STATISTICAL ANALYSIS PLAN

Protocol SYNB1020-CP-002

A Randomized, Double-blind, Placebo-controlled Study to Assess the Safety, Tolerability, and Pharmacodynamics of SYNB1020 in Hepatic Insufficiency and Cirrhosis Patients

Protocol Number: (Version Date)	SYNB1020-CP-002
Name of Test Drug:	SYNB1020
Phase:	1b/2a
Methodology:	Randomized, Double-Blind, Placebo-Controlled
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Document Date:	01 April 2019
Document Version:	Version 2.0

SIGNATURE PAGE

Protocol Title:	A Randomized, Double-blind, Placebo-controlled Study to Assess the Safety, Tolerability, and Pharmacodynamics of SYNB1020 in Hepatic Insufficiency and Cirrhosis Patients
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Protocol Number:	SYNB1020-CP-002
Document Date/Version:	27 Nov 2018/Version 6

Cytel, Inc. Author:

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Signature:_____

Date:_____

Sponsor Approval

By signing this document, I acknowledge that I have read the document and approve of the planned statistical analyses described herein. I agree that the planned statistical analyses are appropriate for this study, are in accordance with the study objectives, and are consistent with the statistical methodology described in the protocol, clinical development plan, and all applicable regulatory guidances and guidelines.

I have discussed any questions I have regarding the contents of this document with the biostatistical author.

I also understand that any subsequent changes to the planned statistical analyses, as described herein, may have a regulatory impact and/or result in timeline adjustments. All changes to the planned analyses will be described in the clinical study report (CSR).

Sponsor Signatory:

[redacted]

Signature:_____

Date:_____

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ABBREVIATIONS

Abbreviation	Definition
%CV	Arithmetic percent coefficient of variation
AE	Adverse events
aPTT	Activated partial thromboplastin time
ATC	Anatomic Therapeutic Class
AUC	Area under the curve
AUC_{0-24}	Area under the concentration versus time curve from time 0 to the end of the dosing interval 24 hours later, calculated using linear trapezoid rule
BP	Blood pressure
BQL	Below the quantifiable limit
BSFS	Bristol stool form scale
BUN	Blood urea nitrogen
CBC	Complete blood count
CRF	Case report form
CRO	Contract research organization
CSR	Clinical study report
CTCAE	Common Terminology Criteria for Adverse Events
ECG	Electrocardiogram
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
HE	Hepatic Encephalopathy
IA	Interim Analysis
ICH	International Conference on Harmonisation
[redacted]	[redacted]
INR	International normalized ratio
IP	Investigational product
IRT	Interactive Response Technology
ITT	Intent-to-treat
LFT	Liver function test
LLOQ	Lower limit of quantification
MedDRA	Medical Dictionary for Regulatory Activities
MELD	Model for End-Stage Liver Disease
MK	Microbiotic-kinetic
NCI	National Cancer Institute
PD	Pharmacodynamic
[redacted]	[redacted]
PK	Pharmacokinetic
PPI	Proton pump inhibitor

Abbreviation	**Definition**
PT	Preferred Term (AEs), or prothrombin time (labs)
q_{max}	Maximum observed fecal SYNB1020 qPCR signal
qPCR	Quantitative polymerase chain reaction
QTcF	QT interval corrected for heart rate
SAE	Serious adverse event
SAF	Safety
SAP	Statistical analysis plan
SD	Standard deviation
SMC	Safety Monitoring Committee
SOC	System/Organ/Class
TEAE	Treatment-emergent adverse event
T_{lag}	Time to first detectable fecal SYNB1020
T_{last}	Time to last detectable fecal SYNB1020
T_{ss}	Time to steady-state
[redacted]	[redacted]
ULN	Upper limit of normal
WHO	World Health Organization

1. INTRODUCTION AND OBJECTIVES OF ANALYSIS

1.1. Introduction

This document presents the statistical analysis plan (SAP) for Synlogic, Protocol SYNB1020-CP-002: A Randomized, Double-blind, Placebo-controlled Study to Assess the Safety, Tolerability, and Pharmacodynamics of SYNB1020 in Hepatic Insufficiency and Cirrhosis Patients.

This SAP is based on the final protocol (version 6) dated 27 November 2018.

1.2. Objectives of Statistical Analysis

Primary Objective

- To evaluate the safety and tolerability of SYNB1020 following multiple doses in hepatic insufficiency and cirrhosis patients. The endpoints related to this objective include the nature and frequency of adverse events (AEs), measurement of vital signs and laboratory assessments (e.g., blood chemistry, complete blood count [CBC], liver function tests (LFTs), international normalized ratio [INR], and urinalysis), and electrocardiograms (ECGs)

Secondary Objectives

- To evaluate SYNB1020 kinetics in feces (measured with qualitative and quantitative polymerase chain reaction [qPCR] fecal assays) following dosing
- To evaluate change from baseline of venous ammonia, including fasting and 24-hour area under the curve (AUC), following SYNB1020 dosing in subjects with hyperammonemia

[redacted]

This SAP is designed to outline the methods to be used in the analysis of study data in order to answer the study objective(s). Populations for analysis, data handling rules, statistical methods, and formats for data presentation are provided. The statistical analyses and summary tabulations described in this SAP will provide the basis for the results sections of the clinical study report (CSR) for this trial.

This SAP also will outline any differences in the currently planned analytical objectives relative to those planned in the study protocol.

2. STUDY DESIGN

2.1. Synopsis of Study Design

SYNB1020-CP-002 is a Phase 1b/2a, randomized, double-blinded placebo-controlled study to evaluate the safety, tolerability, and PD of SYNB1020 in hepatic insufficiency and cirrhosis patients, with dosing of the investigational product (IP) administered in an inpatient unit and subsequent outpatient follow-up for SYNB1020 clearance. Part 1 comprises of a sentinel open-label cohort of 6 subjects with cirrhosis and Model for End-Stage Liver Disease (MELD) score <12 to establish the safety and tolerability of SYNB1020. Once the safety and tolerability have been established in Part 1, enrollment will be opened to subjects in Part 2, a randomized, double-blinded, placebo-controlled study in subjects with cirrhosis and hyperammonemia. Approximately 20 to 40 evaluable subjects are anticipated for enrollment in Part 2, with randomization proceeding in a 1:1 ratio to SYNB1020 and placebo.

In Part 1, eligible subjects will be admitted to an inpatient facility for a run-in diet, baseline assessments, IP administration, safety monitoring, and collection of blood, urine, and fecal samples for evaluation of safety, tolerability, and pharmacokinetic (PK) and PD evaluations.

In Part 2, subjects may be pre-screened for eligibility based on medical history and a single fasting spot venous ammonia measurement. Eligible subjects with elevated fasting spot venous ammonia will then undergo full screening within 7 days of pre-screening. Eligible subjects will be admitted to an inpatient facility for a run-in diet and 24-hour ammonia profile, and those with an elevated 24-hour ammonia AUC (>1.2 × upper limit of normal [ULN]) will proceed with randomization, IP administration, safety monitoring, and collection of blood, urine, and fecal samples for PK and PD evaluations.

Due to uncertainty in the assumptions of variability in AUC of ammonia relative to spot measurements in this population, Part 2 of the study may recruit up to a total of 40 subjects guided by the re-evaluation of the sample size based on the standard deviation (SD) of data observed at the IA.

2.2. Randomization Methodology

There is no randomization in Part 1 of the trial; Part 1 is open-label, single-treatment, single-sequence.

In Part 2, all eligible subjects will be randomized via the Interactive Response Technology (IRT) to one of the 2 treatment arms. The investigator will contact the IRT after confirming eligibility, and the IRT will assign a randomization number to each subject. The randomization number links the subject to the treatment arm and specifies a unique medication number for the first package of study drug. Eligible subjects will be randomized to SYNB1020 and placebo at a ratio of 1:1. Randomization will not be stratified.

2.3. Stopping Rules and Unblinding

There is no concern for unblinding in Part 1 of the trial.

Part 2 is a double-blind study. The Investigator, subject, clinical staff and Sponsor's study management team will be blinded to treatment assignments. The pharmacist at the clinical site or Investigator's designee will be responsible for dispensation of study drug according to the

randomization schedule provided by the Sponsor or designee. Study drug will be administered in a blinded fashion.

During Part 2, an unblinded interim analysis (IA) will occur once the first 16 subjects finish their inpatient visits in order to determine if the trial should be stopped for efficacy or futility, or if more subjects should be enrolled. Unblinded data and who will be unblinded prior to and for the IA is defined in a separate unblinding guidance document. In addition to the IA, the Safety Review Committee (comprised of the Lead Investigator, Medical Monitor, pharmacovigilance physician, and as needed an external expert) will meet at predetermined intervals and ad hoc, as needed, to conduct periodic interim reviews of safety. Further details are provided in a separate Safety Review Committee charter. The Safety Review Committee will also meet at the end of Part 1 to examine safety of the study drug.

The Sponsor may stop either part of this study at any time. Investigators will be notified by the Sponsor or its designee if the study is stopped. The occurrence of the following events will require that further enrollment in the study be stopped:

- Any suspected or proven invasive infection (e.g., sepsis or bacteremia) assessed as at least possibly related to the IP.
- Three or more subjects experience the same National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) grade 3 AE assessed as at least possibly related to study treatment, or two or more subjects experience the same CTCAE grade 4 AE assessed as at least possibly related to the IP.
- Two or more subjects experience serious AEs (SAEs) that are considered by the Investigator to be related to the IP.
- Death occurs at any time during the study and is considered by the Investigator to be related to the IP.
- Approximately 6% mortality is observed after 30 days following dosing. This is defined as ≥2 deaths observed in 24 subjects or ≥3 deaths observed in 40 subjects who have received the IP.
- The Investigator, Medical Monitor, or Sponsor determines that an event or current data warrant stopping the study.

The Sponsor's medical staff, the Contract Research Organization (CRO)'s pharmacovigilance physician and the Lead Investigator will review the data concerning these event(s), along with other available data. Based on the results of their investigation, the Sponsor's medical staff, the CRO's pharmacovigilance physician and the Lead Investigator will determine appropriate follow-up and decide whether the study should be stopped.

If the study is stopped, the events will be investigated, and the study will be closed to further enrollment. Subjects already participating in the study at the time the study is stopped will not receive further IP and will continue the follow-up schedule.

2.4. Study Procedures

The schedule of assessments, as outlined in the study protocol, are provided in Tables 1 and 2.

Table 1 Schedule of Assessments

Study Period	**Optional Pre-Screening**	**Screening**	**Inpatient Monitoring**										**Follow-up[a]**	
Study Day	**Within 7 days of Screening**	**-36 to -6**	**-5**	**-4**	**-3**	**-2**	**-1**	**1**	**2-4**	**5**	**6**	**7 EOS/Discharge Visit[b]**	**14 Follow-up Visit**	**Up to 70 days after last dose**
Informed consent for pre-screening	●													
Informed consent		●												
Medical history	●	●[c]	●											
Physical examination		●	●									●[d]	●[d]	
FibroScan						●								
Screening for drugs of abuse (urine drug screen)		●	●											
Screening for infectious disease (HIV/Hepatitis B, C serology)		●												
Laboratory tests (CBC, LFTs, blood chemistry, urinalysis, PT/aPTT)		●				●			●[e]		●	●		
FSH (in females if non-childbearing potential status is equivocal based on medical history)		●												
Record concomitant medications		●	●	●	●	●	●	●	●	●	●	●	●	
Adverse event reporting		●	●	●	●	●	●	●	●	●	●	●	●	
Vital signs[f]		●	●	●	●	●	●	●	●	●	●	●	●	
Electrocardiogram		●				●			●[e]			●		
Fasting spot venous ammonia	●	●[g]				●		●	●	●	●	●		
Admit to inpatient facility (in the morning after an overnight fast)			●											
Required inpatient stay			●	●	●	●	●	●	●	●	●	●		
Subject randomization (Part 2 only)							●							
Dietary control[h]			●	●	●	●	●	●	●	●	●	●		

Study Period	**Optional Pre-Screening**	**Screening**	**Inpatient Monitoring**										**Follow-up[a]**	
Study Day	**Within 7 days of Screening**	**-36 to -6**	**-5**	**-4**	**-3**	**-2**	**-1**	**1**	**2-4**	**5**	**6**	**7 EOS/Discharge Visit[b]**	**14 Follow-up Visit**	**Up to 70 days after last dose**
Administer H2 inhibitor (ranitidine 150 mg BID 30-60 minutes before breakfast and the evening snack)[i]			●	●	●	●	●	●	●	●	●			
Administer assigned IP (SYNB1020 or placebo, TID) with breakfast, lunch, and dinner[j]								●	●	●	●			
24-hour profile for venous ammonia[k]						●				●	●			
[redacted]						■					■			
[redacted]						■					■			
[redacted]						■					■			
[redacted]						■					■			
[redacted]						■					■			
Fecal SYNB1020 PCR[m]			●					●	●	●	●	●	●	●
[redacted]						■					■			
Hepatic encephalopathy grading (West Haven grade)						●					●			
Child-Turcotte-Pugh score		●				●					●			
Model for End-Stage Liver Disease score		●				●			● [e]		●			
BSFS for each bowel movement collected			●	●	●	●	●	●	●	●	●	●		
Plasma collection for exploratory analyses							●				●			
Urine collection for exploratory analyses							●				●			
Review of adverse event diary card														●

Abbreviations: aPTT = activated partial thromboplastin time; BID = twice daily; BSFS = Bristol stool form scale; CBC = complete blood count; EOS = End of Study; FSH = follicle-stimulating hormone; h = hour(s); H2 = histamine-2 receptor; HIV = human immunodeficiency virus; [redacted] IP = investigational product; LFT = liver function test; PCR = polymerase chain reaction; PPI = proton pump inhibitor; PT = prothrombin time; TID = 3 times daily; [redacted]

[a] One week (7± 1 days) after discharge from the inpatient unit, subjects will return for clinical evaluation and bring a 1-week post-dose fecal sample. Feces will continue to be collected weekly for the first 6 weeks following discharge and biweekly until a subject has a negative SYNB1020 fecal test for up to 10 weeks following completion of study dosing. Subjects who remain colonized with SYNB1020 after 10 weeks following the last dose will be treated as described in Section 7.5.2.
[b] At least 12 hours after completion of the dosing period (i.e., on Day 7 for subjects who continue IP dosing per protocol), an EOS/Discharge visit will be performed. For subjects who discontinue study dosing prematurely after having received at least one dose of SYNB1020/placebo, the EOS/Discharge Visit should be completed within 24 hours of discontinuation.
[c] If not obtained during pre-screening.

[d] Symptom-driven physical examinations will be performed on Days 7 and 14.
[e] Day 4 only.
[f] Vital signs will be collected every 8 hours during the dosing period (Days 1 through 6).
[g] Fasting venous ammonia will not be repeated at screening if measured at pre-screening.
[h] Meal times for breakfast, lunch, and dinner should be the same every day ± 30 minutes, unless otherwise specified.
[i] Subjects already on a PPI regimen will not be switched to ranitidine but will continue their prescribed PPI regimen for the duration of the study.
[j] In Part 1, all subjects receive open-label IP. In Part 2, subjects are randomized to either SYNB1020 or placebo. IP will be administered immediately following a meal.
[k] Samples for venous ammonia will be drawn relative to meal times according to Table 4. Following an overnight fast, subjects will have a baseline sample drawn for ammonia at 0h (before breakfast) and will be given a high-protein shake for breakfast, followed by IP administration on dosing days. Samples for venous ammonia will be drawn at +2h, +4h (before lunch), +6h, +9h (before dinner), +11h, +14h, +18h, and +24h the following morning (before breakfast). Two additional samples will be collected on Day 5 at +3h and +5h [redacted]

[m] One baseline fecal sample is required to be collected prior to SYNB1020 dosing (i.e., any time from Day -5 through Day 1). Feces will continue to be collected weekly for the first 6 weeks following discharge and biweekly (every other week) until a subject has a negative SYNB1020 fecal test for up to 10 weeks following completion of study dosing. Subjects who remain colonized with SYNB1020 after 10 weeks following the last dose will be treated with a 3-day course of oral ciprofloxacin, or other antibiotics. On Day 7, if no void has occurred by the time all other assessments have been completed, the subject may be discharged without a fecal sample.

Table 2 Schedule of Plasma Sampling for 24-Hour Ammonia [redacted]

Study Day **Activity**[a]	**Time of Day**										
Day -2	**0 hours**	**+2 hours**	**+3 hours**	**+4 hours**	**+5 hours**	**+6 hours**	**+9 hours**	**+11 hours**	**+14 hours**	**+18 hours**	**+24 hours**
Plasma draw for 24-hour ammonia	●	●		●		●	●	●	●	●	●
[redacted]	■	■		■		■					
Breakfast	●										
Lunch				●							
Dinner							●				
Day 5											
Plasma draw for 24-hour ammonia	●	●	●	●	●	●	●	●	●	●	●
[redacted]	■	■		■		■					
Breakfast	●										
Give IP	●			●			●				
Lunch				●							
Dinner							●				
Day 6											
Plasma draw for 24-hour ammonia	●	●		●		●	●	●	●	●	●
[redacted]	■	■		■		■					
Breakfast	●										
Give IP	●			●			●				

Study Day **Activity**[a]	**Time of Day**										
Day -2	**0 hours**	**+2 hours**	**+3 hours**	**+4 hours**	**+5 hours**	**+6 hours**	**+9 hours**	**+11 hours**	**+14 hours**	**+18 hours**	**+24 hours**
Plasma draw for 24-hour ammonia	●	●		●		●	●	●	●	●	●
[redacted]	▮	▮		▮		▮					
Lunch				●							
Dinner							●				

Abbreviations: IP = investigational product
[a] Plasma draws occur before meals and meals occur before IP administration, as applicable.

2.5. Safety, Pharmacokinetic, and Pharmacodynamic Variables

2.5.1. Primary Safety Endpoints

The primary endpoint variables will be the following: nature and frequency of AEs, measurement of vital signs and laboratory assessments (e.g., blood chemistry, CBC, LFTs, INR, and urinalysis) and ECGs. Safety assessments performed during the study include physical examinations, measurement of vital signs, a baseline FibroScan, 12-lead ECGs, clinical laboratory evaluations including hematology, serum chemistry, and urinalysis, monitoring of adverse events, blood and urine cultures/PCR, HE Severity Grading, [redacted] and cirrhosis severity grading.

2.5.2. Secondary Microbiotic-Kinetic and PD Endpoints

The secondary endpoints are the following: SYNB1020 kinetics in feces and change from baseline of venous ammonia, including fasting and 24-hour AUC, following SYNB1020 dosing.

In Part 1, a 24-hour fecal sample will be weighed, homogenized, and a sample analyzed for SYNB1020 by qPCR. In Part 2, a fecal sample from the first void of the day will be collected using a collection kit. The following variables will be calculated from the serial fecal sampling from each subject, if data support calculation: T_{lag}, T_{ss}, $q_{max,}$ T_{last} and half-life.

[redacted]

3. SUBJECT POPULATIONS

3.1. Population Definitions

The following subject populations will be evaluated and used for presentation and analysis of the data:

Safety Population Part 2 (SAF) and Safety Population Expanded (SAF2):	All subjects in Part 1 who received at least one dose of SYNB1020 and have at least one post-baseline safety assessment. All randomized subjects in Part 2 who received at least one dose of SYNB1020 or placebo and have at least one post-baseline safety assessment. Subjects will be included in the analysis according to the dose and actual treatment received. Expanded population includes both Parts 1 and 2.
Microbiotic-kinetic (MK) Population Part 2 and Microbiotic-kinetic (MK2) Population Expanded:	Subjects in SAF or SAF2 who completed the clinical study and were considered evaluable for analysis of the MK data. Subjects will be included in the analysis according to the dose and actual treatment received. Expanded population includes both Parts 1 and 2.
Pharmacodynamic (PD) Population Part 2 and Pharmacodynamic (PD2) Population Expanded (PDE):	Subjects in SAF or SAF2 who completed the clinical study and were considered evaluable for analysis of the PD data. Subjects will be included in the analysis according to the study part and actual treatment received. PDE population includes both Parts 1 and 2.

Data will be excluded from the MK/MK2 and/or PD/PD2 Populations in case of any major protocol deviation that may influence the MK and/or PD outcome.

The SAF2 Population will be the primary population for the analysis of all safety parameters for both Part 1 and Part 2. All MK/PD analyses will be performed based on the MK/PD Population for Part 2 and on the MK2/PD2 Populations for both Parts combined (where Part 1 and both Parts combined will be reviewed for sensitivity and Part 2 is the Part for reporting of the primary and secondary analyses).

For on-treatment assessments, subjects will be considered “evaluable for analysis of the PD data” if they received all doses during the ammonia AUC interval and for the preceding 2 days (i.e. 9 doses).

3.2. Protocol Violations

At the discretion of the sponsor, major protocol violations as determined by a review of the data prior to unblinding of the study results and the conduct of statistical analyses may result in the removal of a subject’s data from the MK/PD Populations. The Sponsor, or designee, will be responsible for producing the final protocol violation file (formatted as a Microsoft Excel file), in collaboration with the CRO (Cytel) and the data monitoring group as applicable; this file will include a description of the protocol violation, and clearly identify whether or not this violation

warrants exclusion from either the MK or PD Population. This file will be finalized prior to hard database lock.

Major protocol violations are defined as those deviations from the protocol that are likely to have an impact on the subject's rights, safety, well-being, and/or on the validity of the data for analysis regarding primary and secondary outcomes. The Biostatistician may consider some data to be invalid for analysis, which would be defined as a major deviation and would lead to exclusion of that subject from the relevant population.

All protocol violations will be presented in the data listings.

4. STATISTICAL METHODS

4.1. Sample Size Justification

Part 1: The sample size for the sentinel cohort (6 subjects) is primarily designed for empirical evaluation of safety and tolerability in subjects with cirrhosis.

Part 2: A sample of 16 subjects must complete all 3 ammonia AUC intervals (baseline, Day 5, and Day 6) to detect a 20% reduction in average daily ammonia ($AUC_{0\text{-}24}$/24, from a baseline of 70 µmol/L) with an approximate significance level of 10% and an approximate power of 90%. Significance and power are approximate based on the assumptions below. The number of subjects assumes that the SD of spot ammonia is 9 µmol/L, approximately half of the variance is between subject, and that the SD for average daily ammonia is as little as half that of spot ammonia. The spot ammonia SD is based on a weighted average of SD observed in a prior study of ammonia lowering in a similar population. The variability of AUC may be 50% lower than spot ammonia based on the FDA summary basis of approval for glycerol phenylbutyrate for urea cycle disorders.

4.2. General Statistical Methods and Data Handling

4.2.1. General Methods

All output will be incorporated into Microsoft Excel or Word files, sorted and labeled according to the International Conference on Harmonisation (ICH) recommendations, and formatted to the appropriate page size(s).

Tabulations will be produced for appropriate demographic, baseline, PD, microbiotic-kinetic and safety parameters. For categorical variables, summary tabulations of the number and percentage within each category (with a category for missing data) of the parameter will be presented. For continuous variables, the mean, standard deviation, arithmetic percent coefficient of variation (%CV), geometric mean (geo mean), geometric percent coefficient of variation (geo %CV), median, minimum and maximum values will be presented. For discrete numeric variables (e.g. T_{lag}), the median, minimum, and maximum will be presented.

Formal statistical hypothesis testing will be performed on all primary, secondary [redacted] endpoints with all tests conducted at the 1-sided, 0.10 level of significance. Summary statistics will be presented, as well as confidence intervals on selected parameters, as described in the sections below.

Baseline will be defined as the last scheduled measurement prior to the first IP administration (i.e., pre-dose). If a sequence of baseline measurements is taken pre-dose on the same day, time-matched baseline will be used. If multiple measurements are included within a baseline measurement (e.g., repeated measures at the same nominal time), the arithmetic mean of the multiple samples will be considered the baseline. Unscheduled measurements will only be used as baseline upon consultation with the Sponsor.

For each local laboratory where normal reference range of ammonia has not been locally adequately validated, the normal reference range of ammonia will be defined based on approximately 30 healthy volunteers devoid of any chronic or acute medical conditions and medications with normal liver enzymes. Ammonia sampling, sample processing and analysis will be performed as defined in the SYNB1020-CP-002 protocol and lab manual. The 95th percentile will be used as ULN. Outliers based on visual inspection may be excluded on a case-

by-case basis, and if any outliers are excluded, will be reported in the Changes to Planned Analysis section of the CSR with appropriate notes added to tables and figures where outliers have been excluded.

4.2.1.1. Handling of Values below the Quantifiable Limit (BQL)

Results for endogenous compounds below the quantifiable limit (BQL) will be set to ½ the lower limit of quantification (LLOQ).

Results for exogenous compounds (other than SYNB1020) BQL will be set to zero.

For SYNB1020 fecal copy number, BQL before the first quantifiable measurement will be set to 0 for calculations; BQL between two quantifiable measurements will be set to ½ LLOQ; and BQL after the last quantifiable measurement will be set to 0 for calculations. For semi-logarithmic plotting, SYNB1020 fecal copy number BQL will be set to ½ LLOQ for all measurements.

4.2.2. Computing Environment

All descriptive statistical analyses will be performed using SAS statistical software (Version 9.4) or R (Version 3.4.0 or greater), unless otherwise noted. Medical history and AEs will be coding using the Medical Dictionary for Regulatory Activities (MedDRA) version 20.1, and the severity of AEs and laboratory abnormalities will be graded using the NCI CTCAE, version 4.03. Concomitant medications will be coded using World Health Organization (WHO) Drug Version 10DEC01DDE.

4.2.3. Methods of Pooling Data

Not applicable to the present study.

4.2.4. Adjustments for Covariates

No formal statistical analysis that adjusts for possible covariate effects is planned.

4.2.5. Multiple Comparisons/Multiplicity

Multiplicity will be addressed via the Haybittle-Peto stopping boundary method for group sequential designs.

First, the IA will be evaluated against the efficacy p-value of 0.01.

The overall type I error will be fixed at alpha = 0.10, allowing for unequal p-value boundaries at the two looks (IA and final). Further details will be provided in Section 4.6.

4.2.6. Subpopulations

The following subpopulations will be explored for the primary and key secondary endpoints, provided the sample size is adequate for analysis:

- Ammonia level: subjects with ammonia > 1.5 times ULN versus subjects with ammonia ≤ 1.5 times ULN
- Proton pump inhibitor (PPI) use: subjects already on PPI versus subjects given ranitidine
- Hepatitis status: subjects with a history of Hepatitis B/C versus subjects without a history of Hepatitis B/C

If subgroup analyses for the primary endpoint indicate important subgroup-by-treatment interactions, then further exploratory analyses on subgroups and other endpoints may be taken.

4.2.7. Withdrawals, Dropouts, Loss to Follow-up

Subjects who withdrew from the study may be replaced as defined in section 10.5 of the Protocol (Determination of Sample Size).

4.2.8. Missing, Unused, and Spurious Data

In general, there will be no substitutions made to accommodate missing data points. All data recorded on the case report forms (CRFs) will be included in data listings that will accompany the CSR.

If the start date of an AE is partially or completely missing, the date will be compared as far as possible with the date of the start of administration of study drug. The AE will be assumed to be treatment-emergent if it cannot be definitively shown that the AE did not occur or worsen during the treatment-emergent period (worst case approach).

The following general rules will be used:

- If the start date is complete, an AE will only be excluded as being treatment-emergent if the start date is before the date of first study drug administration or if the stop date is before first study drug administration.
- If the start day is missing but the start month and year are complete, an AE will only be excluded as being treatment-emergent if the start month/year is before the month/year of first study drug administration or if the stop month/year is before first study drug administration.
- If the start day and month are missing but the start year is complete, an AE will only be excluded as being treatment-emergent if the start year is before the year of first study drug administration or if the stop year is before the year of first study drug administration.

If the start date is completely missing, an AE will be considered treatment-emergent unless the stop date is before first study drug administration.

4.2.9. Visit Windows

Screening procedures must be performed to determine eligibility for enrollment in both Part 1 and Part 2 within 30 days prior to admission to the inpatient facility.

During inpatient monitoring, visit windows are not a concern.

The protocol-specified visit window for the End of Study (EOS)/Discharge Visit to occur is at least 12 hours after completion of the last dose (Day 7); subjects who withdraw early should have this visit within 24 hours of discontinuation of the study drug. The first follow-up visit is specified in the protocol as occurring 7 ± 1 day after the EOS Visit, and subjects will be followed for up to 10 weeks after this first follow-up visit occurs. For continuation of follow-up visits, subjects will be required to provide weekly fecal samples (7 ± 1 day after the follow-up visit) for the first 6 weeks following discharge, and bi-weekly (14 ± 1 day) until the subject has

a negative SYNB1020 fecal test (up to 10 weeks after completion of study). However, all data will be analyzed regardless of visit windows.

Table 1 Visit Windows

Evaluation	Protocol-Specified Interval
Screening	Day -36 to Day -6
Part 1 Inpatient	Day -5 to Day 7
Part 2 Inpatient	Day -5 to Day 7
EOS/Discharge	Day 7 (12 hours post-dose)
Follow-Up	Day 13 to Day 15

4.3. Interim Analyses

After all N=6 subjects are discharged from the clinic following Part 1, safety data will be examined as an informal review (not a formal IA).

A formal, unblinded IA will be performed on the PD2 population (Part 2 only) after at least 16 subjects have completed dosing in Part 2 and have been discharged from the unit, while enrollment may continue, for a possible final sample size of N=20 (N_{CP}). N_{CP} may be adjusted based on actual enrollment just before the IA decision but will not be less than 20.

It is assumed that baseline average daily ammonia is 70 µmol/L, and the placebo group will show a 0 – 10% reduction from baseline, while the desired reduction for the treatment group is 20% versus placebo. Furthermore, the assumed SD of the difference in the means (desired reduction) is 9 µmol/L. Additional assumptions were examined, and the operating characteristics at the IA for these more pessimistic possibilities are shown in Section 6.4.

If the p-value at the IA is < 0.028 the trial could be stopped for efficacy. Otherwise, conditional power (CP) will be computed at the IA for N=16 subjects. This is the probability that the trial will yield a statistically significant reduction in AUC of average daily ammonia change from baseline greater for the treatment group at the end of the trial if the observed difference at the IA is the TRUE underlying difference and the trial is continued to completion with a total of N_{CP} subjects. If this probability is less than 30%, the Safety Monitoring Committee (SMC) could suggest that the trial be stopped for futility. Otherwise, if the CP is less than 90% the sample size will be boosted to target a conditional power of 90%, up to a maximum of 40 patients. If the CP is greater than 90% and the p-value at the IA is greater than 0.028, the trial will end with N_{CP} subjects.

Conditional power for an active dose versus placebo is derived as follows:

$$C_p(\hat{\theta}) = 1 - \Phi\left\{\frac{c_2 - B(t)/t}{\sqrt{1-t}}\right\}$$

Parameter	Description
$\Phi\{x\}$	cumulative distribution function for a $N(0,1)$ random variable
c_2	Adjusted critical cut-off for the final analysis. Assuming that alpha of 0.028 is spent at the interim analysis for one-sided testing, that the total one-sided alpha

	level is 0.05, and that only one IA occurs at the planned information fraction of 0.80 (=16/20), the adjusted critical cut-off is $c_2 = -1.904$.
t	$= {}^{n}/_{N}$, where N is the *planned* total sample size for the treatment arm and for the placebo arm (N=20), and n is the sample size of the IA (16 total).
$B(t)$	B value, calculated as $Z_n\sqrt{t}$, where Z_n is the Z statistic associated with the linear regression model of observed data on the treatment group versus placebo group at the IA

Because the total number of subjects could potentially be increased in a data-dependent manner, the final analysis does not use the conventional statistic to determine if statistical significance is reached. Instead it uses the weighted statistic proposed by Cui, Hung, and Wang (Cui et al., 1999) (referred to as the "CHW approach") in which the independent increments of the Z statistic of the 2 stages are combined by pre-specified weights that are based on the planned proportion of total number of subjects at which the IA would be taken if there were no change in the design. In the present case, the study was designed for 30 subjects with an interim analysis when 24 subjects complete the study. Upon further consideration, the interim analysis will be performed when 16 subjects complete the study with a planned total sample size of 20 without adaptation. Details on the operating characteristics can be found in Section 6. Therefore, the planned proportion n is 0.80 (16/20) for stage 1 and 0.2 (4/20) for stage 2 (these values will be adjusted for the actual N and N_{CP} at the IA), and the independent incremental Z statistics for the 2 stages are combined with weights that equal $w_1 = \sqrt{0.80}$ for stage 1 and $w_2 = \sqrt{0.20}$ for stage 2. These weights will remain fixed. They will not be modified based on any changes to the final sample size. The final test statistic under the CHW approach is

$$Z_{CHW} = w_1 Z_1 + w_2 Z_2$$

Where $Z_1$is the Z statistic calculated using the IA PD2 population data (i.e., stage 1 data)and Z_2 is the Z statistic calculated using PD2 population data from all remaining subjects not included in IA (i.e., stage 2 data). . Statistical significance is achieved at the final analysis if $Z_{CHW} < c_2$.

The model used for the IA will be a log-scale, linear mixed model with repeated measures with categorical effects for treatment, time (as a categorical variable), and treatment by time and a random effect by subject on the intercept where time will be coded as baseline for pre-dose and treated for both post-dose assessments:

$$\log(AUC_{i,t}) = \beta_t \times \beta_{treatment} + \beta_t + \beta_{treatment} + \eta_i + \epsilon$$

where:

- $AUC_{i,t}$ is the ammonia AUC for subject i at time t,
- βt is the effect of time (baseline or on-treatment),
- $\beta_{treatment}$ is the effect of treatment (placebo or active),
- η_i is the sample from a normal distribution of the random effect of subject i, and
- ε is the log-scale, residual variability.

4.4. Subject Disposition

A tabulation of subject disposition will be created, including the number screened, the number dosed with placebo, the number dosed with SYNB1020, the number in each subject population for analysis, the number that withdrew prior to completing the study, and reasons for withdrawal.

A by-subject listing of study completion information, including the reason for premature study withdrawal, if applicable, will be presented.

4.5. Demographic and Baseline Characteristics

Baseline, demographic and medical history information will be summarized for the SAF and SAF2 Populations using descriptive statistics. No formal statistical comparisons will be performed.

Demographic, baseline data and medical history will be provided in data listings.

Exposure to study drug will be shown in a by-subject listing for the SAF and SAF2 populations.

The demographic, baseline data and medical history data described above will be shown for Part 1 alone, Part 2 (by treatment), overall by treatment (Parts 1 and 2), and study overall (Parts 1 and 2 – all treatments) using descriptive statistics.

4.6. Safety Analyses

Safety analyses will be conducted using the SAF/SAF2 Populations.

4.6.1. Adverse Events

AEs will be coded using MedDRA and severity will be evaluated using the NCI CTCAE version 4.03. These will be displayed in tables and listings using System/Organ/Class (SOC) and Preferred Term (PT). AEs will also be presented by maximum severity.

AEs will be recorded from the time a subject signs informed consent through the safety follow-up period. Analyses will focus on any AE recorded, as well as treatment-emergent AEs (TEAEs), which are those events that have occurred after the start of the study drug.

AEs are summarized by subject incidence rates, therefore, in some tabulations, a subject contributes only once to the count for a given AE (SOC or PT). Several tabulations also show the total number of events, where a subject can contribute more than once.

The number and percentage of subjects, along with total number of events, with any AE, any TEAE, any TEAE assessed by the Investigator as related to treatment (definite, probable, or possible relationship), any AE resulting in discontinuation of study dosing and with any SAE will be summarized by treatment group and overall. In these tabulations, each subject will contribute only once (i.e., the most related occurrence or the most intense occurrence) to each of the incidence rates in the descriptive analysis, regardless of the number of episodes.

Tabulations will also be presented by MedDRA SOC and PT for the following: all TEAEs, related TEAEs, TEAEs by severity and TEAEs leading to permanent study drug withdrawal.

No formal hypothesis-testing analysis of AE incidence rates will be performed.

All AEs occurring on study will be listed in subject data listings.

By-subject listings also will be provided for the following: subject deaths; SAEs; AEs leading to withdrawal and Grade 3 or 4 AEs, and narratives will be shown for these.

4.6.2. Laboratory Data

Clinical laboratory values will be expressed using SI units and will be measured during screening, on Day -2 (Baseline), Day 4, Day 6 and Day 7.

The following will be performed:

- Alkaline phosphatase, total bilirubin, direct bilirubin, CO2, chloride, glucose, potassium, AST, ALT, sodium, albumin, total protein, phosphorous, calcium, activated partial thromboplastin time (aPTT), international normalized ratio (INR), prothrombin time (PT), CBC with differential, C-reactive protein, follicle-stimulating hormone (FSH, for women), blood urea nitrogen (BUN) and creatinine
- Urinalysis

The actual value and change from Baseline to each on study evaluation will be summarized for each clinical laboratory parameter. In the event of repeat values, the last non-missing value per study day/time will be used. Hematology, chemistry and urinalysis will also be displayed by the number and percent of subjects with normal, abnormal and clinically significant abnormal in shift from baseline tables at each visit.

All laboratory data will also be provided in data listings. Clinical significance and abnormality will be indicated within these listings.

4.6.3. Vital Signs and Physical Examinations

Semi-supine vital signs will be collected during screening, every 8 hours during the dosing period (Day -5 – Day 6), Day 7 and Day 14.

The following will be collected: systolic blood pressure (BP), diastolic BP, pulse and body temperature. Subjects are required to remain in the semi-supine position for at least 5 minutes prior to obtaining vital signs.

The actual value and time-matched change from Baseline (or Screening) to each on study evaluation will be summarized for vital signs. Vitals will also be displayed by the number and percent of subjects with normal, abnormal and clinically significant abnormal.

By-subject listings of vital sign measurements will be presented in data listings.

Physical examinations will be performed during screening and Day -5; and symptom-driven on Day 7 and Day 14.

Any abnormal physical examination findings will be recorded as AEs and included in AE outputs.

4.6.4. Electrocardiogram

A semi-supine single 12-lead ECG will be performed during screening, Day -2 (Baseline), Day 4 and Day 7.

The following will be collected: RR, QT, QRS and PR intervals. Fridericia's formula should be used to calculate the QT interval corrected for heart rate (QTcF).

The actual value and change from Baseline (or Screening) to each on study evaluation will be summarized for ECGs.

ECG results will also be summarized descriptively, including the number and percent of subjects with normal, abnormal and clinically significant abnormal results at Baseline and each study visit.

All ECG data for each subject will be provided in data listings.

4.6.5. FibroScan

A FibroScan (vibration-controlled transient elastography) will be performed once at Day -2 (Baseline) to quantify liver fibrosis.

FibroScan results for each subject will be provided in data listings.

4.6.6. Blood and Urine Cultures/Polymerase Chain Reaction

Blood and urine cultures and/or PCR will be performed as needed according to standard clinical practice if clinical presentation warrants. This may include, but is not limited to, cases in which subjects develop persistent (> 24 hours) fever, chills, night sweats, or other symptoms suggestive of local or systemic clinical infection.

4.6.7. Hepatic Encephalopathy Severity Grading

HE severity grading will be collected via the West Haven Grading System on Day -2 (Baseline) and Day 6.

HE grades will be summarized descriptively, including the number and percent of subjects at each grade on Day -2 (Baseline) and Day 6.

All HE data for each subject will be provided in data listings.

[redacted]

4.6.9. Cirrhosis Severity Grading

Severity of cirrhosis will be assessed by the Child-Turcotte-Pugh score, which reflects a composite score incorporating HE grade, extent of ascites, and bilirubin, albumin, and INR values and MELD score calculated based on bilirubin, creatinine, and INR values. This will be collected during screening, Day -2 (Baseline) and Day 6.

Cirrhosis severity results will be summarized descriptively for each class at every time point it was collected. The composite Child-Turcotte-Pugh and MELD scores will also be summarized by their actual score and change from Baseline to each on study evaluation.

All cirrhosis severity data for each subject will be provided in data listings.

4.6.10. Concomitant Medications

Concomitant medications will be coded using the WHO Drug Dictionary. Results will be tabulated by Anatomic Therapeutic Class (ATC) and preferred term.

The use of concomitant medications will be included in by-subject data listing.

4.7. Pharmacodynamic Evaluations

PD analyses will be conducted using the PD Population. Urine and blood samples will be collected at screening and on study both before and after administration of SYNB1020 or placebo for PD measurements.

All PD parameters of SYNB1020 will be summarized in tables, listings, and figures (Section 6); no formal statistical analysis will be performed, with the exception of venous ammonia AUC and fasting spot venous ammonia. Additional post-hoc exploratory PD analyses of interest may be carried out, in addition to those described below.

4.7.1. Venous Ammonia Area under the Curve

Samples for venous ammonia will be drawn relative to meal times. In order to analyze $AUC_{0\text{-}24}$, subjects must complete all 3 ammonia AUC intervals (Baseline, Day 5 and Day 6).

To calculate the AUC over 24 hours ($AUC_{0\text{-}24}$), the linear trapezoidal method will be utilized, with the following formula:

$$AUC_{0-24} = \sum \frac{(t_i - t_{i-1})(A_i + A_{i-1})}{2}$$

Symbol	Definition
$\sum$	Summation over all times, *i*
t_i	Time point, in hours
t_{i-1}	Previous time point, in hours
A_i	Venous ammonia measurement at time *i*
A_{i-1}	Venous ammonia measurement at previous time point, *i-1*

Once $AUC_{0\text{-}24}$ is calculated, this will be divided by 24 to obtain the average daily ammonia.

Statistical analysis will be performed on log-transformed venous ammonia $AUC_{0\text{-}24}$ as a mixed-model with repeated measures with baseline as a covariate, a fixed effect by treatment (Day 5 and 6 treated as repeated measures on treatment, not differentiated by day), and a random effect by subject. Results will be transformed back to the linear scale for reporting; at the Sponsor's option, results may be normalized to the average (or weighted-average based on number of subjects) upper limit of normal across the labs used in the study.

A bar chart will be generated illustrating the mean change from baseline (average of Day 5 and Day 6 minus baseline) in $AUC_{0\text{-}24}$ for ammonia on the y-axis, bar fill color will indicate active (black) or placebo (white), and subjects across all treatment groups will be sorted in order from

greatest mean change from baseline (left) to least mean change from baseline (right). The bar chart will be generated twice, once for the PD population in Part 2 and once for the PD population in the full study (Part 1 and Part 2). (For clarity, this representation is sometimes referred to as a "waterfall plot".)

4.7.2. Fasting Spot Venous Ammonia

In addition to tables, listings, and figures for descriptive statistics (Section 6), statistical analysis will be performed on log-transformed fasting spot venous ammonia (defined as the ammonia measurement during the day) to assess change from baseline and change from placebo. The statistical model for fasting spot venous ammonia will be a mixed-model with repeated measures with baseline as a covariate, fixed effects by day and treatment, and a random effect by subject. Results will be transformed back to the linear scale for reporting; at the Sponsor's option, results may be normalized to the average (or weighted-average based on number of subjects) upper limit of normal across the labs used in the study.

[redacted]

4.8. Microbiotic-Kinetic Evaluations

MK analyses will be conducted using the MK Population. Additional post-hoc exploratory MK analyses of interest may be carried out, aside from what is described below.

MK variables will be calculated from the serial fecal sampling from each subject. One baseline fecal sample is required to be collected prior to SYNB1020 dosing (i.e., any time from Day -5 through Day 1) and daily following dosing through Day 7. Feces will continue to be collected weekly for the first 6 weeks following discharge and every other week until a subject has a negative SYNB1020 fecal test for up to 10 weeks following completion of study dosing.

The variables to be determined and the time points of determination are summarized below:

Table 2 Microbiotic-Kinetic Parameters for Analysis

Parameter	Description
T_{lag}	The time of the first detectable fecal SYNB1020
T_{ss}	The time to steady-state
q_{max}	The maximum observed fecal SYNB1020 qPCR signal
T_{last}	The time of the last detectable fecal SYNB1020
Half-life	The time for the fecal SYNB1020 PCR signal to decline by 50%

A sample will be considered negative for the purposes of SYNB1020 clearance if it is BQL, and therefore SYNB1020 clearance is the same as T_{last}.

Missing values will not be imputed, and if sufficient data are missing for a given subject, that subject may be considered non-evaluable for MK analysis and would not be included in the MK Population.

All MK parameters of SYNB1020 will be summarized in tables and in listings; no formal statistical analysis will be performed.

5. CHANGES TO PLANNED ANALYSES

In Section 10.3 of version 4.0 of the protocol, it is specified that a fecal sample will be considered negative for the purposes of SYNB1020 clearance if it is below the quantifiable limit (BQL) or estimated to be below <0.1% of the administered dose. This is clarified in Section 4.8 of the SAP: a sample will only be considered negative for the purposes of SYNB1020 clearance if it is BQL. Additionally, this is described in as note to file.

In Section 10.1 of version 4.0 of the protocol, the populations for analysis are described without detail of the differences in populations for Part 1 and Part 2. This is expanded upon in Section 3.1 of the SAP.

Protocol version 6 amended the number of subjects required for of the interim analysis from what was planned in the original protocol, as described in Section 10.5.

6. CLINICAL STUDY REPORT APPENDICES

6.1. Statistical Tables to be Generated

Table Number	Description	At Interim Analysis
Table 14.1.1.1	Subject Enrollment and Disposition (SAF)	
Table 14.1.1.2	Subject Demographic and Baseline Characteristics (SAF)	X
Table 14.1.1.3	Medical History (SAF)	X
Table 14.2.1.1	Overview of Adverse Events and Treatment-Emergent Adverse Events (SAF)	X
Table 14.2.1.2	Summary of Treatment-Emergent Adverse Events by System Organ Class, Preferred Term and Treatment Group (SAF)	X
Table 14.2.1.3	Summary of Treatment-Emergent Adverse Events by System Organ Class, Preferred Term, Severity and Treatment Group (SAF)	X
Table 14.2.1.4	Summary of Related Treatment-Emergent Adverse Events by System Organ Class, Preferred Term and Treatment Group (SAF)	X
Table 14.2.1.5	Summary of Treatment-Emergent Adverse Events Leading to Study Drug Withdrawal by System Organ Class, Preferred Term and Treatment Group (SAF)	X
Table 14.2.2.1	Hematology Results: Change from Baseline (SAF)	
Table 14.2.2.2.x	Hematology: Shifts from Baseline by Visit (SAF)	
Table 14.2.2.3	Chemistry Results: Change from Baseline (SAF)	
Table 14.2.2.4.x	Chemistry: Shifts from Baseline by Visit (SAF)	
Table 14.2.2.5	Urinalysis Results: Change from Baseline (SAF)	
Table 14.2.2.6.x	Urinalysis: Shifts from Baseline by Visit (SAF)	
Table 14.2.2.7	Vital Signs: Change from Baseline (SAF)	
Table 14.2.2.8.x	Vital Signs: Shifts from Baseline by Visit (SAF)	
Table 14.2.2.9	Electrocardiogram Results: Change from Baseline (SAF)	
Table 14.2.3.0.x	Electrocardiogram: Shifts from Baseline by Visit (SAF)	
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[redacted]	[redacted]	[redacted]
Table 14.2.3.7	Severity of Cirrhosis: Child-Turcotte-Pugh Score and Components (SAF)	X
Table 14.2.3.8	Severity of Cirrhosis: Model for End Stage Liver Disease (MELD) Score and Components (SAF)	X
Table 14.3.1.1	Average Area Under the Curve of Venous Ammonia over 24 Hours, Change from Baseline and Change from Baseline/Change from Placebo (PD)	X
Table 14.3.1.2	Daily Fasting Spot Venous Ammonia Change from Baseline and Change from Baseline/Change from Placebo (PD)	X
Table 14.4.1.1	Microbiotic-Kinetic Parameters from Fecal Sampling (MK)	

6.2. Data Listings to be Generated

Listing Number	Description	At Interim Analysis
Listing 16.1.1.1	Subject Enrollment and Disposition (All Screened)	X
Listing 16.1.1.2	Demographic and Baseline Characteristics (SAF)	X
Listing 16.1.1.3	Subject Medical History (SAF)	
Listing 16.1.1.4	Exposure to Study Drug (SAF)	X
Listing 16.1.1.5	Concomitant Medications (SAF)	
Listing 16.1.1.6	Inclusion/Exclusion Criteria Not Met (All Screened)	
Listing 16.1.1.7	Protocol Deviations (All Subjects)	X
Listing 16.2.1.1	Adverse Events (SAF)	X
Listing 16.2.1.2	Serious Adverse Events (SAF)	X
Listing 16.2.1.3	Treatment-Emergent Adverse Events Leading to Study Drug Withdrawal (SAF)	X
Listing 16.2.1.4	Adverse Events of Grade 3 or 4 (SAF)	X
Listing 16.2.1.5	Deaths (SAF)	X
Listing 16.2.2.1	Hematology Results (SAF)	
Listing 16.2.2.2	Chemistry Results (SAF)	
Listing 16.2.2.3	Urinalysis Results (SAF)	
Listing 16.2.2.4	Vital Signs (SAF)	
Listing 16.2.2.5	Electrocardiogram Results (SAF)	
Listing 16.2.2.6	FibroScan Results (SAF)	
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Listing 16.2.3.7	Hepatic Encephalopathy Severity Grading (SAF)	X
Listing 16.2.3.8	Child-Turcotte-Pugh Score and Components (SAF)	X
Listing 16.2.3.9	Model for End Stage Liver Disease and Components (SAF)	X
Listing 16.3.1.1	Venous Ammonia 24-Hour Profile (PD)	X
Listing 16.3.1.2	Daily Fasting Spot Venous Ammonia (PD)	X
Listing 16.3.1.3	Venous Ammonia Area Under the Curve over 24 Hours (PD)	X
Listing 16.4.1.1	Bristol Stool Scale Assessment (MK)	
Listing 16.4.1.2	Fecal Sampling and qPCR Results (MK)	

6.3. Figures to be Generated

Figure Number	Description	At Interim Analysis
Figure 15.1.1.x	Venous Ammonia: Time Profiles by Subject (PD)	X
Figure 15.1.2.1	Venous Ammonia Levels: Time Profiles in Part 2 (PD)	X
Figure 15.1.2.2	Venous Ammonia Levels: Time Profiles in Parts 1 and 2 (PD)	X
Figure 15.1.2.3	Venous Ammonia Levels: Change from Baseline by Time and Treatment in Part 2 (PD)	X
Figure 15.1.2.4	Venous Ammonia Levels: Change from Baseline by Time and Treatment in Parts 1 and 2 (PD)	X
Figure 15.1.2.5	Fasting Venous Ammonia by Patient and Treatment	X
Figure 15.1.2.6	Fasting Venous Ammonia Levels in Part 2	X
Figure 15.1.2.7	Fasting Venous Ammonia Levels in Parts 1 and 2	X
Figure 15.1.3.1	Venous Ammonia Levels: Mean Change from Baseline in AUC_{0-24} in Part 2 (PD)	X
Figure 15.1.3.2	Venous Ammonia Levels: Mean Change from Baseline in AUC_{0-24} in Parts 1 and 2 (PD)	X

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6.4. Operating Characteristics of the Interim Analysis

An IA will be carried out at N=12 (with the study ongoing), with a possible sample size re-estimation to a maximum of N=40 subjects, or if no sample size re-estimation, a final sample size of N=16.

The study design was simulated 10,000 times under the assumption that the true AUC of average daily ammonia change from baseline to Day 5/6 shows a 20% reduction (-0.22 on the log scale) in the treatment group versus the control group.

Based on previous data, the standard deviation of average daily ammonia was estimated to possibly be as small as 50% of that of spot ammonia (~13 µmol/L on the log scale). For a more conservative estimate, we assumed that the standard deviation could also be as high as that of spot ammonia (~26 µmol/L on the log scale). Additionally, measurements for healthy volunteers in protocol SYNB1020-CP-001 showed that the total standard deviation was 25 µmol/L and the standard deviation between and within subject was ~20 µmol/L and 15 µmol/L, respectively (64% of the total variation). Thus, a more pessimistic standard deviation from this data will be examined as 29 µmol/L (64% of 36 µmol/L), with a less pessimistic standard deviation as 20 µmol/L.

Analysis of Stage 1 ammonia data indicated a within-subject, log-scale residual standard deviation of 0.1324 from a baseline $AUC_{0\text{-}24}/24$ of 64.8 µmol/L (approximately 9 µmol/L).

For an IA at N=16, with overall type I error (alpha) set at 0.10 and efficacy p-value set at 0.028, conditional power (CP) will be calculated to drive the decision about whether to increase the sample size. The following boundaries were set: futility boundary at CP=0.30 and efficacy boundary at < 0.028 (one-sided). If the trial is not stopped for efficacy or futility, then if CP≥90%, the trial will continue to completion with a total of 20 subjects, and if CP <90%, the sample size will be increased

to target a CP of 90% up to a maximum sample size 40. The operating characteristics of the design can be seen in the below table:

	Pessimistic SD #1 SD=26 $\mu mol/L$	Pessimistic SD #2 SD=29 $\mu mol/L$	Healthy Volunteer SD SD=20 $\mu mol/L$	Stage 1 Observations SD=9 µmol/L (log-scale SD = 0.1324)
Probability to stop for futility at N=16	41.91%	47.19%	25.07%	0.13%
Probability to stop for efficacy at N=16	36.62%	33.03%	55.32%	99.12%
Probability of increasing beyond 20 $20 < N \leq 40$	21.47%	19.78%	19.61%	0.75%
Overall power	54.73%	48.77%	73.48%	99.87%