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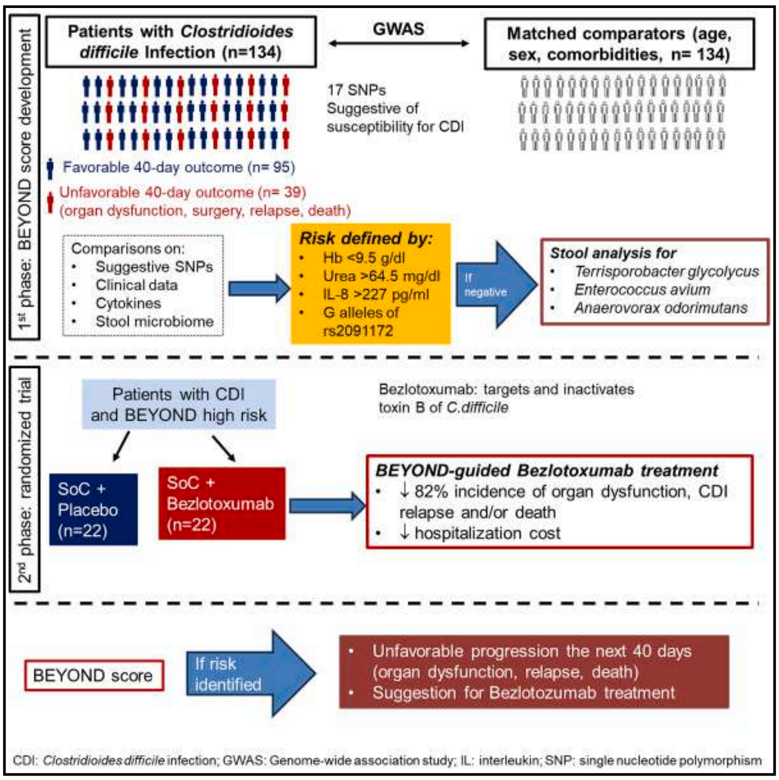
A randomized controlled trial of precision bezlotoxumab treatment for *Clostridioides difficile* infection

Graphical abstract



Highlights

- One early prediction score for unfavorable outcome of infection by *C. difficile* is developed
- The score integrates clinical information with genome, microbiome, and cytokine data
- The score has 84.6% sensitivity and 95.8% specificity
- Patients scoring positive have 82% benefit by bezlotoxumab over placebo treatment

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In brief

Psarrakis et al. describe the BEYOND score for the early prediction of unfavorable outcomes in *Clostridioides difficile* infection. BEYOND integrates data of blood hemoglobin, urea, and interleukin-8 levels; G alleles of rs2091172; and stool carriage of specific bacterial species. Patients with high-risk BEYOND scores received 82% relative protection from bezlotoxumab treatment over placebo.

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A randomized controlled trial of precision bezlotoxumab treatment for *Clostridioides difficile* infection

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SUMMARY

Early risk recognition for organ dysfunction and death by *Clostridioides difficile* infection (CDI) is an unmet need. A prediction score is developed in the BEYOND study (ClinicalTrials.gov; NCT02573571, NCT04725123, and NCT05304715). At the first stage, using 153 patients and 150 comparators, the BEYOND score was developed integrating hemoglobin; blood urea; blood interleukin-8; carriage of G alleles of rs2091172; and presence of *Terrisporobacter glycolicus*, *Enterococcus avium*, and *Anaerovorax odorimutans* in the stool. The score had 84.6% sensitivity and 95.8% specificity for unfavorable outcomes. At the second stage, a double-blind randomized controlled trial was performed, and 44 patients at high-risk by BEYOND score were treated with standard-of-care plus Bezlotoxumab or placebo. The primary endpoint was the incidence of organ dysfunction, CDI relapse, and/or death. This endpoint was met in 72.7% of patients in the placebo arm and 31.8% in the Bezlotoxumab arm ($p = 0.015$). Results suggest that BEYOND score can detect early risk in patients with CDI.

INTRODUCTION

The incidence of *Clostridioides difficile* infection (CDI) ranges between 2.5 and 7.7 cases per 100,000 hospitalizations in the United Kingdom[1] and 1.4 to 8.4 cases per 100,000 hospitalizations in Germany.[2] Global mortality by CDI has increased more than three times from 0.06 per 100,000 population in 1990 to 0.20 per 100,000 population in 2021.[3] CDI is frequently a complication of hospitalization, and death results from progression into sepsis and organ dysfunction.[4] It seems that progression into

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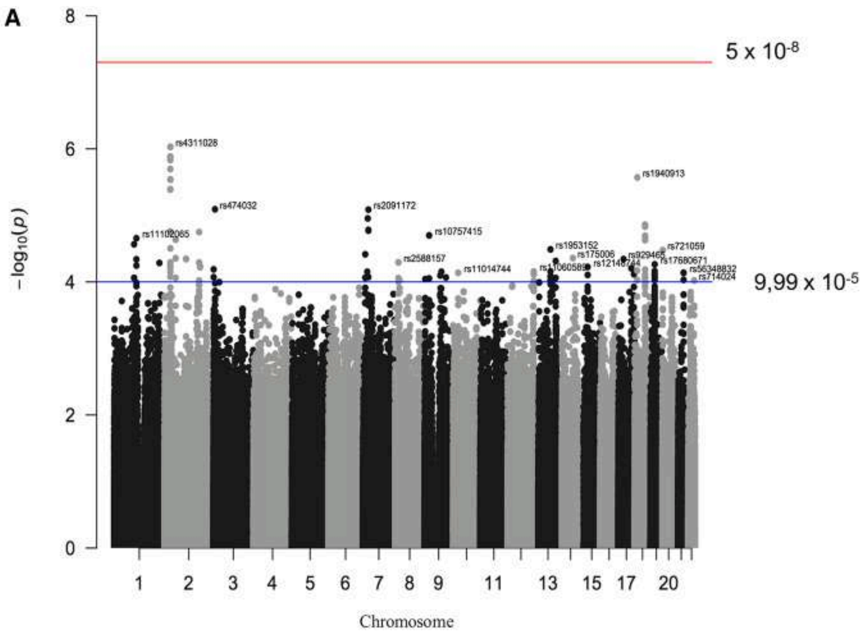
A



B

Carriage of SNPs	b-slope	OR (95% CIs)	p-value
C allele of rs12148744	2.72	15.1 (33.6-64.4)	<0.0001
C allele of rs714024	-2.72	0.07 (0.2-0.23)	<0.0001
C allele of rs721059	-2.92	0.05 (0.2-0.19)	<0.0001
T allele of rs4311028	3.28	26.6 (6.1-116.7)	<0.0001
A allele of rs62183547	-2.55	0.06 (0.02-0.26)	<0.0001
C allele of rs1128226	1.24	3.4 (1.3-9.2)	0.013
A allele of rs4279595	-1.72	0.18 (0.07-0.46)	<0.0001
G allele of rs175006	1.08	2.9 (1.2-7.5)	0.022
T allele of rs3859214	1.68	5.4 (1.9-14.8)	0.001
G allele of rs70028870	1.49	4.4 (1.6-12.2)	0.004
G allele of rs508660	-0.97	0.38 (0.15-0.93)	0.033
T allele of rs7240534	1.27	3.6 (1.2-10.7)	0.023
G allele of rs2091172	1.20	3.0 (1.1-8.2)	0.033
C allele of rs17680671	-1.68	0.18 (0.06-0.55)	0.002

Figure 1. Suggestive SNPs of susceptibility for CDI

(A) Manhattan plot presenting the *p* values of comparisons of SNPs between patients (n = 134) and comparators (n = 134). The cutoffs of statistical significance (red line) and suggestive significance (blue line) are provided.

(B) Final step of the stepwise logistic regression procedure with group (comparators vs. CDI) as the dependent variable; odds ratios less than 1 indicate protective alleles. Abbreviations: CDI, *Clostridioides difficile* infection; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

organ dysfunction results from the interplay between gut microbiome and host response where toxin B of *C. difficile* plays a major role.[5]

Fidaxomicin and vancomycin are first-line treatment options[6]; their efficacy in randomized controlled trials (RCTs) is expressed by sustained clinical response (SCR), and outcomes like progression into organ dysfunction and death are not taken into consideration.[7] SCR is defined as $\leq$2 bowel movements the first 12 days without any relapse for 40 days.[7–9] Bezlotoxumab is a monoclonal antibody that targets and inactivates toxin B of *C. difficile* and substantially decreases the risk of CDI relapse.[9] However, use is restricted by the substantial cost and the lack of profiling of patients who benefit most.[9] Prognostic enrichment tools are required to select patients at risk of unfavorable outcome who benefit from new treatments.

We hypothesized that patients who are at risk for deterioration will benefit from early Bezlotoxumab treatment. Therefore, we designed and conducted the BEYOND (Bezlotoxumab Yielded Outcomes by Addressing Personalized Needs in *Clostridioides Difficile*) study in two stages. At the first stage, we developed the BEYOND score, which integrates host and microbiome information. At the second stage, we conducted the BEYOND RCT to investigate if patients who score for high-risk benefit from Bezlotoxumab adjunctive treatment.

RESULTS

First stage: Development of the BEYOND score

The BEYOND score was developed on the assumption that it should be a composite score integrating clinical routine variables, blood cytokines, and single-nucleotide polymorphisms (SNPs) suggestive of susceptibility for CDI and bacterial stool species. This score should provide sensitivity more than 80% and positive predictive value more than 80% for the prediction of unfavorable outcomes by CDI. Unfavorable outcomes are censored by day 40, and they could be progression into organ dysfunction, CDI relapse, and/or death. The study was originally powered for 150 patients. This total number of participants did not allow to disclose significant genome-wide association study (GWAS) comparisons between patients with favorable and unfavorable outcomes. Two previous studies, one in 96 patients[10] and another in 646 patients,[11] described a strong association between SNPs of genes encoding for proteins involved in the innate and adaptive immune responses and susceptibility to CDI. These publications indicated that carriage of minor frequency of the SNPs was associated with outcome and supported the hypothesis that SNPs suggestive of susceptibility for CDI may be integrated into the BEYOND score.

To discover SNPs associated with susceptibility for CDI, one GWAS was performed. In total, 134 from the 153 patients with CDI and 134 from the 150 matched comparators were analyzed after quality control of the isolated DNA (Figure S1). The first patient was enrolled on 5 September 2015, and the last visit of the last patient was on 11 November 2016. Logistic regression analysis did not disclose any SNP below the level of GWAS

Table 1. Difference in baseline characteristics between patients with CDI and favorable outcome and patients with CDI and unfavorable outcome

	Favorable 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)	1.00
Hemoglobin, g/dL, mean (SD)	10.78 (1.64)	9.9 (1.73)	0.008
Hemoglobin <9.5 g/dL, n (%)[a]	19 (20.0)	17 (43.6)	0.009
Urea, mg/dL, mean (SD)	46.3 (38.1)	88.5 (70.6)	<0.0001
Urea >64.5 mg/dL, n (%)[a]	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 (2v92)	0.117
Previous CDI, n (%)	7 (7.5)	4 (10.3)	0.731
White blood cells, /mm^3 mean (SD)	12,083.0 (8124.5)	12,984.3 (9918.3)	0.586
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)	9.0 (<2–494.7)	16.4 (9–437.9)	0.048
IL-8 >227 pg/mL, n (%)[a]	5 (5.3%)	10 (25.6)	0.002
Eotaxin, pg/mL, median (range)	201.9 (<31–975.5)	152.6 (<31–1753.3)	0.899
Number of ATLAS criteria, mean (SD)	0.88 (0.85)	0.85 (0.85)	0.814
Carriage of SNPs participating in the gene score for CDI susceptibility, n (%)			
G allele of rs2091172	84 (84.4)	25 (64.1)	0.003
C allele of rs12148744	29 (30.5)	16 (41.0)	0.314
C allele of rs714024	34 (25.8)	19 (48.7)	0.178
C allele of rs721059	33 (34.7)	17 (43.6)	0.432
T allele of rs4311028	32 (33.7)	16 (41.0)	0.434
A allele of rs62183547	15 (15.8)	4 (10.3)	0.857
C allele of rs1128226	73 (76.8)	29 (74.4)	0.878
A allele of rs4279595	30 (31.6)	13 (33.3)	0.841
G allele of rs175006	56 (58.9)	28 (71.8)	0.175
T allele of rs3859214	77 (81.1)	36 (92.3)	0.123
G allele of rs70028870	54 (56.8)	16 (41.0)	0.128
G allele of rs508660	31 (32.6)	13 (33.3)	1.00
T allele of rs7240534	80 (84.2)	31 (79.5)	0.615
C allele of rs17680671	55 (57.9)	22 (56.4)	1.00

Abbreviations: CCI, Charlson's comorbidity index; CDI, *Clostridioides difficile* infection; IL, interleukin; SD, standard deviation.
[a]Cutoffs selected to provide specificity >80% by the coordinates of the receiver operator characteristic curve of the indicated variable for unfavorable outcome.

significance (i.e., $5-10^{-8}$). However, 17 SNPs showed suggestive association with CDI susceptibility; 13 SNPs were associated with specific gene expression using publicly available expression-QTL (eQTL) datasets (Tables S1 and S2), mostly associated with the cytoskeleton of the gut and apoptosis. Following stepwise logistic regression analysis, one gene score was developed to provide susceptibility for CDI. The β-slope of each SNP of the logistic model provided the points each SNP provided in the score (Figures 1 and S2).

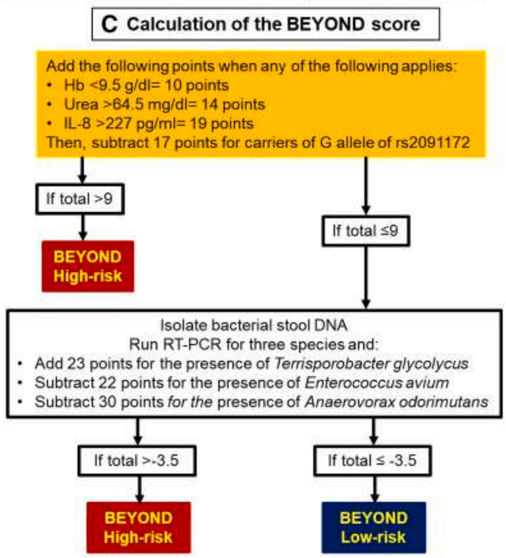
Thirty-nine of the 134 CDI patients (29.1%, 95% confidence interval [CI] 22.1%–37.3%) experienced an unfavorable outcome. The comparisons between the clinical characteristics, cytokines, and suggestive SNPs showed four significantly different variables between patients with favorable and unfavorable outcomes (Table 1), namely hemoglobin, urea, interleukin-8 (IL-8), and carriage of G alleles of rs2091172. Comparisons of the 17 suggestive SNPs for CDI susceptibility between patients with favorable and unfavorable outcomes revealed difference only for carriage of G alleles of rs2091172, which was significantly less frequent in patients with unfavorable outcomes. We next thought that for clinical utility, it would be easier to dichotomize the continuous variables into binomial variables by cutoffs. Cutoffs were defined after plotting one receiver operator characteristic (ROC) curve for each variable and selecting the Youden index coordinate that was the best trade-off to separate between patients with unfavorable and favorable outcomes. The calculated cutoffs were hemoglobin <9.5 g/dL; urea >64.5 mg/dL; and IL-8 >227 pg/mL. These three variables together with carriage of

A Host variables

Variable	β slope	OR (95%CIs)	p-value
Hb<9.5 g/dl	+1	2.9 (1.8-7.5)	0.021
Urea >64.5 mg/dl	+1.44	4.2 (1.5-10.9)	0.003
IL-8 >227 pg/ml	+1.87	6.5 (1.8-23.7)	0.005
G allele of rs2091172	-1.67	0.19 (0.07-0.53)	0.002

B Gut microbiome

Presence of:	β slope	OR (95%CIs)	p-value
Terrisporobacter glycolycus	2.25	9.5 (1.9-48.3)	0.007
Enterococcus avium	-2.18	0.11 (0.02-0.57)	0.008
Anaerovorax odorimutans	-3.05	0.05 (0.00-0.70)	0.027
Parabacteroides merdae	-1.84	0.16 (0.02-1.19)	0.073
Clostridium asparagiforme	-1.11	0.33 (0.05-2.39)	0.272



D Prognostic performance of the BEYOND score

	Progression into unfavorable outcome (n)	No progression into unfavorable outcome (n)	Total (n)
BEYOND High-risk (n)	33 Sensitivity= 84.6% (70.3 to 92.8%) PPV= 89.2% (75.3 to 95.7%)	4	37
BEYOND I Low-risk (n)	6	91 Specificity= 95.8% (89.7 to 98.4%) NPV= 93.8% (87.2 to 97.5%)	97
Total	39	95	134

Figure 2. Development of the BEYOND score to predict the risk of progression into unfavorable outcomes within the first 40 days from the onset of infection by *Clostridioides difficile*

(A) Stepwise logistic regression analysis that identifies four independent host variables associated with unfavorable outcome by CDI.

(B) Stepwise logistic regression analysis that identifies three bacterial species in the stool that are independently associated with unfavorable outcomes by CDI.

(C) Calculation of the BEYOND score. The score is calculated in two steps. At the first step, the presence of the host variables provides individual points per variable. If the sum is more than 9, then the patient is called high-risk for unfavorable outcome. If the first step is negative (i.e., sum ≤9), RT-PCR should run for three bacterial species, and the presence of each species provides separate points. If the sum is more than −3.5, then the patient is called high-risk. If this is less than or equal to −3.5, the patients is called low-risk.

(D) Total prognostic performance of the BEYOND score for unfavorable CI outcome. Abbreviations: CI, confidence interval; n. number of patients; OR; odds ratio; NPV, negative predictive value; PPV, positive predictive value; RT-PCR, real-time PCR.

G alleles of rs2091172 entered a logistic regression model as independent variables. The model confirmed that each of these variables was independently associated with unfavorable outcomes. The fitness of the model was confirmed by the Hosmer and Lemeshow test (a chi-squared value of 2.21; p = 0.697). The β-slopes of the logistic regression were used to develop a score for the host factors (Figure 2A). After ROC curve analysis, it was found that values higher than nine could select for patients with unfavorable outcomes.

The microbiome analysis identified 363 and 280 distinct operational taxonomic units (OTUs) for patients who had and who did not have unfavorable outcomes, respectively (Figure S3). Four species (*Parabacteroides merdae*, *Enterococcus avium*, *Anaerovorax odorimutans*, and *Clostridium aparagiforme*) were abundant in patients with good prognosis and one species (*Terrisporobacter glycolicus*) in patients with unfavorable outcomes (Figure S4). These five species entered as independent variables in one logistic regression model. The model showed that three species, namely, *Terrisporobacter glycolicus, Enterococcus avium*, and *Anaerovorax odorimutans*, were independently associated with unfavorable outcomes. The fitness of the model was confirmed by the Hosmer and Lemeshow test (a chi-squared value of 3.07; p = 0.930). The β-slopes of the logistic regression were used to develop a score for the gut species. After ROC curve analysis, it was found that values higher than −3.5 could select for patients with unfavorable outcomes (Figure 2B).

The abovementioned four independent variables providing information for the host and the three variables providing information for the gut microbiome were integrated into the BEYOND score (Figure 2C). The score is calculated in two steps. The first step takes into consideration the presence of the four host variables: three of the variables add to the score, while the genetic variant G of rs2091172 subtracts points from the score. Patients with scores higher than nine are called high-risk, and there is no need to proceed to the second step. If the score is equal to or lower than nine, then the second step is needed for analyzing the presence of the three bacterial species. If the score of the second step is more than −3.5, the patient is called high-risk. If the score of the second step is ≤ −3.5, the patient is finally called low-risk. The rationale for the two-step approach was to avoid processing stool in case the first step turned positive. The overall sensitivity, specificity, positive predictive value, and negative predictive value of the two-step procedure to predict

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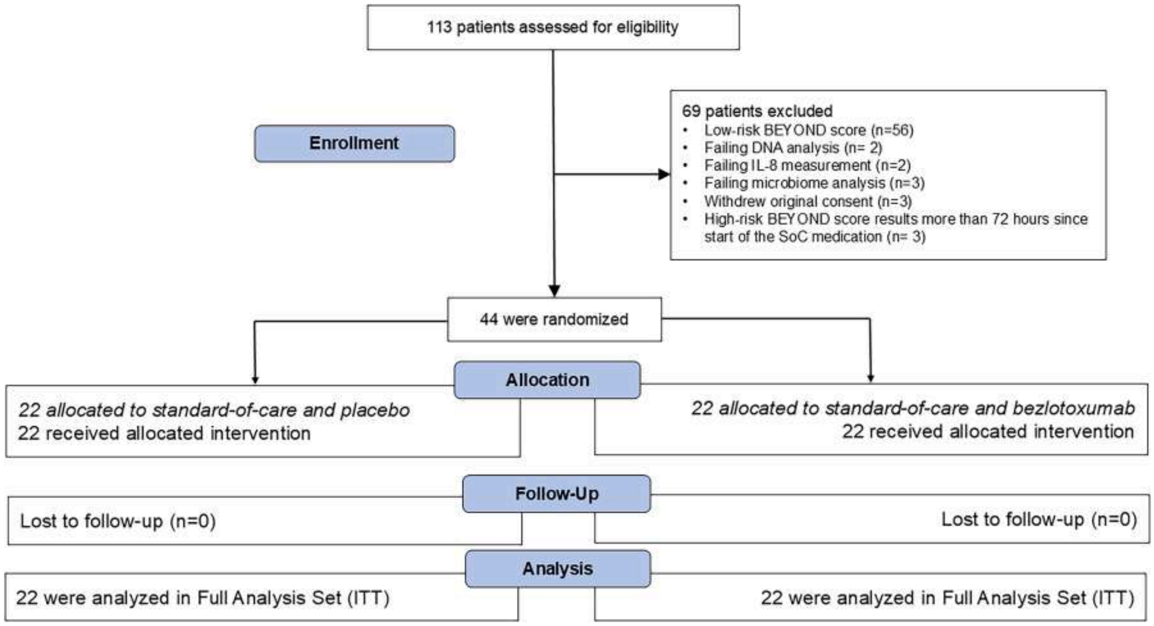

Figure 3. BEYOND trial flow chart
Abbreviations: ITT, intent-to-treat; n, number of patients; SoC, standard of care.

unfavorable outcome are 84.6%, 95.8%, 89.2%, and 93.8%, respectively (Figure 2D). The respective performance of ATLAS score >3[12] is 5.1%, 96.8%, 40.0%, and 71.3%, respectively (Table S3).

Second stage: The BEYOND trial

Patients' characteristics

The rationale of the trial was to apply predictive enrichment using the BEYOND score and to study clinical benefit from bezlotoxumab treatment. The first patient was enrolled on 22 April 2022, and the last visit of the last patient was on 04 June 2023; 113 patients with confirmed CDI were assessed for eligibility by the BEYOND score; 44 patients were randomized: 22 to placebo and 22 to bezlotoxumab. The main reason for exclusion was low-risk BEYOND score (Figure 3). All patients received allocated treatment within 72 h from the start of standard-of-care (SoC) treatment. Baseline characteristics, comorbidities, SoC treatment, and co-administered drugs were similar between the two arms (Table 2).

Follow-up

No patient was lost to follow-up, and all of them were included in the final analysis.

Primary outcome

In the intent-to-treat (ITT) population, the primary endpoint defined as the incidence of unfavorable outcome (including progression into organ dysfunction, CDI relapse, and/or death) by day 40 was met in 72.7% (16/22 patients) receiving placebo (95% CI 51.9%–86.9%) and in 31.8% (7/22 patients) treated with bezlotoxumab (95% CI 16.4%–52.7%) (p = 0.015) (Table 3). SCR has been proposed as clinical meaningful measure of drug efficacy for CDI.[7,8] This was achieved in 45.5% (10/22 patients) and 63.6% (14/22 patients), respectively.

One stepwise logistic regression analysis was done where the primary endpoint was the dependent variable; the variables that were different at baseline between patients who attained and who did not attain the primary endpoint entered the model as independent variables. The analysis confirmed that bezlotoxumab treatment was the only variable significantly associated with the attainment of the primary endpoint (odds ratio [OR] 0.11; 95% CI 0.03–0.049 (p = 0.004) (Table S4).

Secondary outcomes

The incidence of organ dysfunction and the cost of hospital stay were lower in the bezlotoxumab arm (Table 3; Figures S5 and S6).

The BEYOND score was validated among patients who failed screening because of a low-risk score (n = 56); patients with a high-risk score who were not enrolled because the score was provided late (n = 3); and enrolled patients randomized to placebo treatment (n = 22). Nineteen of the 25 patients with a high-risk BEYOND score progressed into unfavorable outcome (76.0%; 95% CI 56.5%–88.5%) compared to 19 of 56 patients with low-risk BEYOND score (33.9%; 95% CI 22.9%–47.0%; p = 0.001) (OR 6.17; 95% CI 2.11–18.01). Comparison of this prognostic performance with the ATLAS score showed that only 22 out of 41 patients who had ATLAS score more than three progressed into unfavorable outcome (53.7%; 95% CI 38.8%–67.9%). The comparisons of the relative abundances of bacterial families in the stool of patients with favorable and unfavorable outcomes were similar between participants of the first and the second stage (Figure 4).

Two post-hoc analyses were done for the need to up-escalate SoC for CDI and the time until live hospital discharge. Both these analyses favored the bezlotoxumab arm (Figures S7 and S8).

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Table 2. Demographics, comorbidities, and clinical characteristics of participants at the BEYOND randomized controlled trial

Variable	Placebo (*n* = 22)	Bezlotoxumab (*n* = 22)	*p* value
Age, years, median (Q_1–Q_3)	77.0 (64.8–84.5)	84.5 (71.5–87.0)	0.169
Male gender, n (%)	9 (40.9)	8 (36.4)	1.00
Female gender, n (%)	13 (59.1)	14 (63.6)	1.00
Infection by SARS-CoV-2, n (%)	4 (18.2)	3 (13.6)	1.00
Days in the general ward before CDI onset, median (Q_1–Q_3)	13.0 (8.8–35.5)	10.5 (8.0–15.0)	0.068
Charlson's comorbidity index, median (Q_1–Q_3)	6.0 (3.0–7.8)	6.0 (4.0–7.3)	0.859
Body weight, kg, median (Q_1–Q_3)	70.0 (61.5–76.8)	75.0 (65.0–80.0)	0.545
White blood cells, (/mm^3), median (Q_1–Q_3)	9,680 (6,700–13,853)	8,940 (5,350–14,947)	0.769
Hemoglobin, g/dL, mean (SD)	10.6 (1.4)	9.7 (1.9)	0.084
Platelets, $\times 10^3/mm^3$, mean (SD)	232.4 (118.0)	254.5 (146.9)	0.585
Urea, mg/dL, median (Q_1–Q_3)	40.0 (21.8–68.3)	37.0 (28.0–53.5)	0.769
Creatinine, mg/dL, median (Q_1–Q_3)	0.86 (0.55–1.47)	0.95 (0.67–1.40)	0.953
ALT, U/L, median (Q_1–Q_3)	11.0 (8.8–14.3)	15.0 (9.8–24.3)	0.086
AST, U/L, median (Q_1–Q_3)	20.5 (13.0–30.3)	17.5 (0.8–24.3)	0.716
Alkaline phosphatase, U/l, median (Q_1–Q_3)	67.0 (53.3–129.3)	66.0 (47.8–101.8)	0.460
γGT, U/L, median (Q_1-Q_3)	24.0 (11.0–34.0)	19.0 (13.5–44.5)	0.688
Total bilirubin, mg/dL, median (Q_1–Q_3)	0.51 (0.30–0.79)	0.46 (0.29–0.76)	0.473
pO_2/FiO_2, median (Q_1–Q_3)	376.0 (306.9–410.0)	352.5 (280.5–410.0)	0.564
CRP, mg/L, median (Q_1–Q_3)	45.1 (25.1–110.6)	79.0 (22.3–134.2)	0.405
SOFA score, median (Q_1–Q_3)	2 (1–3)	2 (1–3)	0.784
Number of bowel movements, median (Q_1–Q_3)	3.0 (3.0–3.3)	3.0 (2.8–4.0)	0.724
Elements of BEYOND score			
Urea >64.5 mg/dL, n (%)	11 (50.0)	6 (27.3)	0.215
Hemoglobin <9.5 g/dL, n (%)	7 (31.8)	11 (50.0)	0.358
SNP rs2091172, n (%)	12 (54.5)	14 (63.6)	0.760
Interleukin-8 > 230 pg/mL, n (%)	17 (77.3)	11 (50.0)	0.116
Terrisporobacter glycolicus in stool, n (%)	9 (40.9)	12 (57.1)	0.366
Enterococcus avium in stool, n (%)	20 (90.9)	19 (90.5)	1.00
Anaerovorax odorimutans in stool, n (%)	4 (18.2)	5 (23.8)	0.721
Comorbidities, n (%)			
Type 2 diabetes mellitus	7 (31.8)	8 (36.4)	1.00
Chronic heart failure	1 (4.5)	5 (22.7)	0.185
Chronic kidney disease	2 (9.1)	5 (22.7)	0.412
Chronic obstructive pulmonary disease	2 (9.1)	2 (9.1)	1.00
Lymphoma/leukemia/multiple myeloma	1 (4.5)	1 (4.5)	1.00
Solid tumor malignancies	7 (31.8)	2 (9.1)	0.132
Liver disease	0 (0.0)	1 (4.5)	1.00
Stroke	4 (18.2)	3 (14.3)	1.00
Atrial fibrillation	4 (18.2)	5 (22.7)	1.00
Coronary artery disease	3 (13.6)	5 (22.7)	0.698
Dyslipidemia	6 (27.3)	5 (22.7)	1.00
Ulcerative colitis	0 (0.0)	1 (4.5)	1.00
Dementia	8 (36.4)	4 (18.2)	0.310
Parkinson disease	3 (13.6)	1 (4.5)	0.607
Hypothyroidism	3 (13.6)	4 (18.2)	1.00
Arterial hypertension	12 (54.5)	11 (50.0)	1.00
Administered antibiotics, n (%)			
3rd generation cephalosporins	1 (4.5)	2 (9.1)	1.00
Piperacillin/tazobactam	2 (9.1)	7 (31.8)	0.132

(*Continued on next page*)

Table 2. *Continued*

Variable	Placebo (n = 22)	Bezlotoxumab (n = 22)	p value
Carbapenems	3 (13.6)	3 (13.6)	1.00
Quinolones	0 (0.0)	2 (9.1)	0.488
Aminoglycosides	1 (4.5)	0 (0.0)	1.00
Linezolid	0 (0.0)	2 (9.1)	0.488
Colistimethate	1 (4.5)	0 (0.0)	1.00
Tigecycline	4 (18.2)	1 (4.5)	0.345
Glycopeptides	1 (4.5)	3 (13.6)	0.607
Azoles	0 (0.0)	2 (9.1)	0.488
Echinocandins	2 (9.1)	0 (0.0)	0.488
SoC treatment, n (%)			
Metronidazole	14 (63.6)	10 (45.5)	0.364
Vancomycin	20 (90.9)	17 (77.3)	0.412
Fidaxomicin	7 (31.8)	5 (22.7)	0.736
Time (hours) to BEYOND score calculation since arrival of samples to the central lab, median (Q1–Q3)	28.5 (21.9–47.3)	30.4 (24.0–50.5)	0.291
Time (hours) from start of SoC treatment until start of study drug, median (Q1–Q3)	54.3 (33.4–65.0)	53.0 (40.3–65.9)	0.834

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; FiO_2, fraction of inspired oxygen; γGT, gamma glutamyl transferase; n, number of patients; OR, odds ratio; pO_2, partial oxygen pressure; Q, quartile; SD, standard deviation; SoC, standard of care; SOFA, sequential organ failure assessment.

Safety analyses

At least one serious TEAE was reported in 4 of 22 patients (18.2%) of the bezlotoxumab arm and in 9 of 22 patients (39.1%) of the placebo arm (p = 0.185; Table S5). The most common serious TEAEs were infections (9.1% and 30.4%, respectively; p = 0.135). No death was related to the study drug. The incidence of the non-serious TEAEs between the two study groups was similar (Table S6).

DISCUSSION

Precision immunotherapy is the vision for management of severe infections,[13] and BEYOND study is one of the first projects that paves the way. BEYOND study delivers a score that integrates information from the human host and the gut microbiome and denotes the likelihood for unfavorable outcome of CDI. The score had a very good capacity for predicting an unfavorable outcome, which subsequently resulted in bezlotoxumab treatment benefit despite the small number of treated patients. The studied primary endpoint was clinically meaningful and reflected patient benefit since bezlotoxumab treatment provided (1) an absolute 18.2% decrease of mortality, (2) an absolute 18.1% increase of SCR, and (3) significantly earlier alive hospital discharge. However, the benefit of bezlotoxumab in mortality and SCR was not statistically significant since the study was not powered for any of these endpoints. Despite the retraction of the drug from the market by the manufacturer in January 2025, the BEYOND score remains a tool of prognostic enrichment.

The results of this project go well beyond our current perception of CDI. So far, analyses of epidemiological series of patients disclose the presence of factors that increase the risk to acquire CDI and/or CDI relapse.[14–16] Gut microbiome research in CDI is focused on alterations over comparators[17] and does not identify prevailing bacterial species in the gut that predispose to host deterioration. However, the real burden of CDI is progression into organ dysfunction and death, and none of the RCTs run so far has studied this dimension of CDI. This problem was highlighted in the meta-analysis of the two registration trials of fidaxomicin pointing out that fidaxomicin treatment prolonged survival over vancomycin.[7] The only realistic approach to suppress the burden of CDI leading to organ dysfunction and sepsis is to recognize early in the disease patients at risk for deterioration who may benefit from immunotherapy. The BEYOND score achieves this goal well before signs of organ dysfunction appear.

The risk for relapse in CDI is usually predicted by the ATLAS source and patient comorbidities.[12,18,19] In the BEYOND project, comorbidities and the ATLAS score did not predict the risk for progression into organ dysfunction. This probably implies that relapse of CDI and progression from CDI to organ dysfunction are two different processes. Two components of the BEYOND score are associated with the function of the gut. More precisely, rs2091172 is associated with the expression of the *HOX* gene involved in gut maturation from an embryonic stage,[20] and IL-8 is excessively produced by intestinal epithelial cells following stimulation by toxins of *C. difficile*.[21]

In the MODIFY trial, patients with CDI at risk of relapse or relapsing CDI received benefit from bezlotoxumab treatment adjunctive to SoC. Treatment substantially reduced the risk for relapse, but no information was reported about further deterioration.[18] Blockade of toxin B at an early stage in patients at risk is the rationale for initiating treatment within the first 72 h. Despite

Table 3. Primary and secondary endpoints of the BEYOND randomized controlled trial

	Bezlotoxumab (*n* = 22)	Placebo (*n* = 22)	% Difference (95% CI)	OR (95%CIs)	*p* value
Primary endpoint					
Incidence of organ dysfunction, CDI relapse, and/or death the first 40 days, n (%)	7 (31.8)	16 (72.7)	40.9 (11.4–61.9)	0.18 (0.05–0.64)	0.015***
Secondary endpoints					
Incidence of organ dysfunction	6 (27.3)	15 (68.2)	40.9 (11.4–61.9)	0.18 (0.05–0.64)	0.015***
Time (days) to organ dysfunction, median	40	20.5	–	0.32** (0.12–0.82)	0.017****
CDI relapse, n (%)	1 (4.5)	0 (0)	4.6 (−10.8 to 24.9)	*	1.00***
Death by day 40, n (%)	6 (27.3)	10 (45.5)	18.2 (−9.8 to 42.6)	0.45 (0.13–1.59)	0.347***
Cost of hospital stay, Euros, mean (SE)	1,825.20 (275.5)	4,218.30 (938.9)	–	–	0.019

*Cannot be calculated because one value is zero; ** hazard ratio, ***Fisher's exact test, **** Cox regression analysis.
Abbreviations: CDI, *Clostridioides difficile* infection; CI, confidence interval; NA, not applicable; OR, odds ratio.

the drug cost, the BEYOND RCT also showed significant financial earnings.

The BEYOND score provides a holistic profiling of the risk of deterioration after CDI and integrates information from the laboratory exams, genetic makeup, immune response, and the gut microbiome. Patients with high-risk BEYOND score receive clinical benefit from bezlotoxumab treatment. BEYOND represents a paradigm for holistic evaluation and precision immunotherapy of severe infections.

Limitations of the study

Five main limitations of the BEYOND project should be acknowledged: (1) the small number of participants in both stages of the study. This limitation is mitigated by the validation of the score at the second stage, which took place 6 years later, and by the proven ability for precise profiling of patients who benefit from bezlotoxumab; (2) the limited power of GWAS to perform direct comparisons between patients with favorable and unfavorable outcomes and identify some SNP directly linked with unfavorable outcomes. In principle, one large-scale GWAS with equal number of patients with favorable and unfavorable outcomes would be required; (3) the conduct of the study only in Greek patients. As a next step, a larger multinational study is warranted to confirm the prognostic performance of the BEYOND score; (4) the lack of *Clostridioides difficile* ribotyping; and (5) the need to build a point-of-care diagnostic panel in the future, which will integrate the seven components of the score, taking into consideration the implementation in two steps. With the existing technology, the time until calculation of the BEYOND score goes up to a maximum of 50 h, which allows the timely start of bezlotoxumab.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Evangelos J. Giamarellos-Bourboulis (egiamarel@med.uoa.gr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All raw data reported in this paper are available for download from the server of the Hellenic Institute for the Study of Sepsis (https://wshiss.com/) and will be shared by the lead contact upon request. Usernames and passwords would be provided upon request to lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

C.P. and N.-R.Z. participated in data collection, data analysis, and drafting the manuscript. V.M., V.K., and E.S. performed data analysis and revised the manuscript for important intellectual content. C.S., E.T., K.I., M.S., S.M., S.G., K.A., A.B., E.H., A.K., P.P., K.T., H.M., G.A., G.P., M.L., A.S., Z.A., S.S., and G.C. recruited patients, collected data, and revised the manuscript for important intellectual content. M.G.N. analyzed the data and revised the manuscript for important intellectual content. E.J.G.-B. conceptualized the study design, participated in data analysis, drafted the manuscript, and takes full responsibility of data integrity.

DECLARATION OF INTERESTS

K.A. reports receiving honoraria and consulting fees from healthcare companies, including MSD, Pfizer, 3M Hellas, GSK/ViiV and Gilead.

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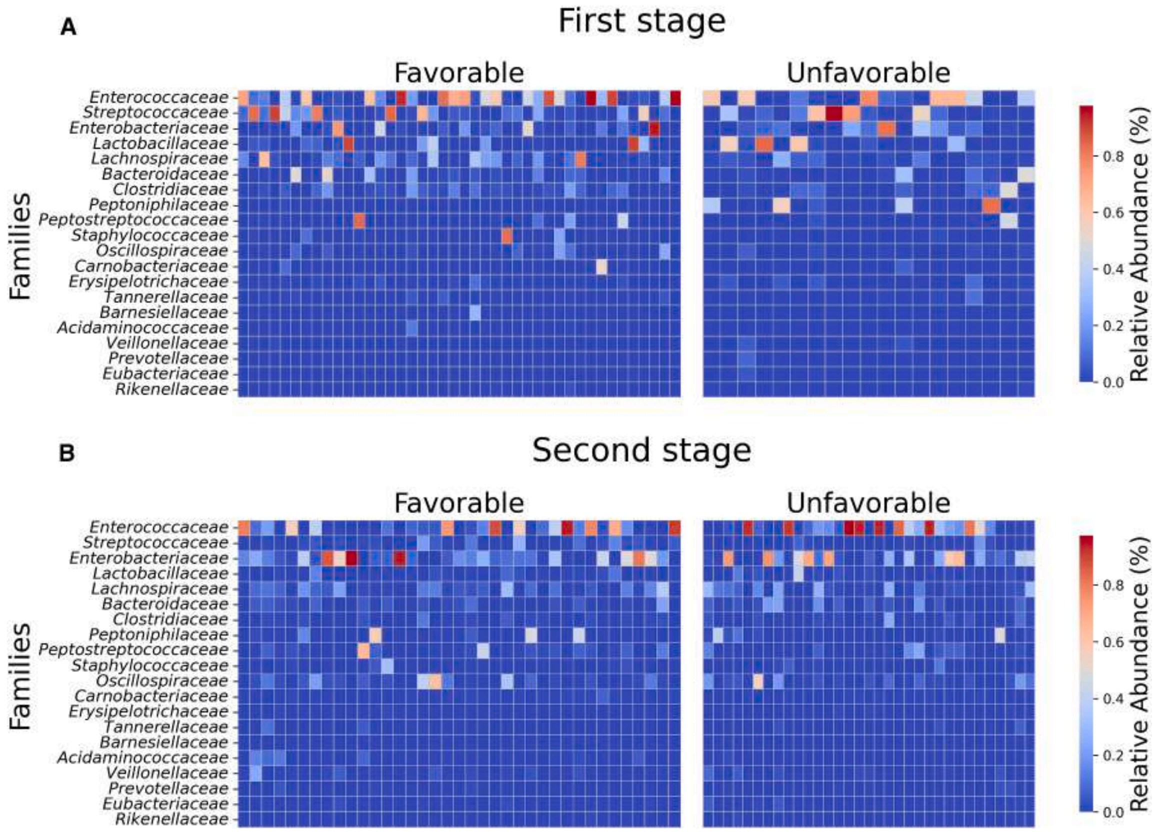

Figure 4. Comparison of the family abundance in the gut microbiome during the two stages of the BEYOND project

The relative abundances of the 20 most common families in stool are shown for participants of the first stage of the project (upper row (A), $n = 61$) and the second stage of the project (lower row (B), $n = 81$). Each row provides separate distribution for patients with favorable and unfavorable outcomes of the *Clostridioides difficile* infection. The analysis of the second stage contains patients who failed screening because of negative BEYOND score and patients who were enrolled in the study and randomized to placebo treatment. The distributions of species between patients with favorable outcome and patients with unfavorable outcome from each stage of the project are similar.

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STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2025.102533.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Rectal swabs	Copan Italia s.p.a., Italy	Catalog#: 519C
Chemicals, peptides, and recombinant proteins		
AllPrep PowerFecal Pro DNA/RNA Kits	QIAGEN GmbH, Hilden, Germany	Catalog#: 80254
Barcoding Kit 1-24	Oxford Nanopore Technologies, Oxford, UK	Catalog#: SQK-16S024
Flow Cell (R9.4.1)	Oxford Nanopore Technologies, Oxford, UK	Catalog#: FLO-MIN106D
Infinium HumanExome BeadChip Kit	Illumina, Inc, San Diego California, USA	Catalog#: 20015247
Mag-Bind® TotalPure NGS	OMEGA, Bio-tek, Inc, Norcross Georgia, USA	Catalog#: M1378-00
QIAamp DNA Blood Mini Kit (50)	QIAGEN GmbH, Hilden, Germany	Catalog#: 51104
Critical commercial assays		
IL-6 enzyme immunoassay	Invitrogen, Waltham, Massachusetts, USA	Catalog#: 88-7066-88
IL-8 enzyme immunoassay	Invitrogen, Waltham, Massachusetts, USA	Catalog#: 88-8086-88
Eotaxin enzyme immunoassay	Wuhan Fine Biotech Co, Wubei, China	Catalog#: EH0121
Deposited data		
https://wshiss.com	Hellenic Institute for the Study of Sepsis	On request
Software and algorithms		
MinKNOW™ v. 22.05.6 & 22.10.7	Oxford Nanopore Technologies, Oxford, UK	N/A
OptiCall v. 0.7.0	Wellcome Trust Sanger Institute, Hinxton, UK	N/A
Python 3.11	SciPy Stats (https://docs.scipy.org/doc/scipy/reference/stats.html)	N/A
IBM SPSS Statistics 26.0	https://www.ibm.com/products/spss-statistics	N/A
Other		
Bezlotoxumab	Merck & Co., Rahway, New Jersey USA	RRID: AB_2911105

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

First stage: development of the BEYOND score

This prospective study took place between September 2015 and December 2016 in eleven departments of Internal Medicine in Greece (ClinicalTrials.gov NCT02573571 and NCT04725123).

Participants with CDI and matched comparators for age, gender and comorbidities, hospitalized at the same time and at the same wards participated after written informed consent. Patients were adults (age ≥18 years) of either gender; with at least three episodes of unformed stool the last 24 h according to the Bristol stool scale; and stool samples positive both for glutamate dehydrogenase (GDH) and toxin A and/or B of *C. difficile*. Patients with any acute organ dysfunction were excluded. Comparators had normal defecation the last seven days (Bristol scale 3 to 5) and stool negative for GDH and toxins of *C. difficile*; they were matched with CDI patients by the Charlson comorbidity index (CCI).

Mean (SD) of participants was 76.2 (13.3) years and of comparators 66.7 (11.7) years. Male/female patients were 80/70 and 71/82 respectively.

Second stage: The BEYOND randomized controlled trial

BEYOND is a phase II, multicenter, double-blind, RCT conducted in 14 study sites in Greece. The study protocol was approved by the National Ethics Committee (approval 195/21) and the National Organization for Medicines (approval IS192-21) (EudraCT number 2021-005473-10, Clinicaltrials.gov NCT05304715). Written informed consent was provided by the patients or legal representatives.

Participants were male and female adults; with at least three episodes of unformed stool the last 24 h; with positive stool for GDH and toxin A and/or B of *C.difficile*; high-risk BEYOND score; and start of randomized treatment the first 72 h from start of SoC treatment. Key exclusion criteria were known allergy to bezlotoxumab; and pregnancy or lactation.

Mean (SD) of participants was 76.4 (13.1) years; 17 were male patients and 27 were female patients.

METHOD DETAILS

First stage: development of the BEYOND score

Approvals were provided by the following Ethics Review Committees: Evangelismos Athens General Hospital, approval 166/2015; ATTIKON University General Hospital, approval 972/2015; G.Gennimatas Athens General Hospital, approval 23962/2015; Thriasio Elefsis General Hospital, approvals 307/2015 and 308/2015; Tzaneion Piraeus Hospital, approval 11815/2015; Sotiria Athens Hospital, approval 1850/2015; Laikon Athens General Hospital; approval 13211/2015; AHEPA Thessaloniki University Hospital, approval 444/2015; Sismanogleion Athens General Hospital, approval 795/2016; and Rion University Hospital, approval 694/2016.

Enrolled patients received standard-of-care (SoC) treatment at the discretion of the attending physician and clinical and laboratory data were recorded daily for 40 days.

Whole blood and stool samples were collected during the first 24 h from diarrhea onset and transported to the Laboratory of Immunology of Infections at the 4th Department of Internal Medicine of ATTIKON University General Hospital. More precisely, 15mL of whole blood was collected under aseptic conditions within the first 24 h of diarrhea onset in the case of CDI patients and the negative stool sample in the case of control patients. The blood was poured into 10 mL in a pyrogen-free tube coated with EDTA (ethylenediamine tetraacetic acid Vacutainer, Becton Dickinson, Cockeysville, Md, USA) and 5mL in a clean and pyrogen free tube (Vacutainer). Ten mL of liquid stool sample in a clean, leak-proof container without disinfectant or detergent residue or one dry rectal swab (Copan Italia s.p.a., Italy; Catalog#: 519C) was collected. The first tube was maintained at −20°C until human DNA isolation and analysis. The second tube was centrifuged and the serum was kept refrigerated at −80°C until cytokines were measured. Stool samples were stored immediately at −80°C until DNA extraction was performed.

The isolation of total genomic DNA was done from the white blood cells using the QIAamp Blood Mini Kit (QIAGEN Inc, Valencia, CA, USA). The amount of isolated DNA was calculated with the NanoDrop ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE, USA). The isolated samples were kept at −80°C until analysis. The samples were plated and analyzed in one HumanCore-chip using BeadArray Reader (Illumina). Genotype calling was performed using Opticall 0.7.0.[22,23] The associations between the genome-wide imputed variants and susceptibility to CDI were tested by logistic regression using PLINKv1.9 (www.cog-genomic.org/plink/1.9/)[24] with age, gender and CCI as confounders. A *p*-value significance threshold less than 5.0 x 10^{-8} was set to call genome-wide significant associations. We considered SNPs with a *p*-value between 9.99 x 10^{-5} and 5 x10^{-8} as variants showing a suggestive association with CDI susceptibility.

Concentrations of interleukin (IL) −6 (Affymetrix Inc., San Diego, CA, USA), IL-8 (Affymetrix Inc, San Diego, CA, USA) and eotaxin (Wuhan Fine Biotech Co, Wubei, China) were measured in duplicate in serum by an enzyme immunoassay. The lower detection limits were 2, 2, and 20 pg/mL respectively. These proteins were selected based on published associations with unfavorable CDI outcomes.[25]

Bacterial DNA was isolated from the samples and 30ng qualified DNA template and the 16S rRNA fusion primers were added for Polymerase chain reaction (PCR). All PCR products were purified by Agencourt AMPure XP beads, dissolved in Elution Buffer and eventually labeled to finish library construction. Library size and concentration were detected by Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on HiSeq platform according to their insert size. Raw data were filtered to obtain the high-quality clean data, after which clean reads that can overlap with each other are merged to tags and further clustered to operational taxonomic units (OTU). Taxonomic classifications were assigned to OTU representative sequence using Ribosomal Database Project database. Analysis like alpha diversity, beta diversity, differential species analysis, network and model prediction were carried out based on OTU profile table and taxonomic annotation results. Raw data were filtered to generate high quality clean reads as follows:

- Reads with average Phred quality values lower than 20 over a 25 bp sliding window were truncated.
- Reads that are contaminated by adapter sequences (default parameter:15 bases overlapped by reads and adapter with maximal 3 bases mismatch allowed) were removed.
- Read with ambiguous base were removed.
- Reads with low-complexity reads (default: reads with 10 consecutive same base) were removed.

To ensure the removal of barcode sequences from pooling libraries, clean reads were assigned to corresponding samples through alignments (0 base mismatch) against barcode sequences by in-house scripts. If paired-end reads overlapped with each other, then a consensus sequence was generated by FLASH (Fast Length Adjustment of Short reads, v1.2.11). OTU were defined as a unified marker for analyzing a taxon unit (seven taxonomy levels) in the research of phylogeny or population genetics. To quantify the abundance of bacteria on every level in each sample, it was necessary to cluster the sequences into OTU with 97% similarity. Tags were clustered to OTU with USEARCH (v7.0.1090). For 16S rDNA and ITS sequences, chimeras in OTU were screened and filtered by mapping to gold database (v20110519), UNITE (v20140703) respectively whereas *de novo* chimera screening was carried out for 18S rDNA sequences. OTU representative sequences were aligned against the database for taxonomic annotation by RDP classifier (v2.2) software (sequence identity is set to be 0.6). Annotation results were filtered as follows.

- Not annotated OTUs were removed;
- Taxonomies not matching with the project's research background were removed.

OTU rank curve visualizes the diversity of species (species richness and species evenness) in samples/groups. The relative abundance of each OTU in each sample was calculated, and the species were ranked by their abundance in descending manner. Taxonomic analysis of OTU representative sequences was carried out by RDP classifier Bayesian algorithm to identify the composition of microbial structure. Abundances of species on seven levels (Phylum, Class, Order, Family, Genus, Species) was calculated after annotation. Approximately-maximum- likelihood algorithm was used to generate phylogenetic trees and the visualization of phylogeny tree is carried out by software R as shown below for the analyzed samples.

The primary endpoint was the development of a score to predict unfavorable outcome of CDI with sensitivity and positive predictive value (PPV) 80% or more. Unfavorable outcome was defined as the incidence of any of organ dysfunction, progression to severe infection, CDI relapse, and/or death the first 40 days. The secondary endpoint was SNPs associated with CDI susceptibility.

Second stage: The BEYOND randomized controlled trial

Analysis was done using similar methods to the first stage. Screening for the BEYOND score was done for clinical criteria and after collection of whole blood and one stool sample or rectal swab. To analyze for the presence of the G allele of rs2091172 genomic DNA was prepared from peripheral blood leukocytes using the protocol of a QIAamp DNA Blood Mini Kit (QIAGEN Inc. Valencia, CA, USA). The total DNA yield was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE). 50-500μg of DNA extract was used in one 25-μL PCR reaction mixture consisting of dNTPs, Taq polymerase (New England BioLabs, Ipswich, MA, USA) and 10 pmol of each intergenic primer: 5′-TGA GAA GGA AGT CCA GTC ACA TC-3’ (forward) and 5′-TGG CAC TGT TGA TAG AAA GAT GAA T-3’ (reverse). Amplification was performed in a thermal cycler (SensoQuest, Gottingen, Germany) using the following cycle conditions: initial denaturation step at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 1 min and extension at 68 for 1 min. Then another cycle of 68°C for 5 min was carried out before termination. Before sequencing, unincorporated primers and deoxynucleotides were removed from the reaction mixtures with Monarch PCR & DNA Clean up Kit (New England BioLabs). Sanger Sequencing was carried out bi-directionally using a 3730 DNA Analyzer (Applied Biosystems, ThermoFisher, USA) and BigDye Terminator DNA sequencing kit (Applied Biosystems), according to the manufacturer’s instructions. Data was analyzed using Analysis Module Variant Analysis (VA) Software (Applied Biosystems, ThermoFisher, USA) and by visual inspection of the electropherograms.

IL-8 was measured using the enzyme immunosorbent assay described above.

For the analysis of the stool samples, total DNA was extracted using the Allprep PowerFecal Pro DNA/RNA Kit (Qiagen, MD, USA; Catalog#: 80254) following manufacturer’s instructions, to obtain high molecular weight DNA required for long-read metagenomics (LRM). The quantity and quality assessment DNA were carried out using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA; Model G2939B) and ND-1000 spectrophotometer, V3.3.1 (Nanodrop Technologies, Inc., Wilmington, DE 19810, USA). Barcoding Kit 1–24 (ONT, Oxford Nanopore Technologies, Oxford, UK; Catalog#: SQK-16S024) protocol for the 16S rRNA gene, was carried out as described by the manufacturer. All reactions contained the same components described in the manufacturer’s protocol, but temperature and cycling parameters were modified slightly. DNA amplification was performed using a SensoQuest thermal cycler (Göttingen, Germany; Catalog#: 011–103). After the PCR amplification step, the 16S rRNA barcoded amplicons were bound to Mag-Bind TotalPure NGS (OMEGA, Bio-tek, Inc, Norcross, USA; Catalog#M1378-00), washed twice with 200 μL of 70% ethanol and eluted in 20 μL of Water for molecular biology (AppliChem, Darmstadt, Germany; Catalog#A7398). About 60 μL (1.2X ratio required) of Mag-Bind beads (OMEGA) were used for the cleaning. The purified amplicon concentration and quality were again checked using an Agilent 2100 Bioanalyzer (Agilent) and an ND-100 Spectrophotometer V3.3 (Nanodrop). Suitable volumes from each amplicon were pooled, and thoroughly mixed by rigorous pipetting at a final concentration of 10 ng/μL, and 10 μL of this pool was used to prepare the mix that was eventually loaded into the Flow Cell for sequencing. The 16S rRNA barcoded amplicons were loaded on a Flow Cell R9.4.1 (FLO-MIN106D, ONT), with ≤24 samples for each run. The Flow Cell was fitted to the MinION device ensuring good thermal contact. 800 μL FLT (ONT) buffer was loaded into the priming port using a P1000 pipette and left for 5 min to prime the flow cell. This priming process was repeated a second time with 200 μL. Base calling was performed on the MinION Mk1C platform (ONT), using MinION control software, MinKNOW versions 22.05.6–22.10.7, using Guppy barcoder (versions 6.1.5–6.4.2). The sequence duration for all samples ranged from 16 to 24 h until the desired number of reads was achieved or the sample was exhausted. SB analyser was used to demultiplex the samples, and discard reads <200 bp. The 16S sequencing data from the Fastq files generated, were then analyzed in the cloud-based platform EPI2ME Agent version 3.5.7 using the Fastq 16S workflow (v2022.01.07). The quality control of the generated reads was employed using Phred quality score (Q-score) ≥7. For (sub)species or strain identification from the reads, BLAST was run in the standard National Center for Biotechnology (NCBI) Taxonomy database[26] with E-value ≤0.01, >30% coverage and >77% identity. Taxonomic classification from Kingdom level up to (sub)species or strain level was then assigned based on the taxonomic ids of the given taxa with the ETE Toolkit 3.0.

Following successful screening, patients were blindly 1:1 randomized by a centralized, web-based system to treatment with SoC and single doses of Bezlotoxumab or placebo. The dose of Bezlotoxumab was 10mg per kg of body weight dissolved in either normal saline 0.9% or 5% dextrose water up to a maximum dose of 1000mg. Placebo was an equal volume of normal saline 0.9% or 5% dextrose water. Study drug and matched placebo were prepared by an unblinded pharmacist and administered by a blinded study nurse. Preparations were similar in appearance; investigators, study nurses and patients were blind to the administered treatment. SoC was selected according to local guidelines and included oral treatment with metronidazole, vancomycin, fidaxomicin or combinations.

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Demographics, comorbidities, daily SOFA (sequential organ failure assessment) scores, lab values, radiology, need for operation, and admission in the Intensive Care Unit (ICU) were recorded. Disposition by day 40 for enrolled and screening-failing patients was captured by physical visits or phone calls. The study was monitored by separate monitors unblinded and blinded to the allocated treatment. All serious and non-serious treatment-emergent adverse events (TEAEs) were captured and reported by MedRA 25.0.

The primary endpoint was the incidence of unfavorable outcome by day 40 defined as any of: organ dysfunction; CDI relapse; and/or death. Organ dysfunction was defined as $\geq$2-point increase of the baseline SOFA score[27] or need for colectomy or ICU admission.

Main secondary outcomes were a) time to organ dysfunction; b) CDI relapse; c) survival by day 40; and d) cost of hospital stay. The prognostic performance of the BEYOND score was also validated.

QUANTIFICATION AND STATISTICAL ANALYSIS

First stage: development of the BEYOND score

The study was powered with 80% power at the 10% level of significance for 150 patients on the assumption that 10% would have an unfavorable outcome. Clinical variables, suggestive loci of susceptibility for CDI, serum cytokines, and microbial species were compared between patients with favorable and unfavorable outcomes. Quantitative variables were transformed into qualitative variables using the Youden index of the Receiver Operator Characteristics curves which could discriminate between patients with favorable and unfavorable outcome. In order to confirm which of these variables were independently associated with unfavorable outcome, one stepwise logistic regression analysis model was applied. Unfavorable outcome was the dependent variable. Clinical variables, SNPs, cytokines and microbial species different between patients with favorable and unfavorable outcomes were the independent variables. The beta-slope of each variable provided the points each variable should represent in the BEYOND score.

Second stage: The BEYOND randomized controlled trial

With the assumption that 60% of placebo-treated patients and 20% of Bezlotoxumab-treated patients would meet the primary endpoint, it was calculated that 44 patients should be enrolled (22 in each arm) to provide 80% power at the 10% level of significance. Analysis was done by the intent-to-treat (ITT) principle. Comparisons for qualitative endpoints were performed by the Fisher's exact test; odds ratios (OR) and 95% confidence intervals (CI) were calculated by Maentel-Henzel's statistics. Confirmatory stepwise logistic regression analysis was done with the primary endpoint as dependent variable and as independent variables the group of treatment and all baseline variables which were different in the univariate analysis between patients meeting and not meeting the primary endpoint. Time event endpoints were compared by Cox regression analysis. The cost of drugs, laboratory and radiology exams, accommodation and interventions was calculated in one itemized approach. To compare relative abundances of microbiome families patients with favorable and unfavorable outcome of the *Clostridioides difficile* infection were selected across the two stages of the BEYOND project. The cohort of the second stage contained patients who failed screening because of negative BEYOND score and patients who were enrolled in the study and were randomized to placebo treatment. Since the gut microbiome of the two stages was analyzed in different platforms and with different sequencing methods (Illumina sequencing for the first stage and Nanopore sequencing for the second) the family level was chosen for comparisons, because OTUs coming from Illumina do not always reach to the species level. The relative abundances of the 20 most common families in stool for participants of the first stage of the project were compared to the abundances of participants in the second stage of the project. They were also compared between the patients with favorable and unfavorable outcome in each stage. Mann-Whitney U rank test was used for these comparisons. Since 80 comparisons were performed in total, the Bonferroni corrected alpha was 0.05/80 = 0.000625 and thus, only two-tailed p values <0.000625 were considered significant.

ADDITIONAL RESOURCES

This two-stage study has been registered with ClinicalTrials.gov NCT02573571, NCT04725123 and NCT05304715.