BEZLOTOXUMAB YIELDED OUTCOMES BY ADDRESSING PERSONALIZED NEEDS IN *CLOSTRIDIOIDES DIFFICILE* INFECTION: THE *BEYOND* DOUBLE-BLIND RANDOMIZED CLINICAL TRIAL

STUDY PROTOCOL

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EudraCT number: 2021-005473-10
Protocol version: 1.0
Protocol date: 5 October 2021

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DISCLOSURE OF PRINCIPAL INVESTIGATOR

Protocol Study Title: BEZLOTOXUMAB YIELDED OUTCOMES BY ADDRESSING PERSONALIZED NEEDS IN *CLOSTRIDIOIDES DIFFICILE* INFECTION: THE *BEYOND* DOUBLE-BLIND RANDOMIZED CLINICAL TRIAL

The herein protocol became known to myself by the Study Sponsor. I understand that the protocol remains as yet unpublished; I certify that all disclosed information to myself for this protocol will remain strictly confidential.

The Principal Investigator,

Print Name

Signature Date

TABLE OF CONTENTS

STUDY SYNOPSIS

Objective	Previous data from our group have shown that integrated information from SNPs of the host DNA, IL-8 and the enrichment of the stool microbiome can indicate the patients with infection by *Clostridioides difficile* (CDI) at risk for unfavorable outcome. This integrated information is forming the BEYOND score. The aim of the BEYOND RCT is to investigate if adjunctive bezlotoxumab treatment to the current standard-of-care may decrease the likelihood of unfavorable outcome for patients who score positive by the BEYOND score.
Study design	Double-blind randomized clinical study powered by the primary endpoint for 44 patients.
Inclusion criteria	1. Age ≥18 years 2. Both genders 3. Written informed consent provided by the patient or by their legal representative in case of patients unable to consent 4. In case of non-menopausal women, unwillingness to become pregnant during the study period. Women of child-bearing potential will be screened by a urine pregnancy test before inclusion in the study. 5. Diarrhea defined as at least 3 episodes of unformed stool in the past 24hours. 6. Positive stool for *C.difficile*. This is defined as any stool sample positive for the presence of glutamate dehydrogenase (GDH) and for the presence of toxin A and/or B. 7. Positive BEYOND score i.e. meeting any of the following: Gene score for susceptibility to CDI more than 53. The score is provided by the following equation: (Carriage of C allele of rs12148744 x 27) – (carriage of C allele of rs714024 x 27) - (carriage of C allele of rs721059 x 29) + (carriage of T allele of rs4311028 x 33) – (carriage of A allele of rs62183547 x 25)

	+ (carriage of C allele of rs1128266 x 12) - (carriage of T allele of rs4279595 x 17) + (carriage of G allele of rs175006 x 11) + (carriage of T allele of rs3859214 x 17) + (carriage of G allele of rs7222870 x 15) – (carriage of G allele of rs5086600 x 9) + (carriage of T allele of rs7240534 x 12) + (carriage of G allele of rs20911172 x 11) - (carriage of C allele of rs17680671 x 17) OR Score provided by the following equation more than 9= [Hemoglobin <9.5 g/dl x 10] + [serum urea >64.5 mg/dl x 14] + [serum interleukin-8 >227 pg/ml x 19] – [carriage of G allele of rs2091172 x 17] OR More than 3log10 of gammaproteobacteria or Enterobacteriaceae or Enterobacteriales in the stool
Exclusion criteria	• Age below 18 years • Denial for written informed consent • Known allergy to bezlotoxumab • Pregnancy or lactation. Women of child-bearing potential will be screened by a urine pregnancy test before inclusion in the study
Intervention	Patients will be blindly assigned to adjunctive treatment with placebo or bezlotoxumab at a 1:1 ratio. All patients will receive standard-of-care treatment for CDI at the discretion of their attending physicians.
Primary endpoint	The superiority of bezlotoxumab over placebo to reduce the incidence of unfavorable outcome. Unfavorable outcome is defined as any of the following: progression into organ dysfunction; relapse of CDI; and death. The primary endpoint is tested on Day 40 from start of blind treatment. Organ dysfunction is defined as any increase of the baseline total SOFA score by at least 2 points. Need for colectomy or admission in the Intensive Care Unit counts as organ dysfunction.
Secondary endpoints	The superiority of bezlotoxumab over placebo when given for CDI of high likelihood for unfavorable outcome on the following:

	• Incidence of organ failure and time to first organ failure • Time to relapse of CDI • Survival time • Cost of hospitalization • Validation of the BEYOND score
Power of the study	This is calculated for the primary endpoint. Power calculation is based on the hypothesis that the difference between bezlotoxumab and placebo will be at least 40%. It is anticipated that 60% of patients allocated to the placebo arm and 20% of patients allocated to the bezlotoxumab arm will achieve the primary endpoint of unfavorable outcome. To demonstrate this difference at the 10% level of significance with 80% power, 22 patients should be enrolled into each arm (44 patients in total). It is anticipated that one out of three screened patients with CDI will meet all inclusion criteria, so that a total of 132 patients need to be screened.
Duration	2 years

ABBREVIATIONS

AE: adverse event

bid: two times daily

CCI: Charlson's comorbidity index

CDI: *Clostridioides difficile* infection

CI: confidence interval

CRF: case report form

eQTL: expression qualitative trait locus

ICU: intensive care unit

IL: interleukin

IRB: institution review board

GDH: glutamate dehydrogenase

GWAS: genome-wide association study

q6h: every 6 hours

OR: odds ratio

OTU: operational taxonomic unit

PPV: positive predictive value

SAE: serious adverse event

SOFA: sequential organ failure assessment

SNP: single nucleotide polymorphism

RCT: randomized clinical trial

RT-PCR: real time polymerase chain reaction

TEAE: treatment-emergent adverse event

tid: three times daily

INTRODUCTION

Clostridioides difficile infection (CDI) is an emerging infection with increased point-prevalence per year as reported both for the United States and for European countries (1, 2). Current evidence suggests that CDI is a complex interaction between the host and the offending pathogen where physician intervention plays a pivotal role. Although originally conceived to be a hospital-acquired infection, it becomes more and more recognized that many cases are community-acquired. This underscores the significance of the host in the pathogenesis of CDI. CDI is traditionally considered to derive from the direct oral transmission from one host to the other in the hospital environment. However, a recent survey in three large hospitals in Wales analyzing the whole genome *C.difficile* isolated from 499 cases of diarrhea positive for glutamate dehydrogenase (GDH) revealed that the vast majority of isolates of *C.difficile* had different nucleotide sequences suggesting against the direct transmission from one patient to the other in the vast majority of cases (3). The complex interaction between the host and *C.difficile* ending to infection is modulated by environmental factors like antibiotic consumption, proton pump inhibitor intake and prolonged hospitalization (4).

The main endpoint of all randomized clinical trials (RCTs) of new antimicrobials targeting *C.difficile* is sustained clinical response that is defined as the composite of the resolution of the infection at the end of treatment without any CDI relapse within the first 40 days (5). This endpoint is mainly targeting to demonstrate if newly developed agents are superior over the comparator vancomycin in the minimization of relapse risk. However, recent data outscore the lack of substantial merit of this endpoint as many cases of CDI bear an intrinsic danger of unfavorable outcome without this being incorporated in the RCT endpoints. More precisely, a substantial rate of patients dies after the end of oral treatment between days 12 and 40 probably as a result of emerging organ dysfunction in the field of CDI colitis (6). Recent epidemiological data coming from the United States challenge even more the traditional way of our thinking of CDI. More precisely, in a prospective survey of 30,326 patients it was shown that the risk of unfavorable outcome and death was greater among first-time infected patients than in relapsed cases. This survey reports that mortality from the first CDI case may be as high as 28% (1). These cases of CDI associated with an unfavorable outcome leading to surgery and

hospitalization in an intensive care unit are associated with a tremendous cost mounting to $34,149 per case (7). Surprisingly when data of the two registration RCTs of fidaxomicin were combined reduced CDI mortality was shown over the comparator vancomycin treatment (6). This observation strengthens the belief that the substantial merit of new agents is not in preventing CDI relapse but in saving lives.

The pivotal RCTs of fidaxomicin influenced the design of the MODIFY studies in a way that the inclusion criteria of these studies comprised not only cases of CDI infection of moderate severity but also patients with high chance of relapse. Half of the enrolled patient population was aged equal to or above 65 years and another quarter of enrolled patients had history of at least one episode of CDI. In these trials, patients with CDI receiving oral treatment with metronidazole, vancomycin or fidaxomicin were randomized to one single dose of adjunctive treatment with placebo or the monoclonal antibody targeting the toxin B of *C.difficile* bezlotoxumab, or the monoclonal antibody targeting the toxin A of *C.difficile* actoxumab or both antibodies. Results revealed that bezlotoxumab treatment reduced considerably the risk of relapse whereas the intake of actoxumab did not affect the clinical outcome of CDI. (8). However, the efficacy of bezlotoxumab treatment on other variables of unfavorable outcome was not analyzed.

One main argument against new treatments targeting *C.difficile* is the substantial cost. This argument is based on the fact that there are no tools that can discriminate between patients at high risk for unfavorable outcome that are in need of new treatments from patients at low risk from unfavorable outcome that will benefit from standard-of-care vancomycin treatment.

AVAILABLE UNPUBLISHED DATA

SPECIFY (Scoring personalized needs in *Clostridium difficile* infections for fidaxomixin therapy) was a prospective study that was sponsored by the Hellenic Institute for the Study of Sepsis. The study was conducted during the period September 2015 to November 2016 in 11 departments of Internal Medicine in Greece (ClinicalTrials.gov NCT02573571) with the endpoint of the development of a score that could comprise clinical signs, biomarker and genetic elements and that could provide positive predictive value (PPV) equal to or more than 80% for the prediction of unfavorable outcome of CDI.

Unfavorable outcome was defined as any of the following: presentation or progression into severe CDI, relapse; and death. The SPECIFY study was conducted in two phases. In the first phase, a total of 153 patients with CDI and 150 comparators matched for their Charlson's Comorbidity Index (CCI) were enrolled. All patients and comparators were of Caucasian origin. DNA was extracted from whole blood. Genome-wide association study (GWAS) was performed using one HumanCore-chip comprising 4,330,811 single nucleotide polymorphisms (SNPs). Following quality control of imputed data 134 comparators and 134 CDI cases were analyzed. After Manhattan plotting, 17 single nucleotide polymorphisms (SNPs) were found to be strongly associated with CDI. These 17 SNPs entered forward step-wise logistic regression analysis and 14 SNPs remained to be independently associated with CDI so as to construct a prediction score for the development of CDI when CCI was high. In the second phase of the SPECIFY study, these 14 identified SNPs were analyzed along with clinical and biomarker data to develop a score that could predict the risk of unfavorable outcome within the population of the 134 patients who also participated in the first phase. Among these 134 patients, unfavorable outcome was found in 39 (29.1%). Using logistic regression analysis, a prediction score to early detect unfavorable outcome was developed. This score comprised only one of the 14 SNPs, namely carriage of G alleles of rs2091172. This score is provided by the following equation:

[Hemoglobin <9.5 g/dl x 10] + [serum urea >64.5 mg/dl x 14] +
[serum interleukin-8 >227 pg/ml x 19] – [carriage of G allele of rs2091172 x 17]

Any score more than 9 was associated with PPV 80% for the prediction of unfavorable outcome meeting successfully the study primary endpoint. At that cut-off, the prediction score has specificity 95.8% and negative predictive value 79.8% for the early detection of unfavorable outcome by CDI. Although the specificity of the score is fully satisfactory, the score has limited sensitivity mounting to 41.0% and mandating further improvement (full clinical study report of the SPECIFY study provided in APPENDIX I).

In order to improve the sensitivity of the score, full microbiome analysis was done in the stool of patients. Unpublished results showed that the enrichment of the gut flora with gammaproteobacteria or Enterobacteriaceae or Enterobacteriales at an LDA score more than 3 log10 was associated with unfavorable outcome.

With this information, we introduce a score which is called BEYOND. In this score, patients with CDI is at increased likelihood for unfavorable outcome if they meet any of the following:

- Gene score for susceptibility to CDI more than 53. The score is provided by the following equation:

(Carriage of C allele of rs12148744 x 27) – (carriage of C allele of rs714024 x 27) - (carriage of C allele of rs721059 x 29) + (carriage of T allele of rs4311028 x 33) – (carriage of A allele of rs62183547 x 25) + (carriage of C allele of rs1128266 x 12) - (carriage of T allele of rs4279595 x 17) + (carriage of G allele of rs175006 x 11) + (carriage of T allele of rs3859214 x 17) + (carriage of G allele of rs7222870 x 15) – (carriage of G allele of rs5086600 x 9) + (carriage of T allele of rs7240534 x 12) + (carriage of G allele of rs20911172 x 11) - (carriage of C allele of rs17680671 x 17)

- Score provided by the following equation more than 9. [Hemoglobin <9.5 g/dl x 10] + [serum urea >64.5 mg/dl x 14] + [serum interleukin-8 >227 pg/ml x 19] – [carriage of G allele of rs2091172 x 17]
- More than 3log10 of gammaproteobacteria or Enterobacteriaceae or Enterobacteriales in the stool

AIM OF THE PROJECT

BEYOND (BEzlotoxumab Yielded Outcomes by addressing personalized Needs in *Clostridioides Difficile* infection) is aiming to investigate if adjunctive treatment with bezlotoxumab may benefit patients who are scoring positive for the BEYOND sore and prevent them from unfavorable outcome.

STUDY DESIGN

This is a prospective, proof-of-concept, double-blind, RCT to compare the efficacy of bezlotoxumab over placebo among patients with CDI and high-risk for unfavorable outcome. The study will be conducted according to the Helsinki declaration in Internal Medicine Departments (see APPENDIX V) participating at the network of the HSSG

(www.sepsis.gr). Patients will be enrolled after written informed consent provided by themselves or by their legal representatives in case of patients not able to consent. The study protocol will be approved by the National Organization for Medicines of Greece and by the National Ethics Committee of Greece. The study will be registered at www.clinicaltrials.gov before inclusion of the first patient. The study will be provided one EudraCT number and will be registered at the EudraCT public site of the EU (https://eudract.ema.europa.eu).

The study shall be governed by:

- The international standards for Good Clinical Principal (GCP) developed by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
- Directive 2001/20/EC for Clinical trials
- General Data Protection Regulation 679/2016 (EC)

Inclusion criteria

Enrolled patients should meet ALL the below inclusion criteria:

1. Age ≥18 years
2. Both genders
3. Written informed consent provided by the patient or by their legal representative in case of patients unable to consent
4. In case of non-menopausal women, unwillingness to become pregnant during the study period. Women of child-bearing potential will be screened by a urine pregnancy test before inclusion in the study.
5. Diarrhea defined as at least 3 episodes of unformed stool in the past 24hours.
6. Positive stool for *C.difficile*. This is defined as any stool sample positive for the presence of glutamate dehydrogenase (GDH) and for the presence of toxin A and/or B.
7. Positive BEYOND score i.e. meeting any of the following:

Gene score for susceptibility to CDI more than 53. The score is provided by the following equation:

(Carriage of C allele of rs12148744 x 27) – (carriage of C allele of rs714024 x 27) - (carriage of C allele of rs721059 x 29) + (carriage of T allele of rs4311028 x 33) – (carriage of A allele of rs62183547 x 25) + (carriage of C allele of rs1128266 x 12) - (carriage of T allele of rs4279595 x 17) + (carriage of G allele of rs175006 x 11) + (carriage of T allele of rs3859214 x 17) + (carriage of G allele of rs7222870 x 15) – (carriage of G allele of rs5086600 x 9) + (carriage of T allele of rs7240534 x 12) + (carriage of G allele of rs20911172 x 11) - (carriage of C allele of rs17680671 x 17)

OR

Score provided by the following equation more than 9= [Hemoglobin <9.5 g/dl x 10] + [serum urea >64.5 mg/dl x 14] + [serum interleukin-8 >227 pg/ml x 19] – [carriage of G allele of rs2091172 x 17]

OR

More than 3log10 of gammaproteobacteria or Enterobacteriaceae or Enterobacteriales in the stool

Exclusion criteria

Patients meeting ANY of the following criteria CANNOT be enrolled in the study:

- Age below 18 years
- Denial for written informed consent
- Known allergy to bezlotoxumab
- Pregnancy or lactation. Women of child-bearing potential will be screened by a urine pregnancy test before inclusion in the study

The study comprises a screening stage and a treatment stage.

Screening stage

The screening stage starts from the time patients or their legal representatives sign the written informed consent form and it can be extended until up to 72 hours from the start of empirical treatment for CDI by the attending physicians. Study sites will be equipped with the quick *C.DIFFICILE* Complete test to run GDH and toxins A/B detection on the study site. If patients meet inclusion criteria 1 to 6 and they do not meet any

exclusion criteria, 5ml of blood is collected after venipuncture of one forearm vein under aseptic conditions; 3ml are collected into one EDTA-coated tube to be used for DNA isolation; and the remaining 2ml into one pyrogen-free tube to be used for centrifugation and serum collection. Then either 10ml of liquid stool or one rectal swab are collected. Samples are transported via courier at the central lab of the Laboratory of Immunology of Infections at the 4th Department of Internal Medicine of ATTIKON University hospital of Athens. The central lab provides service 24hours by 7 days. There, transported samples will be processed and the following will be done:

- Isolation of patient DNA and RT-PCR for the presence of G alleles of rs2091172
- Measurement of serum IL-8 by enzyme an immunosorbent assay
- Measurement of the stool microbiome diversity by the RT-PCT OF gammaproteobacterial, Enterobacteriaceae and Enterobacteriales

Lab results will be provided to the study site and the investigators may calculate the BEYOND score using the hemoglobin and urea biochemistry. If the BEYOND score is positive, the patient can be enrolled in the study.

Patients who fail screening because they score negative for the BEYOND score will be visited/contacted after 40 days to capture information on progression into organ dysfunction; relapse of CDI; and/or death (see below section of Primary endpoint for full definition).

All lab samples will be destroyed immediately after completion of procession.

Treatment stage

Once a patient is considered eligible for enrolment, they will be blindly allocated at 1:1 ratio to adjunctive treatment with placebo or bezlotoxumab. The allocated sequence of the study drug (placebo or bezlotoxumab) will be generated by a biostatistician. The preparation of the study drug will be done in each study site by the un-blinded pharmacist investigators. The administration of the study drug will be done in each study site by the blinded investigators.

Placebo or bezlotoxumab **will be given as single intravenous infusions of one hour** within 72 hours from the start of standard-of-care treatment. The dose of

bezlotoxumab will be 10mg per kg of body weight dissolved in normal saline 0.9% or 5% dextrose water up to maximum of 1000mg. Placebo-treated patients will receive an equal volume of normal saline 0.9% or 5% dextrose water. Standard-of-care treatment will be prescribed to all patients at the discretion of the attending physicians according to local guidelines or to their own decision. This standard-of-care treatment may be oral fidaxomicin 200 mg bid or oral vancomycin 125 mg q6h for 10 days or oral metronidazole 500mg tid for 10 days or equivalent.

Study visits

The following study visits will be done:

Visit 1 (baseline or day 1)

The following procedures are done:

- Recording of: a) demographics; b) vital signs including blood pressure, heart rate and oxygen saturation; c) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); d) co-administered drugs and antimicrobials; e) underlying comorbidities, calculation of the Charlson's Comorbidity Index (CCI) (10, see APPENDIX III) and predisposing illnesses; f) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Administration of the study drug (placebo/bezlotoxumab)

Visit 2

This visit is taking place on day 2. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.

- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 3

This visit is taking place on day 3. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 4

This visit is taking place on day 4. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 5

This visit is taking place on day 5. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 6

This visit is taking place on day 6. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 7

This visit is taking place on day 7. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 8

This visit is taking place on day 8. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 9

This visit is taking place on day 9. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 10

This visit is taking place on day 10. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 11

This visit is taking place on day 12. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

Visit 12

This visit is taking place on day 40. The following procedures are done:

- Recording of: a) vital signs including blood pressure, heart rate and oxygen saturation; b) laboratory findings including white blood cells, creatinine and liver biochemistry (if available); c) co-administered drugs and antimicrobials; d) results of coloscopy if available; g) SOFA score (see APPENDIX II); h) any organ failure; i) need for colectomy or admission in the Intensive Care Unit; k) number of bowel movements; l) clinical cure or relapse; and m) survival or death.
- Recording of any treatment-emergent adverse event (TEAE) or serious TEAE

If a patient is in need colectomy or admission in the Intensive Care Unit or dies between visits 11 and 12, this information and the date of the event are recorded.

In case of patient discharge prior to Day 40, information on final outcome is collected by phone calls. All information is recorded in one case report form (CRF).

The cost of hospitalization of every patient comprising drugs, laboratory and radiology exams, accommodation and interventions will be provided at the end of day 40 in Euros according to the itemized cost for each of the above given by the Greek state.

Clinical cure is defined by the resolution of diarrhea (i.e. fewer than three unformed bowel movements per day for 2 days consecutively) for the duration of treatment and no further need for treatment.

Relapse is defined as the return of more than three unformed bowel movements in 24 hours with a positive stool toxin test necessitating retreatment (5, 11, 12).

Study drugs

Bezlotoxumab will be provided to each site in the form of vials. Bezlotoxumab needs to be stored at 2-8°C at the study site at a refrigerator with recording of temperature. In case recording indicates deviation of temperature below 0°C or above 10°C for more than a day, stored drugs need to be replaced. The placebo (N/S 0.9%) will be provided to

each site in the form of bottles and needs to be stored at room temperature with recording of temperature. The un-blinded site pharmacists are responsible for the recording of the storage conditions of the study drugs. They also need to prepare the test article according to instructions provided at Investigator's Brochure (IB). The IB will be provided by the Sponsor. The bottles with active drug or placebo will be covered to conceal the identity of the test article. Covering materials will be provided by the Sponsor. At the exterior of each bottle after coverage, a label will be placed with the prefix "BEY-", a letter and a 2-digit number. The prefix "BEY-" refers to the study code "BEYOND". The letter refers to the study site. The 2-digit number refers to the serial number of the enrolled patient at the respective study site. For example, the code BEY-A-07 refers to study site A and patient number 07 at that study site. The un-blinded pharmacist will provide the covered bottles to the blinded nurse or blinded investigator who will administer the infusion.

BLINDING PROCEDURES

This study is designed to maintain blinding from participants, site investigators and their teams until completion of the study. At each center, there will be an unblinded pharmacist who will be in charge of randomizing and preparing the study drug for each participant according to the randomized intervention assignment. These pharmacists will not be involved in data acquisition, collection, adjudication of outcomes or adverse events, or any other study procedures. They will not disclose the treatment assignment to the study team members unless it is via a formal process of early unblinding as described below.

An independent biostatistician will generate the assignment to blinding treatment.

Under normal circumstances, all the treatment assignments of participants will remain blinded until the completion of the trial (completion of enrollment and follow-up or early termination of the trial).

Circumstances for Urgent Unblinding

- **Urgent** unblinding of an individual participant's treatment assignment can be performed for emergency medical reasons to protect participant safety when, as

determined by the site Investigator or designee, knowing the participant's treatment assignment would affect immediate medical management of the participant.

- **Non-urgent** early unblinding of an individual participant's treatment assignment for medical/safety reasons, when knowledge of treatment assignment would not affect immediate medical management but may affect other aspects of a participant's medical care/safety.

Reasons and Guidelines for Unblinding

Conventionally, full unblinding of the investigator team takes place after all study data have been recorded and reviewed for all participants, endpoints have been reviewed (if applicable per protocol), and the protocol team has declared the study dataset to be complete and "locked".

It is critical to the objectives of any blinded study that the objectivity of the Investigator team (including site Investigators), other site staff, and participants be maintained. Any unblinding prior to the conventional full unblinding date can result in bias and should therefore be avoided. This includes unblinding the treatment assignments of individual participants as they come off the study, since there is substantial potential for bias in the reporting of results for other participants.

Given that such bias can compromise the integrity or objectivity of the trial, unplanned unblinding prior to the conventional full unblinding date should be undertaken only to protect participant safety or to fulfill safety reporting and other regulatory obligations.

Guidelines for Urgent Unblinding of Individual Participant Treatment Assignments for Medical Reasons

The need for emergency unblinding of individual participant treatment assignments is expected to be extremely rare. Emergency unblinding does not apply for participants who have died, because knowledge of treatment assignment will not affect immediate management in such cases.

Requests for urgent unblinding should be made in writing to the center pharmacist such that he/she can provide the assignment information immediately. In cases of extreme emergency in which it is not possible for the unblinding request to be made in writing, the site investigator or designee may make the request orally but must provide a written

statement of the request within 24 hours, including the reason why the request could not initially be made in writing. In these cases, the center pharmacist can provide the information orally, and provide a written confirmation of the unblinded treatment within 24 hours.

The Site Investigator should alert the Sponsor of the urgent unblinding within 24 hours of the unblinding via email.

Unblinded treatment assignments should be shared with as few individuals as possible on a need-to-know basis. Care should be taken to prevent additional unblinding to maintain study integrity. The site PI and Sponsor are responsible for preventing additional unblinding beyond those who need to know and for protecting information that may identify the participant.

Guidelines for Early Non-Urgent Unblinding of Individual Participant Treatment Assignments for Medical/Safety Reasons

Site PIs or designees may request a participant's treatment assignment before a study is fully unblinded for reasons that are not urgent and do not require immediate (emergency) unblinding but may affect the participant's medical care/safety.

The site PI or designee will consult with the Sponsor via email or teleconference and then submit a written request for unblinding in writing (via email). Early unblinding for this reason should generally not occur until all primary outcome data have been submitted, all queries related to these data have been resolved, and any clinical endpoints have been reviewed by designated reviewers. In cases in which knowledge of a participant's treatment assignment sooner may affect the participant's medical care and/or would otherwise be in the participant's best interest, this requirement can be waived.

STUDY ENDPOINTS

Primary study endpoint

The primary study endpoint is the superiority of bezlotoxumab over placebo, when given for CDI of high likelihood for unfavorable outcome, to reduce the incidence of unfavorable outcome. Unfavorable outcome is a composite endpoint and comprises the incidence of at least of one of the following in each enrolled patient: progression into organ

dysfunction; relapse of CDI; and death. The primary endpoint is tested on Day 40 from start of blind treatment. Organ dysfunction is defined as any increase of the baseline total SOFA (sequential organ failure assessment) score before allocation into blind treatment by at least 2 points. This definition is provided according to the current consensus of the definition of organ dysfunction in critically ill patients (13, 14). The need for colectomy or admission in the Intensive Care Unit count as organ dysfunction.

The study is aiming to demonstrate the superiority of adjunctive bezlotoxumab treatment on the composite endpoint of unfavorable outcome. Patients who do not meet any of the components of this composite endpoint on Day 40 will be considered to have succeeded treatment.

Secondary study endpoints

The comparisons of bezlotoxumab over placebo on the following four endpoints:

- Incidence of organ dysfunction and time to first organ dysfunction. This is defined as the presentation of organ dysfunction in any of the enrolled patients of each group starting from the day of blind allocation until Day 40. For patients with more than two episodes of organ dysfunction, the first one is encountered in the endpoint analysis. Organ dysfunction is defined as any increase of the baseline total SOFA (sequential organ failure assessment) score before allocation into blind treatment by at least 2 points.
- Time to relapse of CDI; the relapse of CDI is under daily follow-up until Day 40. Relapse is defined as the return of more than three unformed bowel movements in 24 hours with a positive stool toxin test necessitating retreatment. The day of CDI relapse from blind allocation will be captured for relapsed patients and compared between groups.
- Survival until Day 40 from study enrolment
- Overall cost of hospitalization; this is calculated as stated above starting from the day of blind allocation until Day 40

- Validation of the BEYOND score; this is done by comparing the incidence of the primary endpoint between patients who have failed screening in the trial and patients who were enrolled in the trial and who were allocated to the placebo arm.

POWER CALCULATION

This is calculated for the primary endpoint using the Power and Sample Size Calculations software version 3.1.2. Power calculation is based on the hypothesis that the difference between bezlotoxumab and placebo will be at least 40% i.e. equal to the one reported in the MODIFY randomized clinical trials (8). It is anticipated that 60% of patients allocated to the placebo arm and 20% of patients allocated to the bezlotoxumab arm will achieve the primary endpoint of unfavorable outcome. To demonstrate this difference at the 10% level of significance with 80% power, 22 patients should be enrolled into each arm (44 patients in total). It is anticipated that one out of three screened patients with CDI will meet all inclusion criteria, so that a total of 132 patients need to be screened.

STATISTICAL ANALYSIS

Regarding the qualitative primary and secondary endpoints, groups of treatment will be compared by the Fisher exact test; odds ratio and 95% confidence intervals (CIs) will be calculated by the Maentel-Henzel's statistics. Secondary endpoints of time to an event will be compared between groups Cox regression analysis.

ADVERSE EVENTS

Treatment-emergent Adverse events (TEAEs) and Serious TEAEs will be collected from baseline until the last patient's evaluation. Investigators should monitor subjects for adverse events and are responsible for recording ALL adverse events and serious adverse events occurring to a patient during the trial. Mortality will not be reported as an SAE since this is the study secondary endpoint.

A TEAE is any undesirable medical occurrence in a subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. The time relationship is defined from the moment the AE occurs during

therapeutic treatment until 30 days or 5 half-lives after treatment discontinuation. The adverse event may be a sign, a symptom, or an abnormal laboratory finding. Reporting to Health Authorities and Ethics Committees will be done by investigator according to the local requirements.

Serious TEAEs. Serious TEAEs must be reported by the investigator to the health authorities and ethics committee according to the local requirements within 24 hours. If an adverse event meets any of the following criteria, it is considered STEAE:

- **Life-threatening situation** The subject was at risk of death at the time of the adverse event/experience. It does not refer to the hypothetical risk of death if the AE were more severe or were to progress.
- **Inpatient hospitalization** or prolongation of existing hospitalization.
- **Persistent or significant disability/incapacity** Any TEAE having an outcome that is associated with a substantial disruption of the ability to carry out normal life functions, including the ability to work. This is not intended to include transient interruption of daily activities.
- **Congenital anomaly/birth defects** Any structural abnormality in subject's offspring that occurs after intrauterine exposure to treatment.
- **Important medical events/experiences** that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event/experience when, based upon appropriate medical judgment, **they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above,** i.e., death, a life-threatening adverse event/experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Examples of such medical events/experiences include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.
- **Spontaneous and elective abortions** experienced by study subject.

A non-TEAE is any untoward medical occurrence in a patient or subject who is administered a pharmaceutical product, and which does not necessarily have a causal

relationship with this treatment. A non-serious adverse event is one that does not meet the definition of a serious adverse event given. **Non-serious TEAEs** must be reported by the investigator to the health authorities and ethics committee according to the local requirements at the end of the trial.

Grading of severity

The severity of the adverse events shall be graded as:

- **Mild** the adverse event is transient and well tolerated by the patient
- **Moderate** the adverse event causes discomfort and affects the usual activities of the patient.
- **Severe** the adverse event affects the usual activities of the patient to an important degree and may cause disability or be life-threatening.

Relationship to the drug

The investigator will use the following definitions to assess the relationship of the adverse event to study drug:

- **Probably Related**: The adverse event has a strong time relationship to the drug or relapses if re-induced, and another etiology is improbable or clearly less probable.
- **Possibly Related**: The adverse event has a strong time relationship to the drug and an alternative aetiology is as probable or less probable.
- **Probably not Related**: The adverse event has a slight or no time relationship to the drug and/or there is a more probable alternative aetiology.
- **Unrelated**: The adverse event is due to an underlying or concomitant disease or to another pharmaceutical product and is not related to the drug (no time relationship and a much more probable alternative aetiology).

If an investigator's opinion of possibly related, probably not related or not related to study drug is given, an alternate etiology must be provided by the investigator. Please note that a severe adverse event/experience is not necessarily serious, as the term severe is a measure of intensity while a serious adverse event is determined based on the

aforementioned regulatory criteria. Individual un-blinding thought to be necessary for the management of an adverse event will be documented in the subject Case Report Form.

All Investigators must report every adverse event and evaluate the severity and possible causality with the study drug according to aforementioned criteria. All adverse events/reactions are reported to Sponsor. The sponsor is responsible for evaluation of all serious TEAEs. All Serious Adverse Events/ Serious Adverse Reactions must be reported within 24 hours by completion of the SAE and faxing/emailing to Hellenic Institute for the Study of Sepsis.

The Sponsor must evaluate whether an adverse event is expected or not. A SAE may qualify for expedited reporting to regulatory authorities if it is determined to be a suspected, unexpected serious adverse reaction (SUSAR). The Sponsor is responsible for submitting expedited safety reports to the appropriate regulatory agency for all confirmed SUSARs. In the case of a fatal or life-threatening SUSAR, the Sponsor will notify the appropriate regulatory agency as soon as possible but in no case later than 7 calendar days after the Sponsor's initial receipt of the information. For a non-life-threatening SUSAR, the report will be submitted no later than 15 days after the Sponsor is made aware of the event.

The Sponsor has the obligation to submit annually a drug safety updated report (DSUR) according to global experience to appropriate regulatory authorities. The electronic submission to Eudravigilance will be performed through the Organisation ID: HISS.

The above pharmacovigilance procedures will be performed for the Sponsor (Hellenic Institute for the Study of Sepsis) by the Consultant Company SustChem Engineering S.A., 144 3rd Septemvriou str, 11251, Athens, and the Qualified Person for Pharmacovigilance (QPPV) will be Ms Areti Voulomenou, MSc (contact details in Appendix V).

QUALITY CONTROL AND ASSURANCE

Quality control and assurance checks are performed by sponsor in order to allow periodic review of adequacy of the study activities and practices and allow for revising such practices as needed so the data and process are maintained, the study meets the protocol and procedural requirements, and is reproducible.

Before enrolling any subject in this study, sponsor personnel and the investigator have to review the protocol, the IB, the CRFs and instructions for their completion, the procedure for obtaining informed consent and the procedure for reporting AEs and SAEs.

A qualified representative of the sponsor will monitor the conduct of the study by visiting the site and by contacting the site by telephone and e-mail. During these site visits, all source documents are reviewed, and information recorded in the CRFs is verified against them.

Besides routine monitoring, quality assurance will be documented through independent auditing of the quality control activities and where applicable, by regulatory authorities through inspections.

ETHICAL CONSIDERATIONS

Prior to the initiation of this study, the study design will receive ethical, scientific, and where applicable, regulatory review. Investigators will conduct this study in accordance with the principles of the Declaration of Helsinki, GCP, and applicable regulatory requirements.

Regarding Informed Consent Form obtaining procedures, before any procedure specified in the protocol is performed, a subject must:

- Be informed of all pertinent aspects of the study and all elements of informed consent
- Be given time to ask questions and to consider the decision to participate
- Voluntarily agree to participate in the study
- Sign and date the updated and approved by IEC/REB ICF version.

DATA HANDLING AND RECORD KEEPING

Each enrollment center will record patient data in Case Report Forms (CRFs). These forms were designed specifically for the trial according to the study protocol and will be completed for every study participant by a blinded member of the study team.

Source documents will be kept under secure file at individual enrollment centers. Delegated members of the Sponsor will have access to all the records from enrolled participants, for monitoring purposes.

FINANCING AND INSURANCE

The BEYOND trial is funded by the Hellenic Institute for the Study of Sepsis (HISS).

PUBLICATION POLICY

The design and primary results of the paper will be published regardless of the study results. HISS will not make final decisions regarding the contents of the publication(s). The results of the trial will also be posted in clinicaltrials.gov and any other public repositories.

PROTOCOL ADHERENCE AND AMENDMENTS

Investigators ascertain that they will apply due diligence to avoid protocol deviations. All significant protocol deviations will be recorded and reported in the clinical study report (CSR). Any change or addition to the protocol can only be made in a written protocol amendment that must be approved and signed by the sponsor, health authorities where required, and the IEC/REB.

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APPENDIX I Final study report of the SPECIFY project

SCORING PERSONALIZED NEEDS IN *CLOSTRIDIUM DIFFICILE* INFECTIONS FOR FIDAXOMIXIN THERAPY: THE SPECIFY PROJECT

FINAL STUDY RESULTS REPORT

Evangelos J. Giamarellos-Bourboulis, MD, PhD

Principal Investigator

Associate Professor of Medicine

Chairman of the Board, Hellenic Institute for the Study of Sepsis

ATHENS 2018

FIRST PART OF THE ANALYSIS

GENETIC SUSCEPTIBILITY TO *CLOSTRIDIUM DIFFICILE*

The first patient was enrolled on September 5 2015 and the last patient on November 11 2016. During this study period, 153 patients and 150 comparators were enrolled from 11 study sites. Comparators were patients hospitalized in the same wards during the same study period. The comparators were fully matched to patients regarding demographics and the Charlson's Comorbidity Index (CCI) (Table 1).

Table 1 Comparison between patients with *Clostridium difficile* infection (CDI) and comparators.

	CDI (n=153)	**Comparators (n=150)**	**p-value**
Age (years, mean ± SD)	76.2 ± 13.3	66.7 ± 11.4	<0.0001*
Male gender (n, %)	71 (46.6)	80 (53.3)	0.251**
CCI (mean ± SD)	5.79 ± 2.44	6.03 ± 2.88	0.443*
Study site (n, %)			
Infections Unit, Evangelismos Hospital	31 (20.3)	30 (20.0)	
4th Department of Internal Medicine, ATTIKON Hospital	29 (19.0)	28 (18.7)	
1st Department of Internal Medicine, G.Gennimatas Hospital	21 (13.7)	20 (13.3)	
1st Department of Internal Medicine, Thriasio Hospital	20 (13.1)	20 (13.3)	
Infections Unit, Tzaneion Hospital	19 (12.4)	19 (12.7)	
2nd Department of Internal Medicine, Thriasio Hospital	17 (11.1)	17 (11.3)	
3rd Department of Internal Medicine, Sotiria Hospital	6 (3.9)	6 (4.0)	

1st Department of Internal Medicine, Laiko Hospital	4 (2.6)	4 (2.7)	
3rd Department of Internal Medicine, AHEPA Hospital	3 (2.0)	3 (2.0)	
2nd Department of Internal Medicine, Sismanogleion Hospital	2 (1.3)	2 (1.3)	
Department of Internal Medicine, Rion Hospital	1 (0.7)	1 (0.6)	

*Student's t-test; **Fisher exact test

DNA was extracted from all samples by the end of December 2016 and it was of appropriate quality. Then GWAS was performed with one HumanCore-chip. Running of the GWAS was completed by the end of April 2017. Bioanalysis started in May 2017 and the first round of results were provided end of July 2017. The quality control of imputed data is shown in Figure 1. Analysis was done for 4,330,811 SNPs in 134 remaining controls and 134 remaining cases.

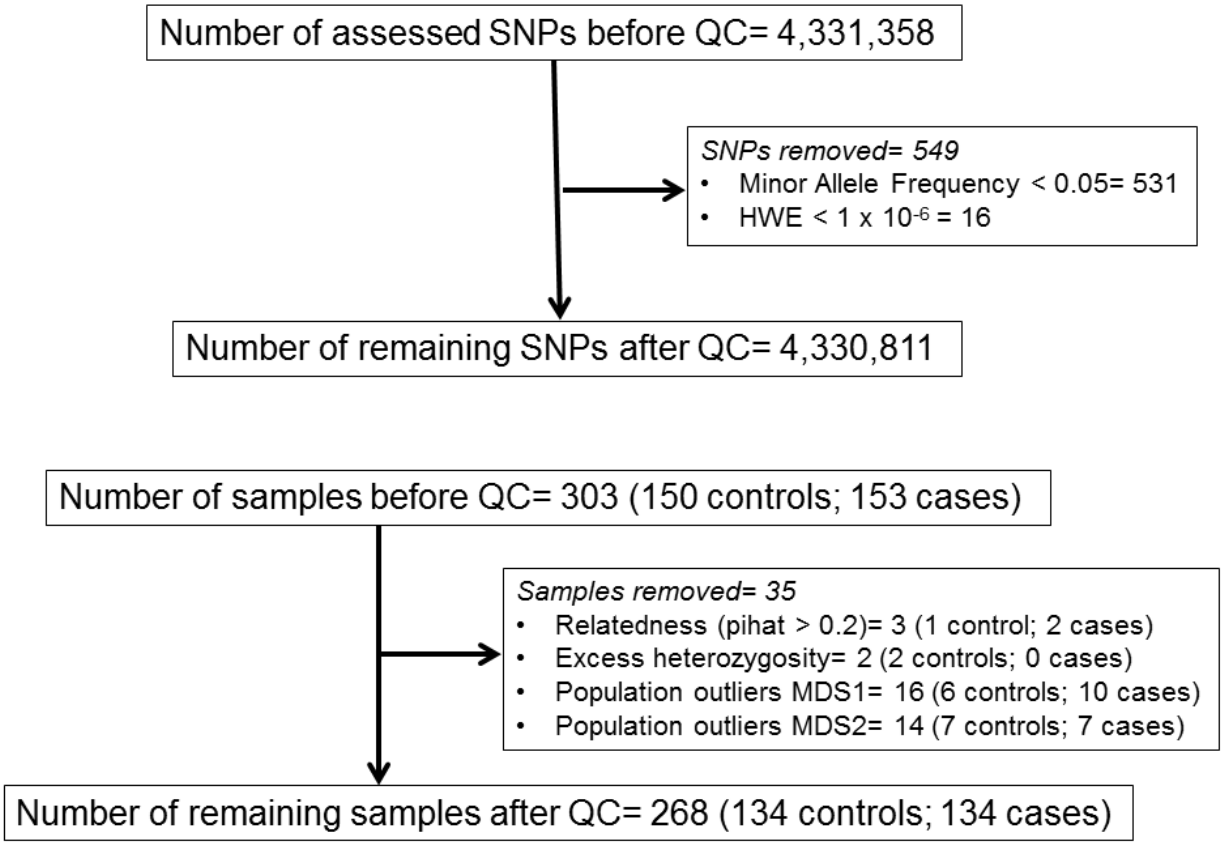

Figure 1 Quality control of imputed data

Abbreviations HWE: Hardy Weinberg equilibrium; MDS: multidimensional scaling; QC: quality control; SNP: single nucleotide polymorphism

Logistic regression analysis taking into consideration age, gender and Charlons' comorbidity index (CCI) did not disclose any SNP below the p level of $5x10^{-8}$ of significance. It was decided to focus on SNPs that according to the Manhattan plot were strongly associated with susceptibility for CDI (Figure 2). Among these four SNPs (rs4311028, rs1940913, rs474032 and rs2091172) were detected at a level of significance lower than $9x10^{-6}$ (Table 2). For the other SNPs, association with expression qualitative trait loci (eQTLs) was analyzed and another 13 SNPs were found to strongly influence the expression of several gene loci as shown in Table 3. The

frequencies of the total of the 17 gene SNPs were compared between controls and cases as shown in Tables 4 and 5.

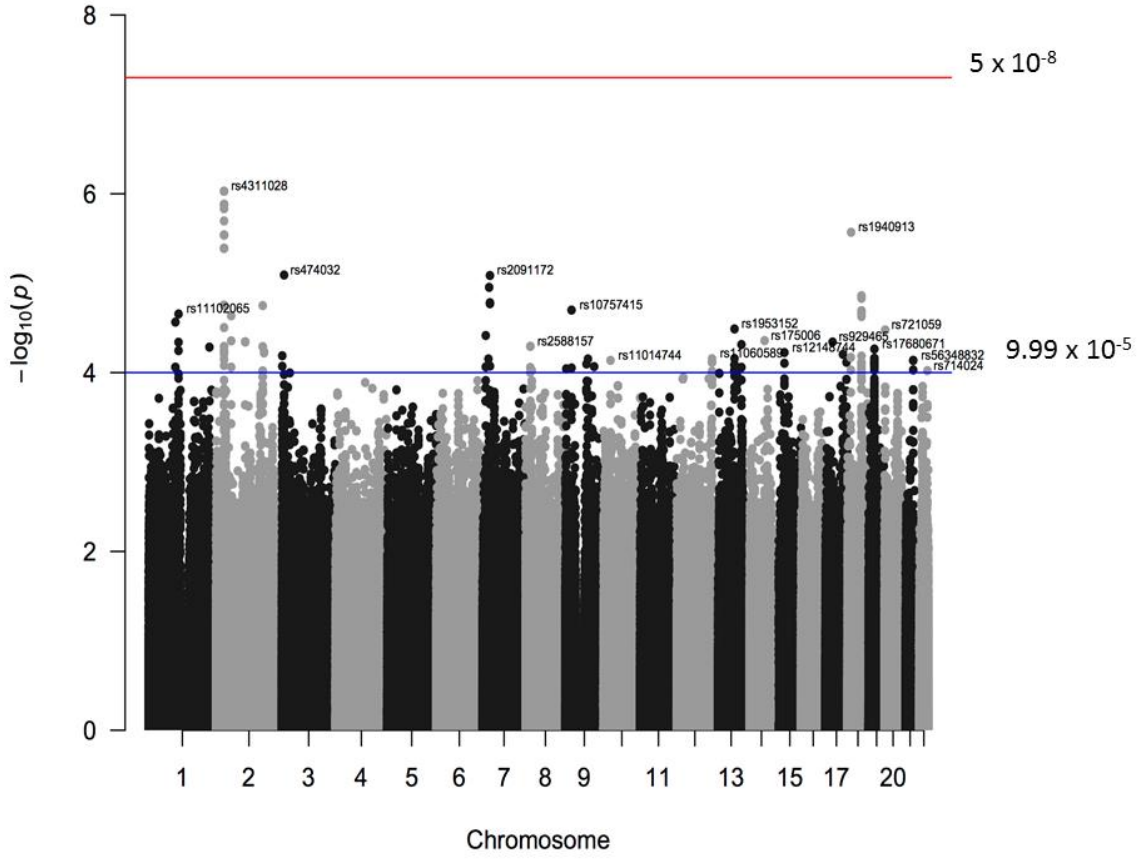

Figure 2 Manhattan plot indicating loci that show suggestive association with susceptibility to *Clostridium difficile* infection

Table 2 The 4 SNPs showing the strongest association with *Clostridium difficile infection*

Chromosome	SNP ID	Position	p-value*	Known gene	Gene function
2	rs4311028	29706981	9.35×10^{-7}	*ALK*	Anaplastic lymphoma kinase (ALK) or ALK tyrosine kinase receptor or CD246; oncogenic kinase
3	rs474032	8076409	8.1×10^{-6}	*LOC101927394*	uncharacterized
7	rs2091172	27515580	8.2×10^{-6}	*HOX genes*	Homeobox; transcription factor
18	rs1940913	13572220	2.69×10^{-6}	*LDLRAD4*	Low-density lipoprotein receptor class A domain-containing protein 4; negative regulator of TGF-beta signaling and thereby probably plays a role in cell proliferation, differentiation, apoptosis, motility, extracellular matrix production and immunosuppression

*after logistic regression analysis

Table 3 The 13 SNPs that are associated with an effect on gene expression loci

				GTEx-eQTLs		BIOS-eQTLs			
Chromosome	**SNP ID**	**Position**	**p-value***	**Gene**	**p-value**	**Gene**	**p-value**	**Tissue**	**Gene function**
2	rs6735120	55961771	2.32×10^{-5}			*SMEK2*	1.4×10^{-11}	Whole blood	Protein Phosphatase 4 Regulatory Subunit 3B/ Regulates the activity of PPP4C at centrosomal microtubule organizing centers
2	rs62183547	172372438	1.78×10^{-5}			*CYBRD1*	2.0×10^{-9}	Whole blood	Cytochrome B Reductase 1/ Plays a role in dietary iron absorption
7	rs1128226	21941669	7.03×10^{-5}			*CDCA7L*	7.2×10^{-9}	Whole blood	Cell Division Cycle Associated 7 Like/ Transcriptional regulation as a repressor of monoamine oxidase A (MAOA)
8	rs4279595	22907330	9.45×10^{-5}	*RP11-875O11.3*	8.2×10^{-14}	*RP11-875O11.3*	5.4×10^{-94}	Whole blood	TNF Receptor Superfamily Member 10b/ Promotes the activation of NF-kappa-B. Essential for ER stress-induced apoptosis.
14	rs175006	75445499	4.38×10^{-5}			*EIF2B2*	4.5×10^{-122}	Whole blood	Eukaryotic Translation Initiation Factor 2B Subunit Beta/ Involved in protein synthesis
15	rs12148744	41315357	5.96×10^{-5}			*CHP1*	1.6×10^{-22}	Whole blood	Calcineurin Like EF-Hand Protein 1/ Essential cofactor of NHE family members and mitogenic regulation of NHE1
17	rs3859214	64460807	6.24×10^{-5}	*PRKCA*	9.4×10^{-10}	*No*		PBMCs	Protein Kinase C Alpha/ Tegulation of cell proliferation, apoptosis, differentiation, migration and adhesion, tumorigenesis, cardiac hypertrophy, angiogenesis, platelet function and inflammation, by directly phosphorylating targets such as RAF1, BCL2, CSPG4,

									TNNT2/CTNT, or activating signaling cascade involving MAPK1/3 (ERK1/2) and RAP1GAP
17	rs7222870	77652955	7.65×10^{-5}			*CTD-2116F7.1*	1.7×10^{-17}	Whole blood	Long Intergenic Non-Protein Coding RNA 2078
18	rs7240534	51885897	1.38×10^{-5}	*POLI*	1.8×10^{-10}	*POLI*	2.2×10^{-137}	Whole blood/ transverse colon	Encodes for error-prone DNA polymerase involved in DNA repair
18	rs508660	12391134	6.75×10^{-5}			*RP11-861E21.2*	2.4×10^{-19}	Whole blood	
19	rs17680671	21576563	5.44×10^{-5}	*RP11-678G14.2*	4.4×10^{-10}	*ZNF429, RP11-678G14.2*	2.9×10^{-77}	Whole blood	Zinc Finger Protein 429/ Involved in transcriptional regulation
20	rs721059	2524927	3.34×10^{-5}			*NOP56*	3.0×10^{-8}	Whole blood	NOP56 Ribonucleoprotein/ Involved in early to middle stages of 60S ribosomal subunit biogenesis
22	rs714024	47013535	9.51×10^{-5}	*GRAMD4*	2.5×10^{-6}	*GRAMD4*	1.5×10^{-140}	Whole blood	GRAM Domain Containing 4/ Mitochondrial effector of E2F1 (MIM 189971)-induced apoptosis

*after logistic regression analysis

Table 4 Comparison of the frequencies of the 4 SNPs that are associated with the strongest association between patients with *Clostridium difficile* infection (CDI) and controls.

	Controls (n=134)	CDI (n=134)	p-value*	OR (95%CIs)**
rs4311028 (n, %)				
CC	119 (88.8)	86 (64.2)		
TC	15 (11.2)	46 (34.3)	<0.0001	
CC		2 (1.5)		
Carriage of C allele	15 (11.2)	48 (35.8)	6.0×10^{-6}	4.43 (2.32-8.42)
rs474032 (n, %)				
GG	58 (43.3)	85 (63.4)		
AG	58 (43.3)	43 (32.1)	<0.0001	
AA	18 (13.4)	6 (4.5)		
Carriage of A allele	76 (56.7)	49 (36.6)	0.001	0.44 (0.69-0.71)
rs2091172 (n, %)				
AA	54 (40.3)	25 (18.7)		
CA	59 (44.0)	70 (52.2)	<0.0001	
CC	21 (15.7)	39 (29.1)		
Carriage of C allele	80 (59.7)	109 (81.3)	1.5×10^{-4}	2.94 (1.68-5.12)
rs1940913 (n, %)				
GG	35 (26.1)	57 (42.5)		
CG	65 (48.5)	64 (47.8)	<0.0001	
CC	34 (25.4)	13 (9.7)		
Carriage of C allele	99 (73.9)	77 (57.5)	0.007	0.48 (0.29-0.80)

Table 5 Comparison of the frequencies of the 13 SNPs that are associated with eQTLs between analyzed patients with *Clostridium difficile* infection (CDI) and controls.

	Controls (n=134)	CDI (n=134)	p-value*	OR (95%CIs)**
rs6735120 (n, %)				
AA	117 (87.3)	88 (65.7)		
GA	17 (12.7)	43 (32.1)	<0.0001	
GG	0 (0)	3 (2.2)		
Carriage of G allele	17 (12.7)	46 (34.3)	4.4×10^{-5}	3.59 (1.93-6.69)
rs62183547 (n, %)				
GG	79 (59.0)	115 (85.8)		
AG	52 (38.8)	18 (13.4)	<0.0001	
AA	3 (2.2)	1 (0.7)		
Carriage of A allele	55 (41.0)	19 (14.2)	1.0×10^{-6}	0.24 (0.13-0.43)
rs1128226 (n, %)				
AA	65 (48.5)	32 (23.9)		
CA	59 (44.0)	79 (59.0)	<0.0001	
CC	10 (7.5)	23 (17.2)		
Carriage of C allele	69 (51.5)	102 (76.1)	4.2×10^{-5}	3.00 (1.78-5.06)
rs4279595 (n, %)				
CC	52 (38.8)	91 (67.9)		
AC	70 (52.2)	39 (29.1)	<0.0001	
AA	12 (9.0)	4 (3.0)		
Carriage of A allele	82 (61.2)	43 (32.1)	3.0×10^{-6}	0.30 (0.18-0.49)
rs175006 (n, %)				
CC	81 (60.4)	50 (37.3)		
GC	45 (33.6)	67 (50.0)	<0.0001	
GG	8 (6.0)	17 (12.7)		
Carriage of G allele	53 (39.6)	84 (62.7)	2.3×10^{-4}	2.56 (1.56-4.20)

rs12148744 (n, %)				
TT	119 (88.8)	89 (66.4)		
TC	15 (11.2)	44 (32.8)	<0.0001	
CC	0 (0)	1 (0.7)		
Carriage of T allele	15 (11.2)	45 (33.6)	1.6×10^{-5}	4.01 (2.10-7.65)
rs3859214 (n, %)				
GG	48 (35.8)	21 (15.7)		
GT	67 (50.0)	76 (56.7)	<0.0001	
TT	19 (14.2)	37 (27.6)		
Carriage of T allele	86 (64.2)	113 (84.3)	2.5×10^{-4}	3.00 (1.67-5.39)
rs7222870 (n, %)				
AA	87 (64.9)	64 (47.8)		
GA	46 (34.3)	62 (46.3)	<0.0001	
GG	1 (0.7)	8 (6.0)		
Carriage of G allele	47 (35.1)	70 (52.2)	0.007	2.02 (1.24-3.31)
rs7240534 (n, %)				
CC	44 (32.8)	23 (17.2)		
TC	71 (53.0)	66 (49.3)	<0.0001	
TT	19 (14.2)	45 (33.6)		
Carriage of T allele	90 (67.2)	111 (82.8)	0.005	2.36 (1.32-4.19)
rs508660 (n, %)				
AA	60 (44.8)	90 (67.2)		
GA	54 (40.3)	39 (29.1)	<0.0001	
GG	20 (14.9)	5 (3.7)		
Carriage of G allele	74 (55.2)	44 (32.8)	3.4×10^{-4}	
rs17680671 (n, %)				
GG	35 (26.1)	57 (42.5)		
CG	65 (48.5)	64 (47.8)	<0.0001	
CC	34 (25.4)	13 (9.7)		

Carriage of C allele	99 (73.9)	77 (57.5)	0.007	0.47 (0.28-0.80)
rs721059 (n, %)				
TT	43 (32.1)	84 (62.7)		
TC	66 (49.3)	46 (34.3)	<0.0001	
CC	25 (18.7)	4 (3.0)		
Carriage of C allele	91 (67.9)	50 (37.3)	8.22×10^{-7}	0.28 (0.17-0.47)
rs714024 (n, %)				
TT	46 (34.3)	81 (60.4)		
TC	69 (51.5)	47 (35.1)	<0.0001	
CC	19 (14.2)	6 (4.5)		
Carriage of C allele	88 (65.7)	53 (39.6)	2.9×10^{-5}	0.34 (0.21-0.56)

*by the Fisher exact test for 2 x 2 comparisons or by the Pearson's chi-square test for 2 x 3 comparisons

**Mantel-Haenszel statistics

Abbreviations CI: confidence intervals; OR: odds ratio

As a next step logistic regression analysis was done to identify which of the total of 17 minor frequency SNP alleles (Tables 3 and 4) are independent predictors of CDI among a patient population of hospitalized patients with similar CCI. Results after nine steps are shown in Table 6; only nine of the 17 SNPs remained significant.

Table 6 Final step of the stepwise logistic regression procedure with group (comparators vs CDI) as the dependent variable; odds rations less than 1 indicate protective alleles.

	B-SLOPE	SE	Wald	p-value	Odds ratio	Lower 95%CI	Upper 95%CI
Carriage of at least one C allele of rs12148744	2,721	,737	13,635	,000	15,192	3,584	64,385
Carriage of at least one C allele of rs714024	-2,715	,635	18,278	,000	,066	,019	,230
Carriage of at least one C allele of rs721059	-2,922	,638	20,954	,000	,054	,015	,188
Carriage of at least one T allele of rs4311028	3,280	,755	18,880	,000	26,584	6,054	116,741
Carriage of at least one A allele of rs62183547	-2,545	,618	16,981	,000	,078	,023	,263
Carriage of at least one C allele of rs1128226	1,239	,498	6,199	,013	3,452	1,302	9,155
Carriage of at least one A allele of rs4279595	-1,724	,484	12,680	,000	,178	,069	,461
Carriage of at least one G allele of rs175006	1,084	,473	5,247	,022	2,957	1,169	7,477
Carriage of at least one T allele of rs3859214	1,682	,518	10,531	,001	5,375	1,947	14,844
Carriage of at least one G allele of rs70028870	1,492	,515	8,388	,004	4,444	1,620	12,195

Carriage of at least one G allele of rs508660	-,972	,457	4,520	,033	,378	,154	,927
Carriage of at least one T allele of rs7240534	1,272	,561	5,151	,023	3,570	1,190	10,712
Carriage of at least one G allele of rs2091172	1,099	,516	4,542	,033	3,001	1,092	8,244
Carriage of at least one C allele of rs17680671	-1,675	,547	9,357	,002	,187	,064	,548

Abbreviation CI: confidence interval

Using the values of the B slope of the logistic regression analysis, we developed a gene score to differentiate between comparators and CDI. This is provided in the following equation:

Gene score=

(Carriage of C allele of rs12148744 x 27) – (carriage of C allele of rs714024 x 27) - (carriage of C allele of rs721059 x 29) + (carriage of T allele of rs4311028 x 33) – (carriage of A allele of rs62183547 x 25) + (carriage of C allele of rs1128266 x 12) - (carriage of T allele of rs4279595 x 17) + (carriage of G allele of rs175006 x 11) + (carriage of T allele of rs3859214 x 17) + (carriage of G allele of rs7222870 x 15) – (carriage of G allele of rs5086600 x 9) + (carriage of T allele of rs7240534 x 12) + (carriage of G allele of rs20911172 x 11) - (carriage of C allele of rs17680671 x 17)

At the above equation, carriers of at least one the indicated alleles score 1 point and non-carriers score 0 points.

The received operator characteristics (ROC) curve to differentiate between CDI and comparators is shown in Figure 3.

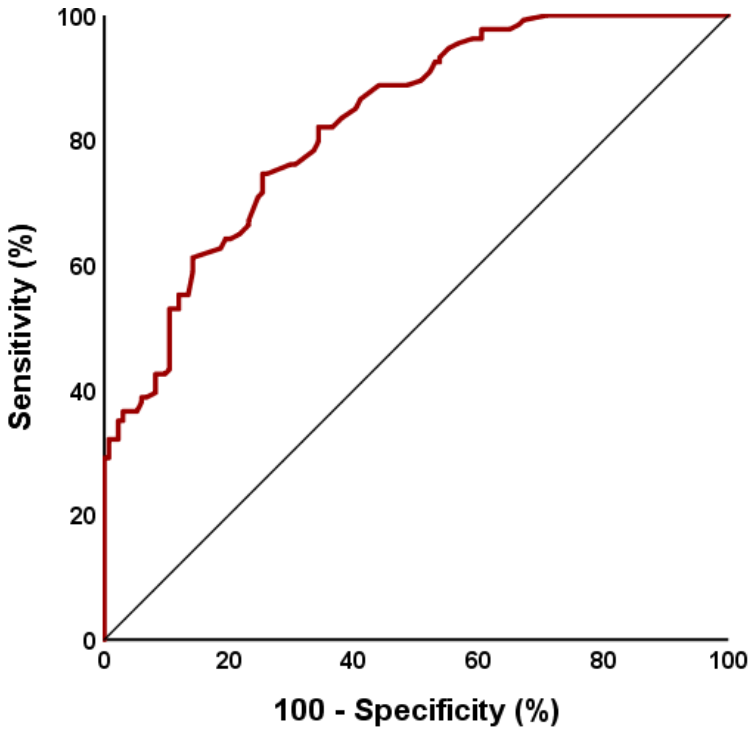

Figure 3 ROC curve of the gene score to discriminate between patients with CDI and comparators

Area under the curve: 0.827 (0.780-0.897), p: 2.05 x 10^{-20}

Using a cut-off of sensitivity greater than 90%, we depicted through the co-ordinate points of the curve a cut-off greater than 53 of the gene score. The diagnostic characteristics of the gene score at that cut-off are provided in Table 7. It is concluded that this gene score at a state of elevated CCI, provides odd ratio 6.89 (3.53-13.42, p: 1.39 x 10^{-8}) for susceptibility to CDI.

Table 7 Diagnostic performance of the gene score at a cut-off greater than 53 to differentiate between CDI and controls among a hospitalized patient population with similar CCI.

	CDI	Comparators	Total
Gene score >53	57 Sensitivity: 42.5% PPV: 81.4%	13	70
Gene score ≤53	77	121 Specificity: 90.3% NPV: 61.1%	198
Total	134	134	268

Abbreviations CDI: *Clostridium difficile* infection; NPV: negative predictive value; PPV: positive predictive value

Special mentioning has to be done to the eQTL of rs7240537 that is associated with the expression of the *PORI* gene in the colon (Figure 4). This gene encodes for error-prone DNA polymerase involved in DNA repair and it is associated with psoriasis and with the interaction of the microbiome with the host in psoriatic arthritis.

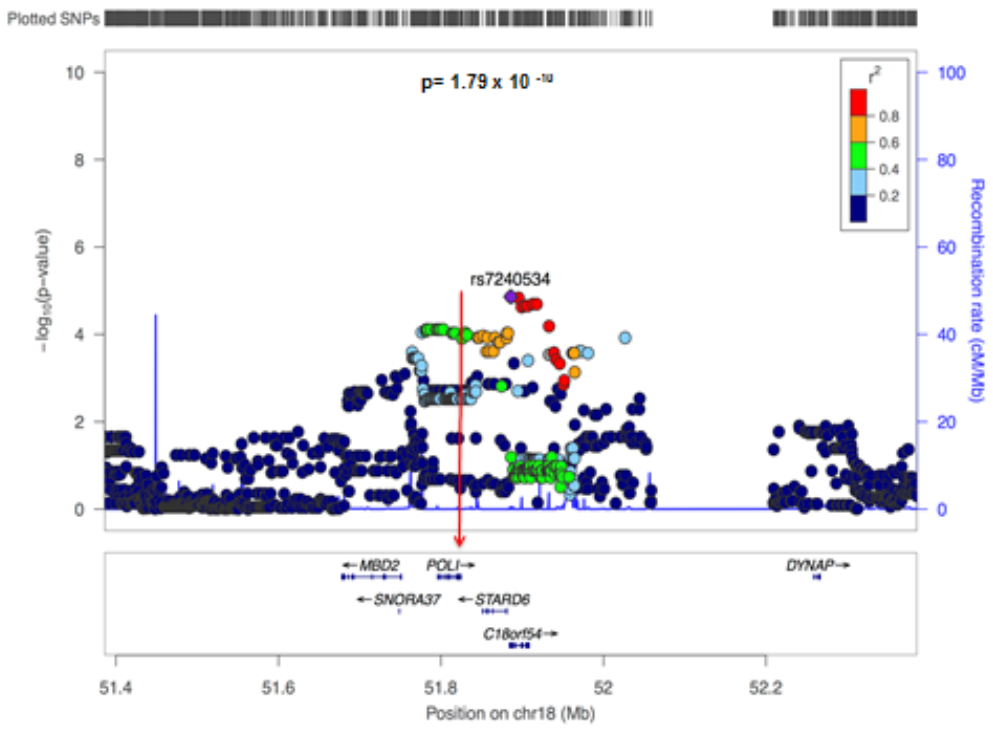

Figure 4 eQTL of rs7240534 associated with the *POLI* gene

SECOND PART OF THE ANALYSIS

SUSCEPTIBILITY TO UNFAVORABLE OUTCOME OF CDI

From the 134 analyzed CDI cases, 39 (29.1%; 95%CIs 22.1-37.3%) met the per protocol primary study endpoint of unfavorable outcome i.e. presenting or developing organ dysfunction and/or CDI relapse and/or death. From these 39 patients, 15 had an extreme CDI phenotype i.e. they had unfavorable outcome with ATLAS score equal to zero. The comparisons between the 95 patients who did not experience the primary endpoint and the 39 patients who met the primary endpoint are shown in Table 8.

Table 8 Difference in baseline characteristics between patients with CDI without unfavorable outcome and patients with CDI and unfavorable outcome

	CDI without unfavorable outcome (n=95)	**CDI with unfavorable outcome (n=39)**	**p-value**
Age (years, mean ± SD)	76.3 ± 12.7	76.1 ± 14.6	0.951**
Male gender (n, %)	50 (52.6)	21 (53.8)	1.000***
Hemoglobin <9.45g/dl (n, %)*	19 (20.0)	17 (43.6)	0.009***
Urea >64.5 mg/dl (n, %)*	18 (18.9)	17 (43.6)	0.005***
Creatinine (mg/dl, mean ± SD)	1.24 ± 1.28	1.83 ± 2.02	0.240**
Hypoalbuminemia (n, %)	7 (7.4)	0 (0)	0.106***
Intake of antimicrobials <3 months (n, %)	68 (71.6)	26 (66.7)	0.678***
CCI (mean ± SD)	5.55 ± 2.17	6.38 ± 2.92	0.117**
Previous CDI	7 (7.5)	4 (10.3)	0.731***
Extreme CDI phenotype (n, %)	0 (0)	15 (38.5)	<0.0001***
White blood cells (/mm^3, mean ± SD)	12083.0 ± 8124.5	12984.3 ± 9918.3	0.586**
C-reactive protein (mg/dl, median-range)	40.0 (<3-331.0)	39.8 (<3-279.0)	0.471****
IL-6 (pg/ml, median-range)	7.0 (7.0-2000)	7.0 (7.0-133.1)	0.779****
IL-8 (pg/ml, median-range)	31.3 (<4-494.7)	16.4 (<4-437.9)	0.877****
Eotaxin (pg/ml, median, range)	201.9 (<31-975.5)	152.6 (<31-1753.3)	0.899****

*selected for specificity >80% by the ROC curve of the indicated variable for unfavorable outcome

**by the Student's t-test

***by the 2-sided Fisher exact test

****by the Mann-Whitney U test

The eQTL of rs714024 is for the *GRAMD4* gene that is a pro-apoptotic protein which binds to Nogo-B and negatively regulates immune responses initiated by nucleic acid–sensing Toll like receptors. Our findings correlate this eQTL with IL-8 and indicate significant differences of circulating IL-8 in relation to the genotype (Figure 5). Following ROC curve analysis, a cut-off of 227 pg/ml is recognized based on which patients with IL-8 greater than 227 pg/ml have odds ratio 6.21 (95%CIs: 1.96-19.64, p: 0.002) for unfavorable outcome.

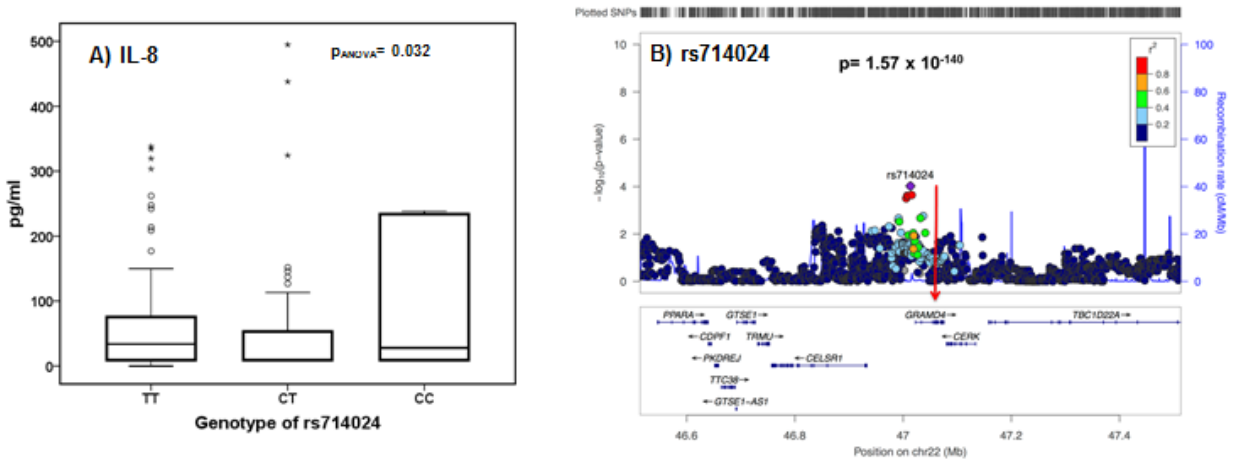

Figure 5 Association between eQTL of rs714024 and circulating levels of IL-8

A) Circulating IL-8 on day 1 in relation to the genotype of rs714024; B) eQTL of rs714024 on TLR signaling. P values of significance are shown.

Since the great majority of patients have positive gene score, it was cross-tabulated which of the nine SNPs of the gene score either as carriers or in homozygosity is associated with unfavorable outcome. Significant results of the cross-tabulations are shown in Table 9.

Table 9 Difference in SNPs/genotypes between patients with CDI without unfavorable outcome and patients with CDI and unfavorable outcome

	CDI without unfavorable outcome (n=95)	**CDI with unfavorable outcome (n=39)**	**p-value**
Carriage of G allele of rs2091172 (n, %)	84 (84.4)	25 (64.1)	0.003
GG genotype of rs6735120 (n, %)	0 (0)	3 (7.7)	0.023

Based on the findings of Tables 8 and 9 and of Figure 4, a logistic regression analysis was done to identify the variables that were independently associated with unfavorable outcome (Table 10).

Table 10 Logistic regression analysis among the variables strongly associated with unfavorable outcome

Variable	**β slope**	**OR**	**95% CIs**	**p-value**
Hb<9.5 g/dl	+1	2.97	1.17-7.46	0.021
Urea >64.5 mg/dl	+1.44	4.23	1.64-10.92	0.003
IL-8 >227 pg/ml	+1.87	6.50	1.79-23.68	0.005
G allele rs2091172	-1.67	0.19	0.07-0.53	0.002

Based on the above Table, a prediction score is formatted from the following equation where the slope of the curves is used:

Prediction score= (Presence of Hb <9.5 g/dl x 10) + (presence of urea >64.5 mg/dl x 14) + (circulating IL-8 >227 pg/ml x 19) – (carriage of G allele of rs2091172 x 17).

In the above equation, the presence of each of the variable is scored as 1 and the absence of each of the variables is scores as 0.

The ROC curve for prediction of unfavorable outcome with this score among patients is shown in Figure 6.

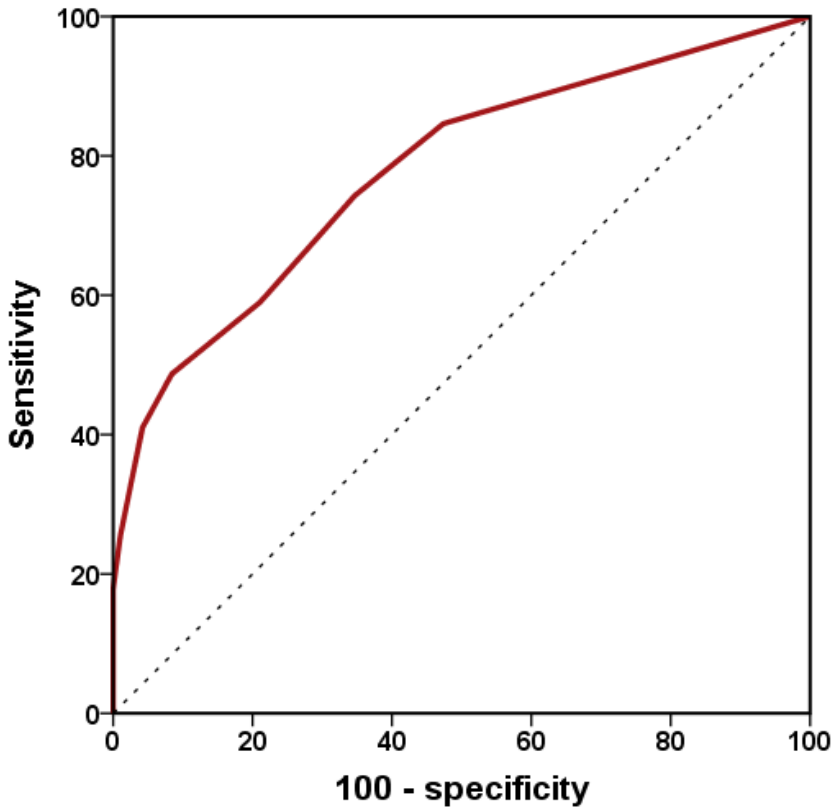

Figure 6 ROC curve of the prediction score to early prognosticate unfavorable outcome among patients with CDI

Area under the curve: 0.777 (0.686-0.869), p: 4.87 x 10^{-7}

Using a cut-off of 9, the positive predictive value of the score to predict unfavorable outcome is 80% (Table 11). The odds ratio for unfavorable outcome among patients with CDI and prediction score more than 9 is 15.82 (95%CIs: 4.83-51.88, p: 5 x 10^{-6}).

Table 11 Diagnostic performance of the prediction score at a cut-off greater than 9 for early prognosis of unfavorable outcome among patients with *Clostridium difficile* infection

	Unfavorable outcome	Favorable outcome	Total
Prediction score >9	16 Sensitivity: 41.0% PPV: 80.0%	4	20
Prediction score ≤9	23	91 Specificity: 95.8% NPV: 79.8%	114
Total	39	95	134

Abbreviations NPV: negative predictive value; PPV: positive predictive value

APPENDIX II The SOFA score

Variable	0 points	1 point	2 points	3 points	4 points
PaO_2/FiO_2 (mmHg)	≥400	<400	<300	<200	<100
Platelets (per mm^3)	≥150	<150	<100	<50	<20
Hypotension	MAP≥ 70 mmHg	MAP< 70 mmHg	Dobutamine whatever dose	Adrenaline ≤0.1* or Noradrenaline≤ 0.1*	Adrenaline>0.1* or Noradrenaline >0.1*
Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Bilirubin (mg/dl)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	≥12
Creatinine (mg/dl) or Urine output	<1.2	1.2-1.9	2.0-3.4	3.5-4.9 or <500 ml/day	≥5.0 or <200 ml/day

*µg/kg/min

Each variable is scored between 0 and 4. The SOFA score is the sum of the score of each variable

APPENDIX III The Charlson's comorbidity index (CCI)

CCI is defined after adding the score for age to the score for co-morbidities.

The score for age is given below:

Age group (years)	Points
0-49	0
50-59	1
60-69	2
70-79	3
80-89	4
90-99	5

The score for comorbidities is given below:

Condition	Points
None	0
• Myocardial infarct (one or more definite or probable myocardial infarctions) • Congestive heart failure (exertional or paroxysmal nocturnal dyspnea treated with digitalis, diuretics, or afterload reducing agents) • Peripheral vascular disease (intermittent claudication or bypass for arterial insufficiency or gangrene or acute arterial insufficiency or an untreated thoracic or abdominal aneurysm ≥6 cm) • Cerebrovascular disease (except hemiplegia) (history of a cerebrovascular accident with minor or no residua and transient ischemic attacks) • Dementia (defined as chronic cognitive deficit) • Chronic pulmonary disease • Connective tissue disease (systemic lupus erythematous, polvmyositis. mixed connective tissue disease, polymyalgia rheumatic and moderate to severe rheumatoid arthritis) • Peptic ulcer disease • Mild liver disease (cirrhosis without portal hypertension or chronic hepatitis) • Diabetes (without complications)	1

• Diabetes with end organ damage (Retinopathy, neuropathy, nephropathy, previous hospitalizations for ketoacidosis, hyperosmolar coma, or juvenile onset or brittle diabetics) • Hemiplegia • Moderate or severe renal disease (moderate defined as serum creatinine of 2-3 mg/dl, severe defined as need for dialysis/transplantation) • Solid tumor (non-metastatic) (without documented metastases, but initially treated in the last five years) • Leukemia/lymphoma/multiple myeloma	2
• Moderate or severe liver disease (Moderate: cirrhosis with portal hypertension; severe: cirrhosis with portal hypertension and a history of variceal bleeding	3
• Metastatic solid tumor • AIDS (acquired immunodeficiency syndrome) (Definite or probable AIDS)	6

APPENDIX IV Study visits

Visits days	Screening	Days 1-10	Day 12	Day 40
Informed consent	x			
Exclusion criteria	x			
Inclusion criteria	x			
quick *C.DIFFICILE* Complete test	x			
Blood sampling	x			
Stool collection or rectal swab	x			
BEYOND score calculation	x			
Study drug administration		x		
AE/SAE		x	x	x
Demographics		x		
Comorbidities and predisposing illnesses		x		
Vital signs		x	x	x
Laboratory findings including white blood cells, creatinine and liver biochemistry (if available)		x	x	x
Co-administered drugs and antimicrobials		x	x	x
Results of coloscopy (if done)		x	x	x
SOFA score		x	x	x
Organ dysfunction		x	x	x
Number of bowel movements		x	x	x
Need for colectomy		x	x	x
ICU admission		x	x	x
Clinical cure/relapse		x	x	x
Mortality		x	x	x

APPENDIX V List of study sites

- 4th Department of Internal Medicine, ATTIKON University General Hospital, (PI: Antonios Papadopoulos, Associate Professor of Internal Medicine and Infectious Diseases)
- 2nd Department of Propaedeutic Internal Medicine, ATTIKON University General Hospital, (PI: Erifili Hatziaggelaki, Professor of Internal Medicine)
- 3rd Department of Surgery, ATTIKON University General Hospital, (PI: Emmanouil Pikoulis, Professor of Surgery)
- 3rd University Department of Internal Medicine, SOTIRIA General Hospital of Chest Diseases of Athens, (PI: Garyfallia Poulakou, Assistant Professor of Internal Medicine)
- 1st University Department of Internal Medicine, LAIKO General Hospital of Athens, (PI: Michael Samarkos, Associate Professor of Internal Medicine and Infectious Diseases)
- 1st Department of Internal Medicine, G. GENNIMATAS General Hospital of Athens, (PI: Georgios Adamis, Director of NHS)
- 1st University Department of Propaedeutic Surgery, IPPOKRATEION General Hospital, (PI: Konstantinos Toutouzas, Associate Professor of Surgery)
- 1st Department of Internal Medicine, THRIASIO General Hospital of Eleusis (PI: Styliani Symbardi, Director of NHS)
- 2nd Department of Internal Medicine, THRIASIO General Hospital of Eleusis (PI: Zoi Alexiou, Director of NHS)
- 2nd Department of Internal Medicine, TZANEIO General Hospital of Piraeus (PI: Chrysos Georgios, Director of NHS)
- 1st Department of Internal Medicine, AHEPA University General Hospital of Thessaloniki (PI: Simeon Metallidis, Associate Professor of Internal Medicine and Infectious Diseases)
- Department of Internal Medicine, University General Hospital of Patras PANAGIA I VOITHIA, (PI: Karolina Akinosoglou, Assistant Professor of Internal Medicine)
- Department of Internal Medicine, University General Hospital of Larissa, (PI: George Dalekos, Professor of Internal Medicine)

- 2nd Department of Internal Medicine, University General Hospital of Alexandroupolis, (PI: Periklis Panagopoulos, Associate Professor of Internal Medicine)
- 1st Department of Internal Medicine, University General Hospital of Ioannina, (PI: Charalampos Milionis, Professor of Internal Medicine)
- 2nd Department of Surgery, G. GENNIMATAS General Hospital of Thessaloniki (PI: Ioannis Koutelidakis, Associate Professor of Surgery)

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