped from about 43 to 34 deaths per 100000 persons.1 The likely cause of the observed increase in death is Clostridioides difficile infection (CDI), the most common cause of antibiotic-associated diarrhoea and healthcare-associated infections.2 In 2017, the estimated number of CDI cases in the USA 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 healthcare 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. 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 (and therefore, consequent CDI treatments). Strategies that target the microbiome—probiotics or faecal transplant, or the toxins (not the bacteria)—tocolvestor or monoclonal antibodies 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 alanine 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. Our preliminary data indicate that AQ may specifically be beneficial for CDI. We found that glutamine and AQ reduced C. difficile toxin A, TcdA, induced apoptosis and that this was associated with inhibition of caspase eight activation in intestinal cell line. Migration of intestinal epithelial cells after injury is also inhibited by both TcdA and TcdB, an effect that is prevented in the presence of glutamine or AQ. Glucosylation of Rho by AQ partially reversed the altered F-actin distribution and increased RhoA expression. In vivo studies confirmed the benefit of AQ in C. difficile-associated diarrhoea. 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. 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 postantibiotic associated relapse, diarrhoea and mortality. 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 44g given orally with standard treatment in seven hospitalised patients, two 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 two 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 diarrhoea and other conditions, we now conduct this double-blind, placebo-controlled randomised 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, randomised, 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 with standard of care, daily AQ supplementation will reduce recurrence (primary outcome) and mortality (secondary outcome) during 60 days post-treatment follow-up. Furthermore, we hypothesise 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 enrolment: the University of Virginia Health, a tertiary academic centre 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, 4mg dose, 24mg dose and 44mg 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 randomised 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 University of Virginia (UVA) site opened for enrolment on January 2021 and the Carilion Clinic on June 2023. The planned end date of the study is June 2025.

**Eligibility, recruitment and enrolment**

Potential participants will be identified through the microbiology reports and limited review of the electronic medical record (EMR) for the enrolment criteria (figure 1). 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 randomised in the trial. The inclusion and exclusion criteria for participant enrolment are summarised in table 1. Schedule of activities are presented in table 2.

Randomisation

This study will employ a randomised, 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 randomisation scheme within each of the study sites using an SAS randomisation algorithm and a block randomisation approach. Because we are enrolling participants over a longer period of time within each of the study sites, we will utilise block randomisation 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 randomised 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.

Figure 1  Study flow chart from screening to final assessment.

Screening
Day -1/0
Screen microbiology laboratory reports and review medical record. Obtain informed consent. N= 260

Randomize

Arm 1: AQ 4g N=65
Arm 1: AQ 24g N=65
Arm 1: AQ 44g N=65
Arm 4: Placebo N=65

Enrollment
Day 1
Perform baseline assessments. Collect blood, urine and stool. Administer initial study intervention.

Days 2-9
Daily clinical assessments for safety. Repeat study intervention.

Day 10
Follow-up assessments of study endpoints and safety. Administer study intervention. Collect blood, urine and stool.

Days 11-69
Weekly follow-up assessments of study endpoints and safety

Day 70
Final Assessment Collect blood, urine and stool
Table 1 Enrolment eligibility

**Inclusion criteria**
- Age 18 years and above, all gender
- Diarrhoea 
- Stool positive for tcdB (toxin B)
- 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 enrolment, 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 aetiology of diarrhoea
- COVID-19 co-infection at the time of CDI diagnosis
- Absolute neutrophil count<500 mcL
- Within 100 days of haematologic or solid organ transplant
- Uncontrolled inflammatory bowel disease (eg, Crohn's disease and ulcerative colitis) or other aetiology of non-infectious diarrhoea
- Enrolment in another investigational drug trial
- Current use of alternative treatment for CDI (eg, antibiotics other than metronidazole, vancomycin or fidaxomicin; IVIg; faecal transplant)
- On probiotics and not willing to discontinue
- Cirrhosis or in participants with ALT (alanine aminotransferase) >3X normal
- End stage renal disease, not on dialysis or creatinine clearance or estimated GFR (glomerular filtration rate) of <30 mL/min even after adequate hydration
- Life expectancy of <6 months

*Diarrhoea is defined as liquid stool or stool that takes the shape of the receptacle, with bowel movements occurring>3X within a 24 hour period.
ALT, alaninaminotransferase; CDI, Clostridioides difficile infection; GFR, glomerular filtration rate; tcdB, toxin B.

If a participant has an adverse event (AE) 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 Data and Safety Monitoring Board (DSMB) or appropriate auditors may request code-breaking. Intentional or unintentional breaking of the blinding will be reported to the Institutional Review Boards (IRB), National Institutes of Health (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, odourless 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 diarrhoea, occurrence of other AEs, new medications or procedures, clinic/urgent care/ER visits or other developments. Diarrhoea is defined as unformed or liquid stool taking the shape of the receptacle and bowel movements>3 within 24 hours. If with recurrent diarrhoea 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 enrolment). 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 randomised participants will be included in the analysis based on the intent-to-treat principle. Standard descriptive statistics will be used to summarise participants’ baseline demographic and clinical characteristics by four treatment groups. Percentages and counts will be used for categorical variables, while mean with SD and IQR 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 CIs. 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 randomised 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. Analysis will use analysis of variance unless statistically significant differences in the distribution of baseline characteristics or features of non-normality are detected and relevant, at which point contingency utilisation of analysis of covariance, 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% CIs. As noted above, we will emphasise 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 non-parametric 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 on request. An investigator may...
discontinue or withdraw intervention or participant from the study for the following reasons: if any clinical 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 recognised) 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 AE 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 AEs. 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 diarrhoea, occurrence of other AEs, new medications or procedures and clinic/urgent care/ER visits or other developments.

The occurrence of an 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 on 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/stabilisation 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 characterised 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 stabilisation.

Safety oversight will be under the direction of a 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, Standard Protocol Items: Recommendations for Interventional Trials reporting guidelines was used.

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 Centre 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. This study involves human participants and was approved by University of Virginia and the Carilion Medical Center Institutional Review Boards (Protocol HSR200046).

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 unauthorised third party without prior written approval of the sponsor.

Data collection and management
Clinical data (including 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 affiliations
1Department of Medicine, Division of Infectious Diseases and International Health, University of Virginia School of Medicine, Charlottesville, Virginia, USA
2Department of Internal Medicine, Virginia Tech Carilion School of Medicine, Roanoke, Virginia, USA
3Department of Public Health Sciences, University of Virginia School of Medicine, Charlottesville, VA, USA
4Department of Biology, University of Virginia College and Graduate School of Arts and Sciences, Charlottesville, VA, USA
5School of Human Development and Health, University of Southampton Faculty of Medicine, Southampton, UK
6Department of Medicine, Division of Gastroenterology and Hepatology, University of Virginia School of Medicine, Charlottesville, VA, USA
7Department of Medicine, Division of General, Geriatric, Palliative & Hospital Medicine, University of Virginia School of Medicine, Charlottesville, VA, USA

Contributors CW and LA-P developed and provided oversight for study design and drafted manuscript. JHS edited the manuscript and participated in study design. PVT and XQW designed the statistical plans and edited the manuscript. MW, JHS, DVDS, WBB, and ENB participated in study design and critically edited the manuscript. JS is from the University of Southampton, 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 CW is a medical advisor for Seres-109 of Aimmune and Seres Therapeutics and site PI for Rebypta Prospective Registry. The rest of the authors have no conflict of interest to disclose.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research.

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ORCID iD Cirle A Warren http://orcid.org/0000-0002-4417-0445

REFERENCES
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 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 and others) will be measured using a commercial 38-plex cytokine profiling kit (R&D Systems) or 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 of 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]).

REFERENCES:


