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 4ºC. 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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