PRECISION BEZLOTOXUMAB TREATMENT FOR *CLOSTRIDIOIDES DIFFICILE* INFECTION

STATISTICAL ANALYSIS PLAN

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LIST OF ABBREVIATIONS

BEYOND: BEzlotoxumab Yielded Outcomes by addressing personalized Needs in *Clostridioides Difficile*

CCI: Charlson's Comorbidity Index

CDI: *Clostridioides difficile* infection

CI: confidence interval

eQTL: expression-QTL

GDH: glutamate dehydrogenase

GWAS: genome-wide association studies

HR: hazard ratio

ICU: intensive care unit

ITT: intent-to-treat

OR: odds ratio

PP: per protocol

PPV: positive predictive value

SAP: statistical analysis plan

SE: standard error

SNP: single nucleotide polymorphism

SoC: standard-of-care

SOFA: sequential organ failure assessment

TEAE: treatment emergent adverse events

INTRODUCTION

This statistical analysis plan (SAP) is developed to guide the statistical analysis of the two-stage BEYOND (BEzlotoxumab Yielded Outcomes by addressing personalized Needs in *Clostridioides Difficile*) project (paper entitled “Precision Bezlotoxumab treatment for *Clostridioides difficile* infection”). More precisely, this SAP is supporting both stages of the project (First stage of the BEYOND project: development of the BEYOND score) with ClinicalTrials.gov NCT02573571 and NCT04725123 and (Second Stage of the BEYOND project: The BEYOND randomized controlled trial) with EudraCT number trial 2021-005473-10, Clinicaltrials.gov NCT05304715.

STUDY ENDPOINTS

Primary endpoint – First stage of the BEYOND project

The primary endpoint was the development of a risk score to predict unfavorable outcome of *Clostridioides difficile* infection (CDI) with sensitivity and positive predictive value (PPV) 80% or more. The BEYOND score is calculated in two steps. At the first step, the presence of the host variables provides points (Hb < 9.5 g/dl, Urea > 64.5 mg/dl, IL-8 > 227 pg/ml, G allele of rs2091172). If the sum is more than 9 (as calculated after receiver operation characteristics curve analysis), then the BEYOND score is positive. If the first step is negative, RT-PCR should run for three bacterial species (*Terrisporobacter glycolycus, Enterococcus avium, Anaerovorax odorimutans*), the presence of each provides separate points. If the sum is more than -3.5 (as calculated after receiver operation characteristics curve analysis), then the BEYOND score is positive.

Unfavorable outcome was defined as the incidence of any of the following the first 40 days of follow-up: organ dysfunction, progression to severe infection, CDI relapse, and/or death.

Secondary endpoint – First stage of the BEYOND project

The secondary endpoint was the identification of Single Nucleotide Polymorphisms (SNPs) which provide susceptibility for CDI.

Primary endpoint – Second stage of the BEYOND project

The primary endpoint was the incidence of unfavorable outcome censored at day 40. Unfavorable outcome was a composite endpoint by the incidence of at least one of the following conditions: progression into organ dysfunction; CDI relapse; and/or death. Organ dysfunction was defined as either any increase by at least 2 points of the baseline sequential organ failure assessment (SOFA) score before start of the study drug or need for colectomy or intensive care unit (ICU) admission.

Secondary endpoints – Second stage of the BEYOND project

The key secondary outcomes were:

- progression into organ dysfunction;
- time to organ dysfunction;
- CDI relapse;
- survival by day 40;
- cost of hospital stay and
- validation of the prognostic performance of the BEYOND score

MAIN FEATURES OF THE STUDY DESIGN

Patient population

<u>Key inclusion criteria for the first stage of the BEYOND project</u>

1. Adults (age ≥18 years) of both genders
2. In case of women, unwillingness to remain pregnant during the study period.
3. Written informed consent provided by the patient. For subjects without decision-making capacity, informed consent is obtained from a legally designated representative following the national legislation in the Member State where the trial is planned.
4. At least three episodes of unformed stool the last 24 hours according to the Bristol stool scale
5. Stool samples positive both for glutamate dehydrogenase (GDH) and toxin A and/or B of *C. difficile*.

Key exclusion criteria for the first stage of the BEYOND project

1. Age less than 18 years
2. Refusal of written consent
3. Pregnancy or lactation
4. Any acute organ dysfunction

Intervention for the first stage of the BEYOND project

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.

Key inclusion criteria for the second stage of the BEYOND project

1. Adults (age ≥18 years) of both genders
2. In case of women, unwillingness to remain pregnant during the study period.
3. Written informed consent provided by the patient. For subjects without decision-making capacity, informed consent is obtained from a legally designated representative following the national legislation in the Member State where the trial is planned.
4. At least three episodes of unformed stool the last 24 hours according to the Bristol stool scale
5. Stool samples positive both for GDH and toxin A and/or B of *C. difficile*
6. Positive BEYOND score
7. Start of randomized treatment the first 72 hours from start of SoC treatment

Key exclusion criteria for the second stage of the BEYOND project

1. Age less than 18 years
2. Refusal of written consent
3. Pregnancy or lactation
4. Any acute organ dysfunction
5. Known allergy to Bezlotoxumab
6. Negative BEYOND score

Intervention for the second stage of the BEYOND project

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-treated patients received an equal volume of normal saline 0.9% or 5% dextrose water. SoC was selected according to local guidelines and it included oral treatment with metronidazole, vancomycin, fidaxomicin or combinations.

NUMBER OF PATIENTS

First stage of the BEYOND project

The calculation of the needed number of patients was based on the assumption that 10% would have an unfavorable outcome. According to this calculation:

- 80% power at the 10% level of significance was used

Final calculation: 150 patients need to be enrolled in total.

Second stage of the BEYOND project

The calculation of the needed number of patients was based on the assumption that 60% of placebo-treated patients and 20% of Bezlotoxumab-treated patients would meet the primary endpoint. According to this calculation:

- 80% power at the 10% level of significance was used
- 1:1 randomization applies (one patient allocated to placebo arm; 1 patient allocated to Bezlotoxumab treatment arm)

Final calculation: 44 patients need to be enrolled in total (22 in the placebo arm and 22 in the Bezlotoxumab treatment arm).

ANALYZED PATIENT POPULATIONS

- Intention-to-treat (ITT) population: this will include all patients who are randomly assigned to treatment. The following patients will be excluded:
  - Patients where a major violation of the inclusion or exclusion criteria took place before the patient was randomized and provided that this was assessed with full objectivity.
  - Patients who withdrew consent and requested withdrawal of data

ANALYSIS OF BASELINE DEMOGRAPHICS

Continuous variables following normal distribution are expressed by means and standard deviation. Continuous variables not following normal distribution are expressed by medians and interquartile range. Binomial variables are expressed as absolute and percentage frequencies with 95% confidence intervals (CI).

All baseline continuous and binomial variables alongside the comparisons between the two arms of treatment are provided in tabular format. These include demographics (gender and age), disease severity, baseline laboratory values, elements of BEYOND score, comorbidities and co-administered treatment.

Demographics, comorbidities and clinical characteristics were compared between the participants at the BEYOND study. Fisher's exact test is used when the data are categorical. When the data are continuous, in case of normal distribution, comparisons between the groups is done by the Student's t-test. In case of linear distribution, comparisons between the groups are done by the Mann-Whitney U test.

STATISTICAL ANALYSIS FOR THE PRIMARY ENDPOINTS

First stage of the BEYOND project

Clinical variables, suggestive loci of susceptibility for CDI, serum cytokines, and microbial species are compared between patients with favorable and unfavorable outcome.

Regarding the microbial species comparisons, the odds ratios (OR) for progression into unfavorable outcome under the presence of each species in the gut microbiota, the respective 95% confidence intervals and the P values are provided and are calculated by Mantel-Haenszel statistics.

Step-wise logistic regression analyses are used to identify SNPs, bacterial species and host variables that were independently associated with unfavorable outcome by CDI. The variables found enter into one multivariable step-wise logistic regression model and the beta-slope is used to build the BEYOND score. The total prognostic performance of the BEYOND score for unfavorable CDI outcome is estimated by calculating the overall specificity and sensitivity, as well as the positive and negative predictive values.

Second stage of the BEYOND project

Analysis is done by the ITT principle. In the ITT population, comparisons for the primary endpoint are performed by the Fisher's exact test; odds ratios (OR) and 95% CIs were calculated by Maentel-Henzel's statistics when all values are not zero. Confirmatory step-wise logistic regression analysis is done with the primary endpoint as dependent variable and as independent variables the group of treatment and all baseline variables which are different in the univariate analysis between patients meeting and not meeting the primary endpoint. The discriminator values for some independent variables (i.e. absolute platelet count, SOFA score) between patients meeting and patients not meeting the primary endpoint by receiver operator characteristics curve analysis are defined by the Youden Index. OR, 95% CIs and P-values are provided when all values are not zero.

KEY SECONDARY ENDPOINT ANALYSIS SUPPORTIVE OF THE PRIMARY ENDPOINT

First stage of the BEYOND project

SNPs showing association with susceptibility to CDI and with specific gene expression using publicly available expression-QTL (eQTL) datasets can be identified through Genome-Wide Association Studies (GWAS) between patients and comparators.

These associations are tested by logistic regression using PLINKv1.9 (www.cog-genomic.org/plink/1.9/) with age, sex and Charlson's Comorbidity Idex (CCI) as confounders. A p-value significance threshold less than 5.0×10^{-8} is set to call genome-wide significant associations. We considered SNPs with a p-value between 9.99×10^{-5} and 5×10^{-8} as variants showing a suggestive association with CDI susceptibility.

In order to develop a gene score that provides susceptibility for CDI, step-wise logistic regression analysis is followed using the β-slope of SNPs. Group (comparators vs CDI) was the dependent variable. Receiver Operator Characteristics curve of the gene score is used to discriminate between patients with CDI and comparators.

SECONDARY ENDPOINTS ANALYSIS

Second stage of the BEYOND project

- *Progression into organ dysfunction*

Comparisons are performed by the Fisher's exact test; OR and 95% CIs are calculated by Maentel-Henzel's statistics.

- *Time to organ dysfunction*

Comparisons are done by Cox regression analysis; the Hazard Ratio (HR) and 95% CIs are provided.

- *CDI relapse*

Comparisons are performed by the Fisher's exact test; OR and 95% CIs are calculated by Maentel-Henzel's statistics.

- *Survival by day 40*

Comparisons are performed by the Fisher's exact test; OR and 95% CIs are calculated by Maentel-Henzel's statistics.

- *Cost of hospital stay*

The cost of drugs, laboratory and radiology exams, accommodation and interventions are calculated in one itemized approach; means and standard errors (SE) are provided.

- *Validation of the prognostic performance of the BEYOND score*

The BEYOND score is validated among patients who fail screening because of negative score; patients with positive score not enrolled because the score is provided late; and enrolled patients randomized to placebo treatment. Comparisons are

performed by the Fisher's exact test; OR and 95% CIs are calculated by Maentel-Henzel's statistics.

EXPLORATORY ANALYSIS

- *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 for participants of the first stage of the project is compared to the abundances of participants in the second stage of the project. They are also compared between the patients with favorable and unfavorable outcome in each stage. Mann-Whitney U rank test is used for these comparisons.

- *Need for change of SoC for CDI the first 40 days from start of the study drug*

Comparison between the two arms of treatment is performed by the Fisher's exact test; 95% CIs are calculated by Maentel-Henzel's statistics.

- *Time to alive hospital discharge the first 40 days from start of the study drug*

Comparisons are done by Cox regression analysis; the Hazard Ratio (HR) and 95% CIs are provided.

SAFETY

The analysis will include all patients who received at least one dose of the study drug. This will be presented in Tabular format and it will contain all Treatment Emergent Adverse Events (TEAE) of both arms. The incidence of each serious TEAE and non-serious TEAE will be expressed as frequency with percentage. Comparisons are performed by the Fisher's exact test.

Statistical analyses are performed with the statistical package SPSS v.26 and with the R statistical software v. 4.3.2.