synlogic

STATISTICAL ANALYSIS PLAN

synpheny
LIVING IN HARMONY WITH PKU

A Phase 3, Double-blind, Placebo-controlled, Randomized Withdrawal Study to Evaluate the Efficacy and Safety of SYNB1934 in Patients with PKU (SYNPHENY-3)

SYNB1934-CP-003

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
AE	adverse event
AraC	arabinose-responsive transcriptional activator
ADLs	activities of daily living
ANCOVA	analysis of covariance
BID	twice per day
CBC	complete blood count
CI	confidence interval
CSR	clinical study report
CTCAE	Common Terminology Criteria for Adverse Events
DAP	diaminopimelate
DEP	dose-escalating, open-label period
DMC	data monitoring committee
ECG	electrocardiogram
EcN	escherichia coli Nissle 1917
eCRF	electronic case report form
FAS	full analysis set
FDA	Food and Drug Administration
GI	gastrointestinal
GPV	genotypic phenotype values
ICH	International Conference on Harmonisation
IMP	investigational medicinal product
IPTG	isopropyl ß-D-1-thiogalactopyranoside
iTD	individually titrated dose
IXRS	interactive response system
LAAD	L-amino acid deaminase
MAR	Missing at Random
MCMC	Markov chain Monte Carlo
MI	multiple imputation
MMRM	mixed-model with repeated measures
MNAR	missing not at Random
mPAL	modified phenylalanine ammonia lyase
NCI	National Cancer Institute
OLE	open-label extension
PAL	phenylalanine ammonia lyase

Phe	phenylalanine
pks	polyketide synthase
PP	phenylpyruvate
PPI	proton pump inhibitor
PPS	per protocol analysis set
PT	preferred term
QD	once per day
RWP	randomized withdrawal period
SAE	serious adverse event
SAS	safety analysis set
SD	standard deviation
SOC	system organ class
TCA	trans-cinnamic acid
TEAE	treatment emergent adverse events
TID	three times per day
Tyr	tyrosine
US	United States

1 INTRODUCTION

SYNB1934 was derived from *Escherichia coli* Nissle 1917 (EcN) in a series of genetic manipulations designed to allow enhanced degradation of Phe within the human gut. The degradation of Phe is carried out by the activity of engineered genes encoding phenylalanine ammonia lyase (PAL), which catalyzes the conversion of Phe to *trans*-cinnamic acid (TCA), and L-amino acid deaminase (LAAD), which catalyzes the conversion of Phe to phenylpyruvate (PP). The deletion of the *pks* island resulted in a modified strain of SYNB1934v1, which is referred to as SYNB1934v1.

Study SYNB1934-CP-003 is a 3-part, Phase 2b/3 study consisting of a dose-escalating, open-label period (DEP; Part 1) of up to 15 weeks, followed by a 4-week, double-blind, placebo-controlled, randomized withdrawal period (RWP; Part 2), and an open-label extension (OLE; Part 3) of up to 36 months.

This Statistical Analysis Plan (SAP) describes data-handling and statistical procedures to be used for the analysis and reporting of efficacy and safety data collected in this Phase 3 study, SYNB1934-CP-003 (protocol version 4.0 01JUL2023), and to be presented in the clinical study report (CSR). The methods are based on those presented in Section 8 of the study protocol. Note that there are multiple country-specific version 4.0 protocols. A notable difference between the United States (US) protocol v4.0 and the non-US v4.0 protocols is that, in the non-US protocols, participants 12 to 17 years of age may be enrolled after the first 20 participants have completed the DEP, with approval of the DMC. Any post-hoc or exploratory analyses not specified in this SAP will be identified as such when they are presented in the CSR.

This SAP was written in accordance with the recommendations outlined in the International Conference on Harmonisation (ICH) E9 Guideline entitled “Guidance for Industry: Statistical Principles for Clinical Trials” and the ICH-E3 Guideline, entitled “Guidance for Industry: Structure and Content of Clinical Study Reports.”

2 STUDY SUMMARY

2.1 STUDY OBJECTIVES AND ENDPOINTS

This study has 3 parts, and each part has a distinct set of objectives, endpoints, and analysis populations. The determination of the primary evidence of efficacy will be the comparison of SYNB1934v1 versus placebo in the randomized withdrawal period (Part 2).

Objectives and endpoints are presented in Table 1 to Table 3 for the respective parts of the study.

Table 1: Objectives and Endpoints: Part 1, Dose Escalating, Open-Label Period (DEP; Part 1)

Objective	Endpoint
Primary	
• To assess the percentage change in blood phenylalanine (Phe) level	• Percent change from baseline in blood Phe level at last measurement of the individually titrated dose (iTD) of SYNB1934v1
Key Secondary:	
• To assess the absolute change in Phe level	• Change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1
• To determine the responder population	• A ≥ 20% reduction from baseline in blood Phe level at any time in the DEP
Other Secondary:	
• To assess proportion of participants achieving blood Phe level ≤ 360 µmol/L	• Blood Phe level ≤ 360 µmol/L at any time of the iTD of SYNB1934v1 • Blood Phe level ≤ 360 µmol/L at any time in the DEP
• To assess the safety and tolerability of SYNB1934v1	• Incidence and severity of adverse events • Changes from baseline in clinical laboratory parameters • Changes from baseline in vital signs measurements
Exploratory:	
• [redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

Note: baseline and week numbering are specific to study Part 1, unless explicitly noted otherwise in the endpoint.

Table 2: Objectives and Endpoints: Part 2, Randomized Withdrawal (RWP; Part 2)

Objective	Endpoint

Primary: To evaluate efficacy of SYNB1934v1 versus placebo in the responder population	• Change from baseline to Week 4 in blood Phe level
Key Secondary:	
• To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to percent change from DEP baseline in blood Phe level	• Percent change from DEP baseline in blood Phe level at Week 4
• To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to change from DEP baseline in blood Phe level	• Change from DEP baseline to Week 4 in blood Phe level
• To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to the proportion of participants with a blood Phe level ≤ 360 µmol/L	• Blood Phe level ≤ 360 µmol/L at Week 4
Other Secondary Objectives:	
• To assess the safety and tolerability of SYNB1934v1	• Incidence and severity of adverse events • Changes from baseline in clinical laboratory parameters • Change from baseline in vital signs measurements
• To evaluate efficacy of SYNB1934v1 versus placebo in the responder population with regard to the proportion of participants with a ≥ 20% reduction from DEP baseline in blood Phe level at Week 4	• A ≥ 20% reduction from DEP baseline in blood Phe level at Week 4
Exploratory:	
• [redacted]	[redacted]
[redacted]	[redacted]

• [redacted]	[redacted]

Note: baseline and week numbering are specific to study Part 2, unless explicitly noted otherwise in the endpoint.

Table 3: Objectives and Endpoints: Part 3, Open-label Extension (OLE; Part 3)

Objective	Endpoint
• To assess the safety and tolerability of SYNB1934v1	• Incidence and severity of adverse events • Changes from DEP baseline in clinical laboratory parameters • Changes from DEP baseline in vital signs measurements
• [redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

Note: baseline and week numbering are specific to study Part 3, unless explicitly noted otherwise in the endpoint.

2.2 STUDY DESIGN

This is a 3-part, Phase 3 study consisting of a dose-escalating, open-label period (DEP; Part 1) of up to 15 weeks, followed by a 4-week, double-blind, placebo-controlled, randomized withdrawal period (RWP; Part 2), and an open-label extension (OLE; Part 3) of up to 36 months. Figure 1 presents a study schematic.

In the DEP, all enrolled participants will maintain a stable diet reflecting their baseline Phe intake and receive escalating doses of SYNB1934v1 from approximately 3 to 15 weeks to determine an iTD, which is defined as the highest dose the participant is able to tolerate. A participant will be defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated.

Blood Phe level will be measured at each dose level after 3 weeks at that level. A responder will be defined as a participant who achieves a ≥ 20% reduction in blood Phe level compared to DEP baseline on their iTD. For inclusion in the RWP primary efficacy analysis population, response will be assessed at the third week of a participant's established iTD for SYNB1934v1.

If at any time during a 3-week dosing interval in the DEP the participant or investigator considers a dose level to be intolerable, the dose may be de-escalated after discussion with the medical monitor. In this case, the participant will restart 3 weeks at the lower level iTD. After de-escalation, participants cannot escalate to a higher dose again during.

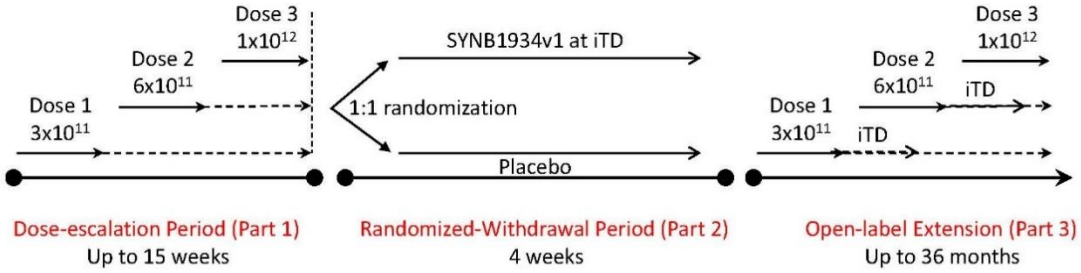
The DEP will contain an optional substudy (DEP weekly substudy) to evaluate the range and variability of [redacted] at each dose level. The [redacted] participants in this substudy will have weekly Phe and Tyr value assessments during the DEP (i.e., with Tyr assessments being primarily assessed for safety).

Once the first [redacted] participants have attained an iTD and completed the DEP or the [redacted] has a baseline DEP and has been in the study for [redacted], whichever occurs earlier, the DEP data will be evaluated by an independent Data Monitoring Committee (DMC). The DMC will use a pre-specified set of criteria based on both safety and efficacy to determine whether the dose regimen is appropriate or whether any doses should be discontinued as well as whether the study should be stopped for futility. Enrollment will not be paused during the interim analysis.

Participants who complete at least 3 weeks at their iTD during the DEP will enter a 4-week RWP. Participants will be randomized 1:1 to receive SYNB1934v1 at their iTD determined in the DEP or placebo TID. Randomization will be stratified on screening Phe level. Blood Phe level will be measured at Weeks 1, 3, and 4 of the RWP. Participants will remain on the same diet they consumed during the DEP.

Participants who complete the 4-week RWP may enter the OLE and receive SYNB1934v1 for up to 36 months at their iTD. (At the sponsor's discretion, enrollment of new participants directly into OLE may be opened.) The iTD in Parts 1 and 3 can differ. Participants will be allowed to modify their standard diet if their blood Phe level is < 240 µmol/L, as determined by either local or central laboratory testing, with guidance from the investigator as outlined in the study-specific Diet Manual. The 3-day dietary intake assessments will be conducted per the Schedule of Events

Figure 1: Study SYNB1934-CP-003 Schematic



2.2.1 Number of Participants

This study plans to enroll approximately [redacted] participants. More participants may be enrolled to achieve [redacted] randomized in Part 2 (RWP).

2.2.2 Randomization and Blinding Procedures

In the RWP, participants will be randomized in a 1:1 ratio to receive either SYNB1934v1 or matching placebo, stratified on screening Phe level ([redacted] Participants, investigators, and the sponsor will be blinded to randomized IMP assignment during the RWP. In the case of an emergency where information regarding treatment assignment would impact the care provided to a participant during the RWP of the study, the investigator will have immediate access to unblind the treatment code in the interactive response system (IXRS). The instructions for unblinding a participant in the IXRS can be found in the IXRS User Guide. In the event unblinding is necessary, the investigator is strongly encouraged to contact the medical monitor to discuss the situation and the participants medical status prior to unblinding. It is mandatory that any personnel involved in the unblinding or who have access to the unblinded treatment assignment maintain the confidentiality of the information.

During the DEP and RWP a participant's blood Phe levels will be blinded to the participant, investigator and sponsor. During the DEP and RWP, the investigator will receive an email alert from the central laboratory if a blood Phe is > 1.5 × the DEP baseline, if Phe is < 30 μmol/L, or if Tyr is < 20 μmol/L. After the receipt of one of these trigger values, the investigator should arrange for a retest of Phe and Tyr. If the trigger value is confirmed to be out of range, the individual participant will be discontinued from the study.

The DEP overall responder rates and DEP baseline Phe values as well as the group-level DEP interim analysis results will be provided to the sponsor by the DMC.

Both the CRO's blinded and unblinded teams will have access to the participant-level blood-Phe levels.

2.2.2.1 De-Escalation in the DEP After Randomization

When a participant in the DEP study completes the 2nd week of their 3-week iTD dosing period, the participant's randomization into the RWP will occur (i.e., the RWP randomization occurs prior to RWP baseline to ensure the participant is provided with the appropriate study medication). If, subsequent to RWP randomization, the participant needs to de-escalate to a lower dose, the participant will not enter the RWP, but instead either discontinue the study or enter the OLE portion of the study. The participant, site, and sponsor will remain blinded to the participant's randomized RWP study medication.

2.2.3 Unblinding after the RWP

Once all participants have completed the RWP, the DEP and RWP parts of the study will be locked, unblinded, and analyzed.

2.3 Efficacy Assessments

The following are the list of efficacy/pharmacodynamic assessments:

2.3.1 Blood Phenylalanine and Tyrosine

Blood Phe and Tyr samples will be drawn 2.5 to 5 hours after the last meal. Phe and Tyr samples will be drawn in duplicate, using separate tubes from the same venipuncture, and analyzed separately. The time of the last meal will be recorded when taking Phe samples.

Baseline samples for DEP and RWP must be drawn prior to the first dose for those respective periods. During the DEP, blood Phe and Tyr will be measured at Week 3 of each completed dose level. During the OLE, blood Phe and Tyr will be measured quarterly. Blood Phe and Tyr will also be measured at early termination.

For all Tyr summaries the average value will be used, with the individual duplicate values being presented in the listings.

The individual duplicate blood Phe values will be used in some analyses, and the average of the duplicate values used for other analyses and summaries (see Section 3.3).

2.3.2 DEP Weekly Substudy

Participants will have the option to enroll in a substudy to evaluate [redacted]. Incentives will be provided to ensure that at least twenty participants are recruited into this substudy. Participants in this substudy will have [redacted] assessments during the DEP. These weekly values may be performed during a 3-day window around the [redacted] These samples will be drawn 2.5 to 5 hours after the last meal. P[redacted] Note: for 3 consecutive days prior to scheduled study visits and blood Phe draws, all participants will have a 3-day dietary intake assessment (see Section 3.10.2).

2.4 Safety Assessments

2.4.1 Adverse Events

Adverse events will be assessed continuously by direct observation and participant interviews. For GI AEs, participants will also fill out a diary card during screening, DEP, and RWP to allow for enhanced capture of these AEs. The severity of AEs will generally be evaluated using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), v5.0, with exceptions described in Table 9. All AEs occurring from the time a participant signs the ICF through the follow-up period will be recorded, regardless of causal assessment to IMP.

2.4.2 Vital Signs, Weight, Height, and Electrocardiograms

Semi-supine vital signs (systolic blood pressure, diastolic blood pressure, pulse, and body temperature), weight, and height will be collected as specified in the Schedule of Events Table 11 and Table 12). Participants are required to remain in the semi-supine position for at least 5 minutes prior to obtaining vital signs. Body mass index (kg/meters^2) (=weight/height^2) will be calculated based on weight and height as specified in the Schedule of Events (i.e., at the Screening visit).

2.4.3 Clinical Laboratory Measurements

Clinical safety laboratory tests will be performed at the time points specified in in the Schedule of Events and Table 11 and Table 12): chemistry panel, CBC with differential, and creatinine clearance. A pregnancy test for women of childbearing potential and a follicle stimulating hormone test for postmenopausal women will be performed at the time points specified in the Schedule of Events. See Appendix 2 for a list of clinical laboratory tests.

Screening results will be assessed by the investigator for inclusion of participants in the study. Additionally, unscheduled clinical laboratory tests may be obtained at any time during the study at the investigator's discretion. The diagnosis corresponding to any clinically significant abnormality or abnormality requiring treatment/intervention must be recorded as an AE.

2.5 Study Estimands

The following are the descriptions of the estimands for the primary and key secondary endpoints in the study, based on the methods described in ICH-E9 (R1).

2.5.1 Part 1: Primary and Key Secondary Efficacy Objective Estimands

The estimands corresponding to the Part 1 DEP primary and key secondary objectives and endpoints are described in Table 4 and Table 5, respectively.

Table 4: Estimand for the Part 1 Primary Objective

Objective	To assess the percentage change in blood Phe level
A. Population	The population is the DEP full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Continuous variable: Percent change from DEP baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1

C. Intercurrent events	• Participant iTD dose is dropped: As described in Section 2.2, the participant's iTD will be the highest dose tolerated. Additionally, as described in Section 3.17, doses may be dropped at the DEP interim analysis. A treatment-policy strategy approach will be taken in that the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • Treatment discontinuation: If a participant permanently discontinues study drug in the DEP, the participant will be asked to have an early termination visit 30 days after last dose of study drug (see Section 5.6 of the study protocol). Treatment discontinuation will follow the treatment-policy approach in that if the participant has reached an iTD (defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated) the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • A Participant does not reach an iTD: A participant will be defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated. A participant who does reach an iTD will not have post-baseline Week 3 blood Phe level assessment. A principal stratum approach will be taken in that the target population of interest is the set of participants who reach an iTD. • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The population-level summary is the least-squares mean for the percent change from baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1 derived from an MMRM using an observed case approach. The MMRM model is described in Section 3.14.1.2.

DEP = dose-escalating, open-label period; iTD = individually titrated dose; MMRM = mixed model with repeated measures; Phe = phenylalanine.

Table 5: Estimands for the Part 1 Key Secondary Objectives

Objective	To assess the absolute change in Phe level
A. Population	The population is the DEP full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Continuous variable: Change from DEP baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1

C. Intercurrent events	• Participant iTD dose is dropped: As described in Section 2.2, the participant's iTD will be the highest dose tolerated. Additionally, as described in Section 3.17, doses may be dropped at the DEP interim analysis. A treatment-policy strategy approach will be taken in that the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • Treatment discontinuation: If a participant permanently discontinues study drug in the DEP, the participant will be asked to have an early termination visit 30 days after last dose of study drug (see Section 5.6 of the study protocol). Treatment discontinuation will follow the treatment-policy approach in that if the participant has reached an iTD (defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated) the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • A Participant does not reach an iTD: A participant will be defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated. A participant who does reach an iTD will not have post-baseline Week 3 blood Phe level assessment. A principal stratum approach will be taken in that the target population of interest is the set of participants who reach an iTD. • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The population-level summary is the least-squares mean for the change from baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1 derived from an MMRM using an observed case approach. The MMRM model is described in Section 3.14.1.2.
Objective	To determine the responder population
A. Population	The population is the DEP full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Dichotomous variable: A ≥ 20% reduction from baseline in blood Phe level at any time in the DEP

C. Intercurrent events	• Participant iTD dose is dropped: As described in Section 2.2, the participant's iTD will be the highest dose tolerated. Additionally, as described in Section 3.17, doses may be dropped at the DEP interim analysis. A treatment-policy strategy approach will be taken in that the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • Treatment discontinuation: If a participant permanently discontinues study drug in the DEP, the participant will be asked to have an early termination visit 30 days after last dose of study drug (see Section 5.6 of the study protocol). Treatment discontinuation will follow the treatment-policy approach in that if the participant has reached an iTD (defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated) the participants DEP blood Phe levels on a dropped dose will be included in the DEP primary efficacy analysis. • A Participant does not reach an iTD: A participant will be defined as having reached an iTD if they tolerate 3 weeks at a dose, regardless of whether other doses are tolerated. A participant who does reach an iTD will not have post-baseline Week 3 blood Phe level assessment. A principal stratum approach will be taken in that the target population of interest is the set of participants who reach an iTD. • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The proportion of participants meeting the ≥ 20% reduction from baseline in blood Phe level at any time in the DEP will be summarized along with [redacted] confidence intervals.

DEP = dose-escalating, open-label period; iTD = individually titrated dose; MMRM = mixed model with repeated measures; Phe = phenylalanine.

2.5.2 Part 2: Primary and Key Secondary Efficacy Objectives Estimands

The estimands corresponding to the Part 2 RWP primary and key secondary objectives and endpoints are described in Table 6 and Table 7, respectively.

Table 6: Estimand for the RWP Part 2 Primary Objective

Objective	To evaluate efficacy of SYNB1934v1 versus placebo in the responder population
A. Population	The population is the RWP responder full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Continuous variable: Change from RWP baseline to Week 4 in blood Phe level

C. Intercurrent events	• Treatment discontinuation: consistent with a treatment-policy strategy, treatment discontinuation will not be considered (i.e., all observed data will be used regardless of treatment discontinuation). • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The population-level summary is the least-squares means and difference in the change from baseline to Week 4 in blood Phe levels between the SYNB1934v1 and placebo treatment groups. An MMRM using an observed case approach is being applied. The post-baseline blood Phe levels at Weeks 1, 3, and 4 will be used. The MMRM model is described in Section 3.14.2.1.

MMRM = mixed model with repeated measures; Phe = phenylalanine; RWP = randomized withdrawal period.

Table 7: Estimands for the RWP Part 2 Key Secondary Objectives

Objective	To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to the percent change from DEP baseline in blood Phe level
A. Population	The population is the RWP responder full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Continuous variable: Percent change from DEP baseline to Week 4 in blood Phe level.
C. Intercurrent events	• Treatment discontinuation: consistent with a treatment-policy strategy, treatment discontinuation will not be considered (i.e., all observed data will be used regardless of treatment discontinuation). • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The population-level summary is the least-squares means and difference in percent change from DEP baseline to Week 4 in blood Phe levels between the SYNB1934v1 and placebo treatment groups. An MMRM using an observed case approach is being applied. The post-baseline blood Phe levels at Weeks 1, 3, and 4 will be used. The MMRM model is described in Section 3.14.2.4.
Objective	To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to the change from DEP baseline in blood Phe level
A. Population	The population is the RWP responder full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Continuous variable: Change from DEP baseline to Week 4 in blood Phe level.
C. Intercurrent events	• Treatment discontinuation: consistent with a treatment-policy strategy, treatment discontinuation will not be considered (i.e., all observed data will be used regardless of treatment discontinuation). • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered. (i.e., all observed data will be used regardless of antibiotic use) • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	The population-level summary is the least-squares means and difference in the change from DEP baseline to Week 4 in blood Phe levels between the SYNB1934v1 and placebo treatment groups. An MMRM using an observed case approach is being

	applied. The post-baseline blood Phe levels at Weeks 1, 3, and 4 will be used. The MMRM model is described in Section 3.14.2.4.
Objective	To evaluate the efficacy of SYNB1934v1 versus placebo in the responder population with regard to the proportion of participants with a blood Phe level ≤ 360 µmol/L
A. Population	The population is the RWP responder full analysis set (defined in Section 3.5) characterized by the inclusion/exclusion criteria provided in Section 4.2 of the study protocol.
B. Variable	Dichotomous variable: blood Phe level ≤ 360 µmol/mL at Week 4
C. Intercurrent events	• Treatment discontinuation: consistent with a treatment-policy strategy, treatment discontinuation will not be considered (i.e., all observed data will be used regardless of treatment discontinuation). • Antibiotic use: consistent with a treatment-policy strategy, antibiotic use will not be considered (i.e., all observed data will be used regardless of antibiotic use). • Prohibited medication use: consistent with a treatment-policy strategy, prohibited medication use will not be considered (i.e., all observed data will be used regardless of prohibited medication use).
D. Population-level summary	Difference in proportions between the SYNB1934v1 and placebo treatment groups. The analysis method is described in Section 3.14.2.4.

DEP = dose-escalating, open-label period; MMRM = mixed model with repeated measures; Phe = phenylalanine; RWP = randomized withdrawal period.

3 STATISTICAL METHODS

3.1 General Methods

3.1.1 Computing Environment

All statistical analyses will be performed using SAS® Version 9. 4 or higher.

3.1.2 Reporting of Numerical Values

All clinical study data will be presented in data listings. Continuous variables will be summarized using the number of observations, mean, standard deviation (SD), median, minimum, and maximum. Categorical and ordinal variables will be summarized using frequency counts and percentages. Confidence intervals (CIs) will also be provided where appropriate.

Means, medians, and CIs will be reported to one decimal place more than the data reported on the electronic case report form (eCRF) or by the laboratory/vendor; standard deviations (SD) to two more places. Minimum and maximum will be reported to the same number of decimal places displayed on the eCRF or by the laboratory/vendor. P-values will be two-sided and reported to 4 decimal places.

Proportions will be presented as percentages.

3.2 Baseline Value and Change from Baseline

Unless otherwise explicitly defined otherwise, the baseline value is defined as the most recent non-missing value obtained immediately prior to administration of first dose.

- Change from baseline will be calculated by subtracting the baseline value from the post-dose assessment for each participant (i.e., post-dose – baseline).
- Percent change from baseline will be calculated by multiplying the change from baseline by 100 and dividing by the baseline value (i.e., =100 × change from baseline /baseline)

3.3 Blood Phe Values at Baseline and Post-Baseline Visits

Baseline for blood Phe level in each of the 3 parts of the study will be defined as the mean of the duplicate blood Phe level measurements at the first visit of that part. If only one blood Phe level measurement is available, then that measure will be used as baseline. If the measurement at the first visit is missing, the most recent non-missing blood Phe levels from the central lab to the visit will be used (i.e., blood Phe levels from the local labs will not be used for analyses but can be used as for the inclusion criterion).

As defined in the schedule of events (Table 11and Table 12), the Part 2 RWP baseline is the End of the DEP visit, and the OLE baseline is the Part 2 RWP Week 4 visit.

Similarly, for the determination of responders and participants who achieve a specific reduction in Phe (e.g., Phe level ≤ 360 µmol/L at any time in the DEP, or a ≥ 20% reduction from baseline), and in situations where a single-Phe value is needed for a for a particular visit (e.g., summary statistics over time), the mean of the duplicate blood Phe level measurements at the applicable post-baseline timepoint (e.g., Week 3) will be used. If only one of the duplicate blood Phe levels is available at a particular post-baseline timepoint, then that measurement will be used. Unless otherwise specified in the SAP, only the MMRM analyses of blood Phe will use the duplicate blood Phe assessments, all other summaries will use the average of the duplicate blood Phe measurements.

3.4 Handling of Missing/Incomplete Values

Unless otherwise specified, missing data will not be imputed; the analyses will be performed on the non-missing values (i.e., this will be referred to as observed-case).

If a non-numeric clinical laboratory value is reported for an endpoint of the form "< X.XX", "≤ X.XX", ">X.XX", or "≥X.XX" and the endpoint is being summarized as a numeric quantitatively (i.e., assuming the endpoint response is numeric) the numeric value portion of the result being reported will be imputed. For example, an endpoint non-numeric values of "<2.00" and ">100.00" may be reported; in these instances, the values of 2.00 and 100.00, respectively, will be imputed.

3.4.1 Missing Dates

The imputation of missing date components (i.e., missing day, month, or year values) for start/stop date of adverse events and prior/concomitant medications are described in the corresponding section where the date is discussed.

3.4.2 Missing Data for Efficacy Analyses

The methods for the handling of missing data for the efficacy analyses are described in the corresponding section where the analysis is discussed.

3.4.3 Calculations for the DEP Responder

As discussed in Section 2.2, a responder is a defined as a participant who achieves a ≥ 20% reduction in blood Phe level compared to DEP baseline. The following are additional details regarding the definition of a responder:

- A participant will be considered a responder if they met the ≥ 20% reduction in blood Phe level at any time in the DEP.
- This will include the scheduled Week 3 blood Phe assessments at the end of each 3-week dosing period.
- This will not include assessments that were made specific to the participants included in the DEP Weekly substudy (Section 2.3.2).

3.5 Analysis Populations

Analysis populations will be defined for each of the 3 parts of the study (DEP, RWP, and OLE).

Full analysis set (FAS): The full analysis sets are based on the intention-to-treat principle. The DEP FAS includes all participants enrolled in the DEP who take at least one dose of DEP study medication.

The RWP FAS is defined as all participants randomized into the RWP who take at least one dose of RWP study medication. Additionally:

- The responder RWP FAS is defined as participants in the RWP FAS who are responders
- The nonresponder RWP FAS is defined as participants in the RWP FAS who are nonresponders.

A responder is defined as a participant who achieves a ≥ 20% reduction in blood Phe level compared to DEP baseline on SYNB1934v1.

The responder RWP FAS will be the primary efficacy analysis population for the RWP. Analyses on the RWP FAS will be performed according to the randomized treatment group.

In the event that, based on the interim analysis results utilizing the criteria provided in Table 10, the DMC recommends dropping either the 1×10^{12} or the 1×10^{12} and 6×10^{11} doses the following will occur:

- The participants in the DEP who have an iTD corresponding to a dropped dose will go directly into the OLE part of the study (i.e., as opposed to being randomized into the RWP).
- The RWP FAS, responder RWP FAS, and nonresponder RWP FAS, will exclude all participants whose iTD dose level was dropped.

These participants who are excluded from the RWP FAS will be summarized separately.

Safety analysis set (SAS): For each of the 3 study parts, the SAS will include all participants who received any amount of IMP in the corresponding study part with treatment assignment based on the treatment received.

For the RWP the safety analyses will be presented for the RWP SAS overall as well as by responder/nonresponder subgroups. Similarly, the OLE safety analyses will be presented for the OLE SAS overall as well as by responder/nonresponder subgroups.

A participant will be considered receiving treatment if the eCRF question "did the patient receive any study drug doses" is answered "Yes" and there is a corresponding non-missing date.

Per-protocol analysis set (PPS): The DEP PPS is defined as all DEP FAS participants, who had no major protocol deviations that would affect efficacy, completed their final 3-week iTD, whose dietary Phe remained within 20% of their DEP baseline value (see Section 3.10.2), and who did not require systemic antibiotic treatment during DEP (see Section 3.12). The DEP PPS will be used as a sensitivity analysis for the DEP primary efficacy endpoint.

The responder RWP PPS is defined as all responder RWP FAS participants, who had no major protocol deviations that would affect efficacy, had at least 75% treatment compliance (see Section 3.13.1), whose dietary Phe remained within 20% of their DEP baseline value (see Section 3.10.2), and who did not require systemic antibiotic treatment during DEP or RWP (see Section 3.12).

The responder RWP PPS will be used as a sensitivity analysis for the RWP primary efficacy endpoint. RWP major protocol deviations will be determined in a blinded manner prior to the RWP database lock. Analyses on the responder RWP PPS will be performed according to the randomized treatment group.

Following the protocol deviations handling plan, major protocol deviations that would affect efficacy will be determined by a review of the functional team members (i.e., the set of functional team members will be defined in the protocol deviations handling plan), which will be blinded for the RWP.

RWP Randomized analysis set: The RWP Randomized analysis set is defined as all participants randomized into the RWP. The responder Randomized analysis set is defined as participants in the Randomized analysis set who are responders. The nonresponder Randomized analysis set is defined as participants in the Randomized analysis set who are nonresponders.

3.5.1 Subgroups

For selected efficacy and safety analyses (see Section 3.14 and Section 3.15, respectively) the summaries will be performed overall and by responder/nonresponder subgroups (i.e., as defined in Section 3.5).

The DEP will contain an optional substudy (DEP weekly substudy) to evaluate the range and variability of Phe and Tyr response at each dose level (see Section 2.3.2). The twenty participants in this substudy will have weekly Phe and Tyr value assessments during the DEP. The weekly Phe and Tyr assessments will be summarized for this subgroup.

The following additional subgroups will also be considered:

- Adolescents: Note that, for non-US sites, participants 12 to 17 years of age may be enrolled after the first 20 participants have completed the DEP, with approval of the DMC.
- Participants on sapropterin dihydrochloride (Kuvan) at the DEP baseline visit (i.e., DEP study day 1).
- Genotypic phenotype values (GPV) subgroups: For the subset of participants who have non-missing results for allele #1 and allele #2 (i.e., from the Genotyping eCRF page), their GPV will be calculated. The GPV will be calculated by entering the allele information into the following website http://www.biopku.org/biopku/search-allele-start.asp. The GPV is a number from 0 to 10; the following 3 subgroups will be considered based on the GPV score: 0-2.7, 2.8-6.6, 6.7-10.0.
- Participants in the DEP who had an iTD of a dose that was discontinued (i.e., this subgroup will only be utilized if a dose is discontinued as per the DMCs recommendation as defined in Section 3.17).
- Participants in the RWP who were excluded from the RWP FAS (i.e., this subgroup will only be utilized if a dose is discontinued as per the DMCs recommendation as defined in Section 3.17).

The analyses to be performed on these subgroups will be discussed in the corresponding efficacy and safety sections.

3.6 Participants Disposition and Evaluability

3.6.1 Participant Disposition

The following participant disposition is summarized using the analysis populations defined in Section 3.5.

- The number of participants who entered the Screening Period (i.e., as defined by signing informed consent)
- The number and percent of participants rescreened (using the number screened as the denominator).

The following percentages will use the DEP FAS as the denominator:

- The number of participants enrolled in DEP (i.e., defined as being assigned study drug)
- The number and percent of participants completing/not completing the DEP (i.e., completed the DEP is defined as having completed the End of DEP/RWP Baseline Visit as indicated in the eCRF)
- The number and percent of participants who achieve an iTD
- The number and percent of responders.

The following percentages will use the RWP FAS as the denominator:

- The number of participants randomized into the RWP overall and by responder/nonresponder status
- The number and percent of participants completing/not completing the RWP (i.e., completed the RWP is defined as having a non-missing RWP Week 4 visit) overall and by responder/nonresponder.

The following percentages will use the OLE SAS as the denominator:

- The number of participants enrolled in OLE (i.e., defined as being assigned OLE study drug)
- The number and percent of participants completing the OLE/not completing the OLE (i.e., completing the study as determined by the eCRF question “Did the subject complete the study per protocol?”).

Note: The number and percent of participants who did not complete each of the 3 study parts will be summarized along with the reason:

  - LOST TO FOLLOW-UP
  - PROTOCOL DEVIATION
  - ADVERSE EVENT
  - LOW LEVELS OF PHE OR TYR OR PROFOUND ELEVATION IN PHE
  - PREGNANCY
  - STUDY TERMINATED BY SPONSOR
  - PHYSICIAN DECISION
  - WITHDRAWAL BY SUBJECT
  - NON-COMPLIANCE WITH STUDY DRUG
  - DEATH
  - OTHER.

The summaries will be overall for Part 1; overall, responder status, and treatment group (i.e., active or placebo) for Part 2; and overall and responder status for Part 3.

3.6.2 Protocol Deviations

The protocol deviations will be shown in a participant listing, by study part and treatment group (for the RWP).

For the RWP, the determination of major protocol deviations and major protocol deviations that would impact efficacy (i.e., a criterion for the RWP PPS as defined in Section 3.5) will be performed in a blinded manner.

3.7 Demographics

Demographics will be summarized for the following analysis Populations:

- DEP: FAS and SAS
- RWP: FAS, SAS, PPS
- OLE: SAS.

For each analysis population the summary will be overall and by treatment group for the RWP (i.e., SYNB1934v1 and Placebo). For the RWP, results will also be presented by responder/nonresponder.

Descriptive statistics for continuous variables will be provided for age (at time of informed consent), height, weight, and BMI. Frequency counts and percentages will be tabulated for sex, fertility status (for females), race, and ethnicity.

3.8 Baseline Characteristics

Baseline characteristics will be summarized for the DEP FAS, DEP SAS, RWP FAS, RWP SAS, and responder RWP FAS analysis populations.

The following information will be summarized for the DEP populations:

- Screening Phe
- DEP Baseline Phe
- DEP Baseline Phe intake
- DEP Baseline tyrosine (Tyr)
- Sapropterin dihydrochloride (Kuvan) at DEP Baseline (Yes/No)
- Current Proton pump inhibitor (PPI) Use at DEP Screening (Yes/No).

The following information will be summarized for the RWP populations:

- Screening Phe
- DEP Baseline Phe
- DEP Baseline Phe intake
- RWP Baseline Phe
- DEP Baseline tyrosine (Tyr)
- RWP Baseline tyrosine (Tyr)
- Sapropterin dihydrochloride (Kuvan) at DEP Baseline (Yes/No)

- Current Proton pump inhibitor (PPI) Use at DEP Screening (Yes/No).

3.9 Medical History

Medical history will be summarized for the DEP SAS. Medical history will be coded using MedDRA version 25.1 (or higher). The coded Preferred Terms (PT) will be summarized overall for the DEP SAS and, for the RWP SAS, by responder/nonresponder within each treatment group, and overall. A participant will be counted only once for each preferred term. The summary will present the results alphabetically by System Organ Class (SOC) and, within SOC, by decreasing frequency for the PT.

3.10 Prior and Concomitant Medications/Procedures

Prior and concomitant medications will be coded using WHO Drug (Version: B3 Global September 2022)

The incidence of prior medication will be presented by therapeutic class, and preferred (generic) drug name for the DEP SAS.

For the DEP and OLE study periods, the incidence of concomitant medication will be presented by therapeutic class, and preferred (generic) drug name for the corresponding SAS. A participant is counted only once for each therapeutic class and for each preferred drug name.

A participant is counted only once for each therapeutic class and for each preferred drug name.

Prior medications are defined as those taken and stopped prior to the date of first study treatment in the DEP. Any medication given at least once on or after the DEP first study treatment date will be defined as a concomitant medication including those which were started before first study treatment date and continued after first study treatment date.

Where a medication start date is partially or fully missing, and it is unclear as to whether the medication is prior or concomitant, it will be assumed that it is a concomitant medication.

Note that PPI and H2 blocker use will be summarized separately (see Section 3.10.1)

3.10.1 PPI and H2 Blocker Use

PPI and H2 blocker use is captured on a specific eCRF page. PPI and H2 use will be summarized (separately) using the same methodology described in Section 3.10 (separate listings as well).

3.10.2 [redacted] Use

[redacted] use is captured on a specific eCRF page. [redacted] use will be summarized (separately) using the same methodology described in Section 3.10 (separate listings as well).

3.11 Dietary Phe

For 3 consecutive days prior to all scheduled study visits and blood Phe draws (see Schedule of Events Table 11 and Table 12), all participants will have a 3-day dietary intake assessment. Participants will follow their usual diet (including Phe, protein, and medical food intake) from baseline in the DEP to the end of the RWP.

The following information will be summarized for the 3-day DEP Baseline Period:

- Calories (KCAL)
- Total Protein (grams)
- Natural Protein (grams)
- Medical Food Protein (grams)
- Phe (grams).

The following information will be summarized for the DEP, RWP, and OLE Periods using the corresponding SAS:

- At each scheduled visit, the percent change from baseline in 3-day dietary Phe levels will be summarized
- For the DEP and RWP, the number and of times a participant's 3-day Phe exceeded the within ± 20% (inclusive) DEP baseline Phe criterion.

Note: the 3-day dietary endpoints to be summarized are the 3-day averages which are provided on the corresponding eCRF page.

For the DEP and OLE, the results will be presented overall. For the RWP, the results will be summarized by treatment group and responder/nonresponder within each treatment group.

3.12 Systemic Antibiotic Treatment

Systemic antibiotic treatment is defined as any concomitant medication antibiotic in which the route is oral, intravenous, or intramuscular. Any topical use is specifically not systemic. This 'topical use' includes antibiotics applied to any reachable area (most often the eye and ear canal).

The number and percent of participants using systemic antibiotic treatment will be summarized for the DEP, RWP, and OLE Periods using the corresponding SAS. For the DEP and OLE, and the results will be presented overall. For the RWP, the results will be summarized by treatment group and responder/nonresponder within each treatment group.

3.13 Treatment Compliance and Exposure

3.13.1 Compliance to Study Treatment

Compliance rates will be calculated for each of the 3 study periods (DEP, RWP, and OLE) using the following methodology:

For a participant, the compliance at each of the 3 individual dose levels received ($3x10^{11}$, $6x10^{11}$, $1x10^{12}$), will be calculated by the following:

$$\% \text{ Compliance} = 100 \times \frac{\#\textit{ of sachets given} - (\#\textit{ of sachets returned} + \#\textit{ of sachets lost or destroyed})}{\textit{Expected \# of sachets to be taken}}$$

Here:

# of sachets given: is determined by the number of kits given for the corresponding dose level

For each dose level kit there are 30 additional sachets provided in case of delayed visits, destroyed sachets, etc. In addition, for dose levels $6x10^{11}$ and $1x10^{12}$ there are 30 additional sachets in each kit for the lower dose levels of $3x10^{11}$ and $6x10^{11}$, respectively, in case a dose reduction is needed. If a participant requires a dose reduction, they will use the additional sachets from the existing kit until a new kit is received. Study drug administration and study drug accountability will be completed at the dose level in EDC.

# of sachets returned: is determined by the "Number of [individual dose level] sachets returned" field on the drug accountability CRF page for the corresponding dose level

# of sachets lost or destroyed: is determined by the "Number of sachets lost or destroyed" field on the drug accountability CRF page for the corresponding dose level

Expected # of sachets to be taken: is determined by taking the difference between the "Start date" and "Stop date" on the study drug administration CRF for the corresponding dose level and accounting for either the dose ramp schedule for the DEP and OLE or three sachets a day for the RWP.

For the DEP and OLE and the expected number of sachets follows the following dose ramp schedule. Dose frequency is linked to an individual sachet (e.g., TID is 3 sachets). De-escalation of dose is possible at any point during the dose ramp. When a participant has de-escalated the expected # of sachets to be taken will be calculated by the actual number of days spent at the dose level.

Table 8: DEP and OLE Dose Ramp Schedule

	Dose Level 1			**Dose Level 2**			**Dose Level 3**		
Timing	D1-9	D10-14	D15-21	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9
Dose (live cells)	[redacted] 3×10^{11}*	3×10^{11}	3×10^{11}	6×10^{11}	6×10^{11}	6×10^{11}	1×10^{12}	1×10^{12}	1×10^{12}
Frequency	QD, D1-3	QD, D10-11	TID	QD × 3 days	TID	TID	QD × 3 days	TID	TID
	BID, D4-6	BID, D12-14		BID × 4 days			BID × 4 days		
	TID, D7-9								

BID = twice per day; D = day; QD = once per day; TID = 3 times per day.
* See Section 4.3.1 of the study protocol for details.

For the RWP Period, the compliance rate will be the compliance rate for the corresponding dose level the participant was on during the RWP (i.e., dose modification is not allowed during the RWP).

For each of the 3 study periods (DEP, RWP, and OLE), compliance will be summarized using the SAS (defined in Section 3.5), with results also being presented by:

- For the DEP: By dose level
- For the RWP: By treatment group, responder/nonresponder subgroups, and treatment group within responder/nonresponder subgroups
- For the OLE: By dose level, responder/nonresponder subgroups

In addition to summary statistics for the compliance rate, the number and percentage of participants whose compliance rate is ≥ 75% will be reported.

3.13.2 Treatment Duration

For each of the 3 study periods treatment duration will be Last dose date – First dose date + 1.

For each of the 3 study periods (DEP, RWP, and OLE), treatment duration will be summarized using the SAS populations (defined in Section 3.5), with results also being presented by responder/nonresponder and treatment group within responder/nonresponder subgroups for the RWP and OLE.

Additionally for the DEP, the duration of time (i.e., days) that a participant was on a dose level that the participant discontinued from will be summarized by dose level and overall.

3.13.3 Distribution of SYNB1934v1 iTD in the RWP

The number and percentage of participants at each of the 3 individual dose levels ($3x10^{11}$, $6x10^{11}$, $1x10^{12}$) will be summarized for the RWP using the FAS population (defined in Section 3.5), with results also being presented by responder/nonresponder and treatment group within responder/nonresponder subgroups.

3.14 Efficacy Analysis

3.14.1 Part 1: DEP

3.14.1.1 Weekly DEP Substudy and Missing Data

Unless otherwise explicitly specified, the additional weekly blood Phe data collected in the subgroup of participants in the DEP Weekly Substudy (see Section 2.3.2) will not be considered when imputing missing blood Phe data in the DEP efficacy analyses.

3.14.1.2 Primary Efficacy Endpoint Analysis

The DEP primary efficacy endpoint is the percent change from DEP baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1. The last measurement is the participant's last Week 3 blood Phe level at the iTD of SYNB1934v1.

The mean percent change from baseline at the participant's iTD will be tested by an MMRM analysis. The MMRM model will have DEP baseline blood Phe level as a fixed effect. The dependent variable is

the percent change from baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1 (i.e., with duplicate blood Phe assessments being made at the last measurement).

Least-squares means for the percent change from baseline, along with the corresponding [redacted] confidence intervals and p-values testing a percent change from baseline equal to 0, will be calculated. The primary efficacy analysis will test the least-squares means percent change from baseline equal to 0 for the blood Phe level at the participants' last week (Week 3) at his or her established iTD.

At each of the scheduled assessments blood Phe will be drawn in duplicate. The MMRM will incorporate the participant repeated/duplicate measures. For the MMRM analysis, the results of the post-baseline duplicate samples from the Week 3 visit will be included in the statistical model (i.e., without averaging the results within a given visit before inclusion into the MMRM).

The MMRM analysis will be based on the restricted maximum likelihood method assuming an unstructured covariance structure to model the repeated-measures. A Kenward-Roger approximation will be used for the denominator degrees of freedom. If there is a convergence problem due to the unstructured covariance matrix, a variance components covariance structure will be used.

The DEP FAS will be the primary efficacy analysis population (defined in Section 3.5). As the primary efficacy analysis is considering only participants who reach an iTD (see Section 2.5.1) missing data for the DEP Week 3 blood Phe will generally be limited to those scenarios where the blood Phe sample is not analyzed. In the scenario that a participant is missing both blood Phe values at the DEP Week 3 for their iTD, the following algorithm will be applied:

- If the participant has a corresponding previous non-missing Week 3 blood Phe value at the iTD dose (e.g., the participant escalates to a higher dose then subsequently de-escalates back to a lower dose, which is their iTD) this value will be used.
- Otherwise, the value will remain missing, and an observed-case approach will be used.

3.14.1.3 Primary Efficacy Sensitivity and Supplementary Analysis

The following sensitivity and supplementary analyses will be performed for the primary efficacy endpoints.

- ***Analysis by iTD***: The primary efficacy endpoint analysis will be performed on each iTD subgroup.

- ***Imputation of Missing Data***: Multiple imputation will be used to impute missing blood Phe data during the DEP. The imputation of missing data analysis will assume the data is missing at random (MAR). The methodology for the multiple imputation is provided in Section 3.18.1. As discussed in Section 3.14.1.1, the imputation will not utilize the data from the DEP Weekly substudy. The analysis model will be the same model as the DEP primary efficacy endpoint analysis. The DEP FAS will be the primary efficacy analysis population for this analysis.

- ***Analysis of Covariance***: The primary efficacy endpoint will be analyzed using an analysis of covariance (ANCOVA) model. The percent change from DEP baseline in blood Phe level at the last

measurement of the iTD of SYNB1934v1 will be the dependent variable is a factor and DEP baseline Phe is a covariate. For this analysis the 2 Phe assessments occurring at the last measurement of the iTD of SYNB1934v1 will be averaged (i.e., if just 1 Phe assessment at this timepoint, that value will be used).

Least-squares means for the percent change from baseline overall dose level, along with the corresponding [redacted] confidence intervals and p-values testing a percent change from baseline equal to 0, will be calculated.

This will be an observed-case analysis using the DEP FAS population, missing data will not be imputed.

- ***Excluding Participants on a Dropped Dose***: The primary efficacy endpoint analysis will be performed on the DEP FAS using an observed case analysis which excludes participants who were.

- ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The primary efficacy endpoint analysis will be performed on the DEP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the DEP: Treatment discontinuation, antibiotic use, prohibited medication use.

- ***RWP PPS Analysis***: The primary efficacy endpoint analysis will be performed using the responder DEP PPS analysis set (defined in Section 3.5).

- ***MMRM Analysis by Dose Level***: The percent change in blood Phe level will be analyzed using an MMRM model. All available Week 3 blood Phe data will be used. The model will have percent change from baseline as the dependent variable. Dose level (as a categorical variable) and baseline blood Phe will be fixed effects. The MMRM analysis will be based on the restricted maximum likelihood method. A Kenward-Roger approximation will be used for the denominator degrees of freedom. The covariance structure for the repeated-measures will be a direct product of unstructured for the multiple Week 3 visits (i.e., a total of 5 possible Week 3 visits, 3 for each dose level and 2 for possible repeats of the 3×10^{11} and 6×10^{11} doses) and compound symmetry for the repeated assessments at a particular visit. As such the covariance structure will use the direct product structure "UN@CS" as defined in SAS PROC Mixed.

  This will be an observed-case analysis, this analysis will be performed using the DEP FAS and also using the responder RWP FAS population, missing data will not be imputed.

  If the MMRM analysis does not converge, the repeated measures at each blood Phe assessment will be averaged together and the average of the repeated measures will be used to calculate the percent change in blood Phe level, the dependent variable. The model will have percent change from baseline as the dependent variable (i.e., using the averaged value at Week 3 visit). The MMRM model will be the same (i.e., same set of fixed effects) with the exception that the covariance structure for the repeated-measures will be unstructured for the multiple Week 3 visits (i.e., a total of

5 possible Week 3 visits, 3 for each dose level). If this model does not converge a compound symmetry covariance structure will be used.

Additionally, histograms illustrating the DEP Week 3 change and percent change from baseline in blood Phe by treatment group will be produced, overall and by iTD.

3.14.1.4 Key Secondary Efficacy Endpoints Analysis

The key secondary efficacy endpoint analyses for the DEP are provided below. These analyses will be performed on the DEP FAS.

- ***Change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1***

  The key secondary endpoint, change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1, will be analyzed using an MMRM analysis similar to the analysis methodology model described for the DEP primary efficacy analysis (Section 8.4), with the dependent variable being the change in blood Phe level from DEP baseline.

- ***A ≥ 20% reduction from baseline in blood Phe level at any time in the DEP***

  The number and proportion of participants meeting this criterion will be summarized along with [redacted]). As discussed in Section 3.14.1.1, this analysis will not consider the additional blood Phe data from DEP Weekly substudy. Note this will consider all Week 3 post-baseline blood Phe values occurring in the DEP. This will be an observed-case analysis approach using the DEP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.

3.14.1.5 Key Secondary Sensitivity and Supplementary Analysis

- ***Change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1***
  - ***Analysis by iTD***: The analysis will be performed on each iTD subgroup**.**
  - ***Imputation of Missing Data***: Multiple imputation will be used to impute missing blood Phe data during the DEP. The imputation of missing data will assume the data is missing at random (MAR). The methodology for the multiple imputation and analysis is provided in Section 3.18.1. The analysis model will be the same model as the DEP key secondary endpoint analysis. This analysis will be performed on the DEP FAS population.
  - ***Analysis of Covariance:*** The change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1 endpoint will be analyzed using an ANCOVA model. The change from DEP baseline in blood Phe level at the last measurement of the iTD of SYNB1934v1 will be the dependent variable, using the average of the duplicate blood Phe levels (i.e., if one of the values is missing, the non-missing value will be used). The ANCOVA has Treatment as a fixed effect and DEP baseline Phe is a covariate. This will be an observed-case analysis using the DEP FAS population, missing data will not be imputed.

- ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The key secondary efficacy endpoint analysis will be performed on the DEP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the DEP: Treatment discontinuation, antibiotic use, prohibited medication use.
- ***RWP PPS Analysis***: The analysis will be performed using the responder RWP PPS analysis set (defined in Section 3.5).
- ***MMRM Analysis by Dose Level***: The change in blood Phe level will be analyzed using an MMRM model. All available Week 3 blood Phe data will be used. The model will have change from baseline as the dependent variable. Dose level (as a categorical variable) and baseline blood Phe will be fixed effects. The MMRM analysis will be based on the restricted maximum likelihood method. A Kenward-Roger approximation will be used for the denominator degrees of freedom. The covariance structure for the repeated-measures will be a direct product of unstructured for the multiple Week 3 visits (i.e., a total of 5 possible Week 3 visits, 3 for each dose level and 2 for possible repeats of the 3×10^{11} and 6×10^{11} doses) and compound symmetry for the repeated assessments at a particular visit. As such the covariance structure will use the direct product structure "UN@CS" as defined in SAS PROC Mixed.

  This will be an observed-case analysis, this analysis will be performed using the DEP FAS and also using the responder RWP FAS population, missing data will not be imputed.

  If the MMRM analysis does not converge, the repeated measures at each blood Phe assessment will be averaged together and the average of the repeated measures will be used to calculate the percent change in blood Phe level, the dependent variable. The model will have change from baseline as the dependent variable (i.e., using the averaged value at Week 3 visit). The MMRM model will be the same (i.e., same set of fixed effects) with the exception that the covariance structure for the repeated-measures will be unstructured for the multiple Week 3 visits (i.e., a total of 5 possible Week 3 visits, 3 for each dose level). If this model does not converge a compound symmetry covariance structure will be used.

- ***A ≥ 20% reduction from baseline in blood Phe level at any time in the DEP***
  - ***Analysis by iTD***: The analysis will be performed on each iTD subgroup**.**
  - ***Imputation of Missing Data***: Multiple imputation will be used to impute missing blood Phe data during the DEP. The imputation of missing data analysis will assume the data is missing at random (MAR). The methodology for the multiple imputation is provided in Section 3.18.1. The number and proportion of participants meeting this criterion will be summarized along with [redacted] based on the imputed data. This analysis will be performed on the DEP FAS population.
  - ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The key secondary efficacy endpoint analysis will be performed on the DEP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the DEP: Treatment discontinuation, antibiotic use, prohibited medication use.

- ***RWP PPS Analysis***: The analysis will be performed using the responder RWP PPS analysis set (defined in Section 3.5).

3.14.1.6 Control of Multiplicity

The overall Type I error rate for the DEP will be controlled at the 2-sided [redacted] for the primary endpoint. If the DEP primary endpoint analysis is significant at the 2-sided [redacted] the key secondary endpoint, change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1, will be tested at the 2-sided [redacted] level.

Note that the dichotomous DEP key secondary endpoint, a ≥ 20% reduction from baseline in blood Phe level at any time in the DEP, does not have an associated inferential test.

3.14.1.7 Secondary Efficacy Endpoints Analysis

The following are the analysis methods for the DEP secondary efficacy endpoints.

- ***Blood Phe level ≤ 360 µmol/L at any time of the iTD of SYNB1934v1***
- ***Blood Phe level ≤ 360 µmol/L at any time in the DEP***

For each of these 2 endpoints, the proportion of participants meeting the endpoint will be summarized along with [redacted] confidence intervals. This will be an observed-case analysis approach using the DEP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.

3.14.1.8 Exploratory Endpoints Analysis

The following are the analysis methods for the set of DEP exploratory endpoints. These analyses will be performed on the DEP FAS. For the dichotomous endpoints, this will be an observed-case analysis approach using the DEP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint. For the continuous endpoint, this will be an observed-case analysis. As discussed in Section 3.14.1.1, these analyses will not consider the additional blood Phe data from DEP Weekly substudy.

- ***A ≥ 20% reduction from baseline in blood Phe level at the third week of a participant's last 3 weeks at the iTD of SYNB1934v1***

  Note that if a participant has 2 Week 3 blood Phe visits at their iTD (i.e., the participant escalated in dose then de-escalated back to their iTD) both Week 3 values will be considered such that the participant can reach the ≥ 20% reduction at either of these assessments.

- ***Blood Phe level ≤ 600 µmol/L at any time of the iTD of SYNB1934v1***
- ***Blood Phe level ≤ 600 µmol/L at any time in the DEP***

  For each of these endpoints, the number and proportion of participants meeting the corresponding criteria will be summarized along with [redacted] For these endpoints using a ≤ 600 µmol/L criterion, the analysis will be performed on those participants with a baseline DEP blood Phe value > 600 µmol/L.

- ***Dose level at first blood Phe level ≤ 360 µmol/L***

  The distribution of the dose levels (i.e., $3x10^{11}$, $6x10^{11}$, $1x10^{12}$) when a participant first had a blood Phe level ≤ 360 µmol/L will be summarized. The percentage will be two presented ways:

  1) Based on the DEP FAS with a category for participants who did not reach a blood Phe level ≤ 360 µmol/L
  2) Based on the sub-set of participants who reached a blood Phe level ≤ 360 µmol/L.

- ***Dose level at first ≥ 20% reduction in blood Phe level***

  The distribution of the dose levels (i.e., $3x10^{11}$, $6x10^{11}$, $1x10^{12}$) when a participant first had a blood Phe reduction of level ≥ 20% will be summarized. The percentage will be presented ways:

  1) Based on the DEP FAS with a category for participants who did not reach a blood Phe level ≥ 20%
  2) Based on the sub-set of participants who reached a blood Phe level ≥ 20%.

- ***Change in weekly blood Phe levels during each step of the dose ramp***

  For the participants that participate in the DEP Weekly blood Phe substudy (see Section 2.3.2). Summary statistics of the Weekly blood Phe, change from baseline in blood Phe, and percent change from baseline in blood Phe will be provided for each week the blood Phe is assessed.

3.14.1.9 Subgroup Analyses

The DEP primary and key secondary efficacy analyses will be performed for the following subgroups (as defined in Section 3.5.1):

- Participants on sapropterin dihydrochloride (Kuvan) at the DEP baseline visit
- Genotypic phenotype values (GPV) subgroups.

Additionally, if a dose was discontinued (see Section 3.17) the DEP primary and key secondary efficacy endpoint analyses will be performed on the subgroup of participants who had an iTD of a discontinued dose. For the DEP primary endpoint analysis, the MMRM analysis would be performed including the participants who had an iTD corresponding to a discontinued dose(s), in addition to the participants with an iTD corresponding to the retained doses.

As a supplemental analysis, summary statistics for the following endpoints will be performed on the DEP FAS adolescent subgroup:

- Change and percent change from baseline in blood Phe level at last measurement of the iTD of SYNB1934v1.
- A ≥ 20% reduction from baseline in blood Phe level at any time in the DEP

3.14.2 Part 2: RWP

The efficacy analyses for the RWP will be based on the responder RWP FAS (defined in Section 3.5) unless explicitly stated otherwise.

3.14.2.1 Primary Efficacy Endpoint Analysis

The RWP primary efficacy endpoint is the change from RWP baseline to Week 4 in blood Phe level. An MMRM analysis will be used to compare the mean change in blood Phe level between the placebo and SYNB1934v1 dose groups. The MMRM model will have treatment group, Screening Blood Phe (as a dichotomous variable: ≤ / > 720 µmol/L), RWP baseline blood Phe level, iTD dose level, visit, and visit × treatment group (interaction effect), visit × iTD dose level (interaction effect), and visit × RWP baseline blood Phe level (interaction effect) as fixed effects. The MMRM will also incorporate the participant's repeated measures at visits Weeks 1, 3, and 4, with visit being treated as a categorical variable. Least-squares means for both treatment groups and the SYNB1934v1 treatment group difference from placebo, along with the corresponding [redacted] confidence intervals and p-values will be calculated for Weeks 1, 3, and 4. The primary efficacy analysis will compare the least-squares means treatment difference between SYNB1934v1 and placebo in the change from baseline in the blood Phe level at Week 4.

At each of the scheduled assessments, blood Phe will be drawn in duplicate. The MMRM will incorporate these participant repeated measures. For the MMRM analysis, the results of each of the post-baseline duplicate samples from a visit will be included in the statistical model (i.e., without averaging the results within a given visit before inclusion into the MMRM).

The MMRM analysis will be based on the restricted maximum likelihood method. A Kenward-Roger approximation will be used for the denominator degrees of freedom. The covariance structure for the repeated-measures will be a direct product of unstructured (i.e., for the visits) and compound symmetry for the repeated assessments at a particular visit (i.e., the subject-level repeated measures has 2 dimensions, post-baseline Weeks 1, 3, and 4, as well as the 2 assessments at each visit. As such the covariance structure will use the direct product structure "UN@CS" as defined in SAS PROC Mixed).

For the dependent variable, this will be an observed-case analysis using the responder RWP FAS population, missing data will not be imputed.

In the event that a participant is missing their RWP baseline blood Phe (i.e., which is their DEP iTD Week 3 blood Phe value), the participant's last non-missing DEP Week 3 blood Phe value (i.e., the most recent non-missing Week 3 blood Phe value from a previous dose level) will be used as the RWP baseline blood Phe covariate value (i.e., as specified in Section 3.3, the baseline RWP covariate value will be the mean of the corresponding duplicate blood Phe assessments). In the unlikely event that the participant does not have any non-missing DEP Week 3 blood Phe values, the blood Phe covariate value will remain missing, and the participant will be excluded from the RWP primary efficacy analysis, which is an observed case analysis. Note that the multiple imputation sensitivity analysis Section 3.14.2.3 will impute missing RWP baseline blood Phe.

3.14.2.2 Primary Efficacy Analysis Model Does Not Converge

If the RWP primary efficacy MMRM analysis does not converge, the repeated measures at each blood Phe assessment will be averaged together and the average of the repeated measures will be used to calculate the change from baseline in blood Phe level, the dependent variable. The model will have change from baseline as the dependent variable. The model will have percent change from baseline as the dependent variable (i.e., using the averaged value at each of the Week 1, 3, and 4 visits). The MMRM model will be the same (i.e., same set of fixed effects as described in Section 3.14.2.1) with the exception that the covariance structure for the repeated-measures will be unstructured to account for the within-subject Week 1, 3, and 4 assessments. If this model does not converge, a compound symmetry covariance structure will be used.

3.14.2.3 Primary Efficacy Sensitivity and Supplementary Analysis

The following sensitivity and supplementary analyses will be performed for the RWP primary efficacy endpoint.

- ***Imputation of Missing Data***: Multiple imputation methods will be used to impute missing blood Phe data during the RWP. The imputation of missing data analysis will be performed twice, once assuming the data is missing at random (MAR), and also assuming missing not at random (MNAR). The methodology for the multiple imputation is provided in Section 3.18.2. The analysis model will be the same model as the RWP primary efficacy endpoint analysis.

- ***Analysis of Covariance***: The primary efficacy endpoint will be analyzed using an analysis of covariance (ANCOVA) model. The change from RWP baseline to Week 4 in blood Phe level is the dependent variable, using the average of the duplicate Week 4 blood Phe levels (i.e., if one of the values is missing, the non-missing value will be used). The ANCOVA has treatment as a fixed effect and RWP baseline Phe is a covariate. This will be an observed-case analysis using the responder RWP FAS population, missing data will not be imputed.

- ***Responder RWP PPS Analysis***: The primary efficacy endpoint analysis will be performed using the responder RWP PPS analysis set (defined in Section 3.5).

- ***Responder Randomized set Analysis***: The primary efficacy endpoint analysis will be performed using the responder Randomized analysis set (defined in Section 3.5). Missing data will be imputed assuming the data is missing at random (MAR). The methodology for the multiple imputation is provided in Section 3.18.2.

- ***Responder RWP FAS Analysis Including Participants in a Dropped Dose***: The primary efficacy endpoint analysis will be performed using the responder RWP FAS analysis set (defined in Section 3.5) with the modification that participants whose dose was dropped at the DEP interim analysis are included in the analysis (i.e., this analysis will only be performed if one or more doses are dropped at the DEP interim analysis (see Section 3.17).

- ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The primary efficacy endpoint analysis will be performed on the responder RWP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the RWP: Treatment discontinuation, antibiotic use, prohibited medication use.

Additionally, histograms illustrating the Week 4 change and percent change from baseline in blood Phe by treatment group will be produced.

3.14.2.4 Key Secondary Efficacy Endpoint Analyses

The key secondary efficacy endpoint analyses for the RWP are provided below. These analyses will be performed on the responder RWP FAS.

- ***Percent change from DEP baseline in blood Phe level at Week 4***

  The percent change from DEP baseline to Week 4 in blood Phe level, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the percent change in blood Phe level from DEP baseline, and the DEP baseline blood Phe level as the baseline blood Phe level covariate. This will be an observed-case analysis using the DEP FAS population, missing data will not be imputed. Note that if the MMRM model does not converge, the same methodology as described for the RWP primary efficacy analysis (Section 3.14.2.1) will be applied.

- ***Change from DEP baseline to Week 4 in blood Phe level***

  The change from DEP baseline to Week 4 in blood Phe level, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the change in blood Phe level from DEP baseline, and the DEP baseline blood Phe level as the baseline blood Phe level covariate. This will be an observed-case analysis using the responder RWP FAS population, missing data will not be imputed. Note that if the MMRM model does not converge, the same methodology as described for the RWP primary efficacy analysis (Section 3.14.2.1) will be applied.

- ***Blood Phe level ≤ 360 µmol/L at Week 4***
  The proportion of participants meeting the key secondary endpoint, Blood Phe level ≤ 360 µmol/L at Week 4, will be tested between the placebo and SYNB1934v1 treatment groups using a Fisher's Exact test. This will be an observed-case analysis approach using the responder RWP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.

3.14.2.5 Key Secondary Sensitivity and Supplementary Analysis

  - The following are the set of sensitivity and supplementary analysis for the RWP Key secondary endpoints:

- ***Percent change from DEP baseline in blood Phe level at Week 4***

- ***Imputation of Missing Data***: Multiple imputation methods will be used to impute missing blood Phe data during the RWP. The imputation of missing data analysis will be performed twice, once assuming the data is missing at random (MAR), and also assuming missing not at random (MNAR). The methodology for the multiple imputation and analysis is provided in Section 3.18.2. The analysis model will be the same model as the corresponding key secondary endpoint analysis.
- ***Analysis of Covariance***: The percent change from DEP baseline to Week 4 in blood Phe level endpoint will be analyzed using an ANCOVA model. The percent change from DEP baseline to Week 4 in blood Phe level will be the dependent variable, using the average of the duplicate Week 4 blood Phe levels (i.e., if one of the values is missing, the non-missing value will be used). The ANCOVA has treatment as a fixed effect, and DEP baseline Phe is a covariate.
- ***RWP PPS Analysis***: The key secondary efficacy MMRM analysis will be performed using the responder RWP PPS analysis set (defined in Section 3.5).

  Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication: The key secondary efficacy endpoint analysis will be performed on the responder RWP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the RWP: Treatment discontinuation, antibiotic use, prohibited medication use.

- ***Change from DEP baseline to Week 4 in blood Phe level***
  - ***Imputation of Missing Data***: Multiple imputation methods will be used to impute missing blood Phe data during the RWP. The imputation of missing data analysis will be performed twice, once assuming the data is missing at random (MAR), and also assuming missing not at random (MNAR). The methodology for the multiple imputation and analysis is provided in Section 3.18.2. The analysis model will be the same model as the corresponding key secondary endpoint analysis.
  - ***Analysis of Covariance***: The change from DEP baseline to Week 4 in blood Phe level endpoint will be analyzed using an ANCOVA model. The change from DEP baseline to Week 4 in blood Phe level is the dependent variable, using the average of the duplicate Week 4 blood Phe levels (i.e., if one of the values is missing, the non-missing value will be used). The ANCOVA has treatment as a fixed effect, and DEP baseline Phe is a covariate. This will be an observed-case analysis using the responder RWP FAS population, missing data will not be imputed.
  - ***RWP PPS Analysis***: The key secondary efficacy MMRM analysis will be performed using the responder RWP PPS analysis set (defined in Section 3.18.2).
  - ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The key secondary efficacy endpoint analysis will be performed on the responder RWP FAS using an observed case analysis which excludes observations after the following intercurrent events occur in the RWP: Treatment discontinuation, antibiotic use, prohibited medication use.

- ***Blood Phe level ≤ 360 µmol/L at Week 4***
    - **Exact Logistic Regression**: The Blood Phe level ≤ 360 µmol/L at Week 4 endpoint will be tested between the placebo and SYNB1934v1 treatment groups using an exact logistic regression with treatment group as a factor and RWP Baseline Blood Phe (≤360/>360 µmol/L) as a dichotomous covariate. This will be an observed-case analysis approach using the responder RWP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.
    - **RWP PPS Analysis**: The Fisher's exact test will be performed using the responder RWP PPS analysis set.
    - ***Excluding Observations after Treatment Discontinuation, Antibiotic use, or Prohibited Medication***: The Blood Phe level ≤ 360 µmol/L at Week 4 endpoint will be tested via Fisher's Exact Test using an observed case analysis which excludes observations after the following intercurrent events occur in the RWP: Treatment discontinuation, antibiotic use, prohibited medication use. This will be an observed-case analysis approach using the responder RWP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.

3.14.2.6 Control of Multiplicity

The overall Type I error rate for the RWP will also be controlled at the [redacted] for the primary endpoint (i.e., DEP, and RWP, will each have a 2-sided Type I error rate of [redacted]). The control of multiplicity at [redacted] within each study part is consistent with this study having 3 parts, with each study part having a distinct set of objectives, endpoints, and analysis populations.

For the RWP, the 3 key secondary endpoints will be tested at the [redacted] level using a fixed sequential testing methodology in this order:

1) Percent change from DEP baseline to Week 4 in blood Phe level.

2) Change from DEP baseline to Week 4 in blood Phe level

3) Blood Phe level ≤ 360 µmol/mL at Week 4

These key secondary endpoints will be tested following this prespecified order only if the RWP primary efficacy endpoint null hypothesis is rejected. An endpoint analysis will be considered statistically significant if the corresponding test for that analysis and all previous endpoint analyses are statistically significant using a [redacted] Type I error rate [redacted]

3.14.2.7 Secondary Efficacy Endpoints Analysis

The additional secondary efficacy endpoint for the RWP is:

- ***A ≥ 20% reduction from DEP baseline in blood Phe level at Week 4.***

The proportion of participants achieving a ≥ 20% reduction in blood Phe level from baseline to Week 4 will be tested between the placebo and SYNB1934v1 treatment groups using a Fisher's Exact test.

This will be an observed-case analysis approach using the responder RWP FAS such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint.

3.14.2.8 Exploratory Endpoints Analysis

The following are the analysis methods for the set of Exploratory endpoints.

For the dichotomous endpoints, this will be an observed-case analysis approach such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint. For the continuous endpoint, this will be an observed-case analysis.

The following analyses will be performed on the responder RWP FAS

- ***Change from baseline to Week 1 in blood Phe level***

  The analysis of the change from RWP baseline to Week 1 in blood Phe level will be a part of the RWP primary efficacy analysis, described in Section 3.14.2.1 for responder RWP FAS.

- ***Percent change from baseline to Week 1, and Week 4 in blood Phe level***

  The percent change from RWP baseline to Week 1, Week 3, and Week 4 in blood Phe level, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the percent change in blood Phe level from RWP baseline, and the RWP baseline blood Phe level as the baseline blood Phe level covariate.

- ***Blood Phe level ≤ 600 µmol/L at Week 4***

  The proportion of participants meeting the Blood Phe level ≤ 600 µmol/L at Week 4 criteria, will be tested between the placebo and SYNB1934v1 treatment groups using a Fisher's Exact test.

The following analyses will be performed on the nonresponder RWP FAS.

- ***Change from baseline to Week 1 and Week 4 in blood Phe level***

  The analysis of the change from RWP baseline to Weeks 1 and 4 in blood Phe level will be analyzed as described in Section 3.14.2.1 using the nonresponder RWP FAS.

- ***Percent change from baseline to Week 1 and Week 4 in blood Phe level***

  The percent change in blood Phe level from RWP baseline to Week 1 and from RWP baseline to Week 4, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the percent change in blood Phe level from RWP baseline, and the RWP baseline blood Phe level as the baseline blood Phe level covariate.

- ***Blood Phe level ≤ 360 µmol/L at Week 4***

  The proportion of participants meeting the Blood Phe level ≤ 360 µmol/L at Week 4 criteria, will be tested between the placebo and SYNB1934v1 treatment groups using a Fisher's Exact test. The analysis will be repeated using a criterion of ≤ 360 µmol/L.

- ***A ≥ 20% reduction from DEP baseline in blood Phe level at Week 4***

  The proportion of participants achieving a ≥ 20% reduction in blood Phe level from baseline to Week 4 will be tested between the placebo and SYNB1934v1 treatment groups using a Fisher's Exact test.

- ***Change from DEP baseline to Week 4 in blood Phe level***

  The change from DEP baseline to Week 4 in blood Phe level, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the change in blood Phe level from DEP baseline, and the DEP baseline blood Phe level as the baseline blood Phe level covariate.

- ***Percent change from DEP baseline in blood Phe level at Week 4***

  The percent change from DEP baseline to Week 4 in blood Phe level, will be analyzed using an MMRM analysis similar to the model described for the RWP primary efficacy analysis (Section 3.14.2.1), with the dependent variable being the percent change in blood Phe level from DEP baseline, and the DEP baseline blood Phe level as the baseline blood Phe level covariate.

3.14.2.9 Subgroup Analyses

The RWP primary and key secondary efficacy analyses will be performed for the following subgroups (as defined in Section 3.5.1):

- Participants on sapropterin dihydrochloride (Kuvan) at the DEP baseline visit
- Genotypic phenotype values (GPV) subgroups.

Additionally, if a dose was discontinued (see Section 3.17) the RWP primary and key secondary efficacy endpoint analyses will be performed on the subgroup of participants who had been excluded from the RWP FAS.

As a supplemental analysis, summary statistics for the following endpoints will be performed on the RWP FAS adolescent subgroup:

- Change and percent change from RWP baseline to Week 4.
- Change and percent change from DEP baseline to Week 4.
- Blood Phe Level ≤ 360 µmol/L at Week 4.

3.14.3 Part 3: OLE

The following set of OLE endpoints will be performed separately for the responder and nonresponder OLE FAS. For the dichotomous endpoints, this will be an observed-case analysis approach such that if a participant is missing the data needed to determine the endpoint, they will be considered as not having met the endpoint. For the continuous endpoint, this will be an observed-case analysis.

- ***Change from DEP baseline in blood Phe levels***

At each scheduled OLE assessment timepoint, summary statistics for change from DEP will be provided.

Additionally, the change from DEP baseline in blood Phe levels will be analyzed using MMRM. The dependent variable is the change from DEP baseline in blood Phe level at each of the scheduled assessment timepoints in the OLE. Least-squares means for the change from baseline, along with the corresponding [redacted] intervals and p-values testing a change from baseline equal to 0, will be calculated.

Note that the least-squares means will use the observed margins approach (i.e., as opposed to equal weighting across the 3 iTD dose levels, the weighting will be proportional to the number non-missing percent change from baseline observations in each of the 3 iTD dose levels).

- ***Blood Phe level ≤ 360 µmol/L***
  At each scheduled OLE assessment timepoint, the number and proportion of participants meeting the corresponding criteria will be summarized along with [redacted] (i.e., [redacted]

  In addition to the analysis being performed on the responder/nonresponder OLE FAS populations, this analysis will be performed stratified on if a participant had a had an increase in 3-day dietary Phe (i.e. as discussed in Section 3.10.2) relative to DEP Baseline at each particular visit (i.e., at each timepoint summaries will be performed for the following groups:
    - Responder OLE
    - Nonresponder OLE
    - Responder OLE participants who had an increase in 3-day dietary Phe*
    - Responder OLE participants who did not have an increase in 3-day dietary Phe*
    - Nonresponder OLE participants who had an increase in 3-day dietary Phe*
    - Nonresponder OLE participants who did not have an increase in 3-day dietary Phe*

    * Note: if a participant has a missing value for the 3-day dietary Phe at the OLE timepoint or at DEP baseline, the participant will be excluded from the analysis.

- ***Change from DEP baseline in blood Phe levels over time***
  At each scheduled OLE assessment timepoint, summary statistics for change from DEP will be provided overall and stratified on the participants increase in dietary Phe (see Section 3.10.2) using the following categories:
    - 0 mg/kg/day (i.e., no increase)
    - 1-20 mg/kg/day
    - 21-40 mg/kg/day
    - >40 mg/kg/day.

- ***Change from DEP baseline in blood Phe level over time until first protocol-specified increase in dietary protein intake.***
  At each scheduled OLE assessment timepoint, summary statistics for change from DEP in blood Phe level will be provided. This analysis will exclude observations which occur after the participant had their first protocol-specified increase in dietary protein.
- ***Change from DEP baseline in dietary Phe intake***

  As discussed in Section 3.10.2, at each scheduled OLE assessment timepoint, participants will have a 3-day dietary intake assessment. At each scheduled visit, the percent change from baseline in 3-day dietary Phe levels will be summarized.

3.15 Safety Analysis

Safety will be evaluated by scheduled monitoring of AEs, vital signs, clinical laboratory measurements, ECGs, and PEs. By-participant listings of all measurements and parameters will be presented in tabular format, including absolute values and changes from baseline (if applicable), by study period, dose cohort (as applicable), and study day.

Safety results will be presented separately for each of the 3 study Periods (DEP, RWP, and OLE). For the DEP, safety analyses will be performed on the DEP SAS. For the RWP and OLE study parts, safety analyses will be performed on the corresponding SAS, with results being presented for the responder and nonresponder populations as well as the overall population.

Additionally, adverse events will also be presented across the 3 Study Periods (i.e., DEP, RWP, and OLE) as discussed in Section 3.15.1.7.

3.15.1 Adverse Events

Adverse events will be coded using the Medical Dictionary for Regulatory Activities (MedDRA) Version 25.1 (or higher) and will be classified using SOC and PT (with the exception of diarrhea and nausea AEs as discussed in Section 3.15.1.1). Severity of AEs and laboratory abnormalities will be graded using the National Cancer Institute Common Terminology Criteria for Adverse Events. Adverse events causality will be dichotomous: Related or Unrelated (i.e., as defined in Section 6.1.2 of the study protocol).

Treatment emergent adverse events (TEAEs) are any adverse events occurring or worsening in severity after the first administration of SYNB1934v1 study medication.

TEAEs will be tabulated by study Part, treatment group (for Part 2), system organ class, and preferred term. Incidence tables of participants with TEAEs will be presented for all TEAEs by maximum severity, serious AEs (SAEs), TEAEs assessed as related to IMP, and TEAEs resulting in discontinuation of study dosing.

The number and percentage of participants who experienced at least one TEAE as well as the number and percentage of participants who experienced each specific SOC and PT will be presented. For the

presentation of TEAE incidences, the SOCs will be sorted alphabetically, and within SOC, the preferred term (PT) will be used and presented by decreasing total frequency.

3.15.1.1 Adverse Events Severity

The severity rating of an AE refers to its intensity. The severity of each AE will be categorized using the NCI CTCAE. For any term that is not specifically listed in the CTCAE scale, intensity should be assigned a grade of 1 through 5 using the following CTCAE guidelines:

Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2: Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living.

Grade 3: Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activities of daily living.

Grade 4: Life-threatening consequences; urgent intervention indicated.

Grade 5: Death related to AE.

Exceptions to CTCAE criteria will be implemented for grading certain gastrointestinal-related TEAEs, as detailed in Table 9.

Table 9: Severity Assessment of Diarrhea and Nausea

Grade	Diarrhea[a]	Nausea
1	Increase of diarrhea, < 3 liquid stools a day over baseline; mild increase in ostomy output compared to baseline	Loss of appetite without alteration in eating habits and/or with use of planned anti-nausea medication
2	Diarrhea ≥ 3 liquid stools daily and limiting instrumental ADLs	Use of unplanned concomitant medications to increase oral intake
3	Diarrhea requiring hospitalization or limiting self-care ADLs	Inadequate oral caloric or fluid intake; tube feeding, TPN or hospitalization indicated (unchanged from CTCAE)
4	Life-threatening consequences; urgent intervention indicated (unchanged from CTCAE)	Not applicable
5	Death (unchanged from CTCAE)	Not applicable

ADLs = activities of daily living; CTCAE = Common Terminology Criteria for Adverse Events, TPN= Total parenteral nutrition.

[a] Diarrhea is defined as liquid stools without consistency in shape.

3.15.1.2 Adverse Events Causality

An adverse event will be assessed a causality of Related/Not Related to each the following:

- SYNB1934v1
- PPI.

3.15.1.3 Adverse Events of Special Interest

As discussed in Section 6.3 of the study protocol, adverse events of special interest (AESIs) for SYNB1934v1 are as follows:

- Hypersensitivity reactions due to SYNB1934v1
- Infections due to SYNB1934v1
- Grade ≥ 3 gastrointestinal AEs per protocol specified grading criteria, including nausea, vomiting, diarrhea, and abdominal pain.

3.15.1.4 Adverse Event Missing Date Imputation

The following rules apply when determining if an AE is treatment-emergent in the scenario where the start date is missing or partially missing. These rules provide an algorithm to "impute" a complete AE start date which will then be used to determine if the AE is treatment emergent.

AE Start Date Imputation

AE start date missing day and month:

- If the year is the same as the year of the treatment start date, the day and month of the date of treatment start date will be assigned to the missing fields
- If the year is prior to the year of the treatment start date, December 31 will be assigned to the missing fields
- If the year is after the date of the treatment start date, January 1 will be assigned to the missing fields.

AE start date missing month only:

- The day will be treated as missing, and both month and day will be replaced according to the above procedure.

AE start date missing day only:

- If the month and year are the same as the month and year of the treatment start date, the day of the treatment start date will be assigned to the missing day
- If either the year is before the year of the date of the treatment start date or if both years are the same, but the month is before the month of the treatment start date, the last day of the month will be assigned to the missing day

- If either the year is after the year of the treatment start date or if both years are the same, but the month is after the month of the treatment start date, the first day of the month will be assigned to the missing day.

AE start date completely missing:

- If the AE end date is complete and after the treatment start date, the treatment start date will be assigned to the missing start date
- If the end date is complete and before the treatment start date, the end date will be assigned to the missing start date
- Otherwise, the AE start date will be assigned the treatment start date.

If the end date is complete and the imputed start date as above is after the end date, the start date will be imputed by the end date.

AE End Date Imputation

The following is the algorithm to impute a missing AE end date.

AE end date year is missing or AE is "ongoing":

- If the AE end date year is missing or is indicated as "ongoing" the AE end date will not be imputed.

AE end date missing day and month:

- If the AE end date day and month is missing the month and day should be set to the earliest of the following: 31DECYYYY, date of death, participant's last visit date.

AE end date missing month only:

- The day will be treated as missing, and both month and day will be replaced according to the above procedure.

AE end date missing day only:

- If the AE end date day is missing the day should be set to the earliest of the following: last day of the month, date of death, participant's last visit date.

3.15.1.5 Adverse Event Summaries

The following are the set of adverse event summaries.

A. Overall summary of TEAEs

B. Incidence of TEAEs by SOC and PT

C. Incidence of TEAEs by SOC, PT, and Severity

D. Incidence of TEAEs by SOC, PT, and Related to SYNB1934v1

E. Incidence of TEAEs by SOC, PT, and Related to PPI

F. Incidence of TEAEs Leading to Drug Interruption by SOC and PT

G. Incidence of TEAEs Leading to Dose Reduction by SOC and PT

H. Incidence of TEAEs Leading to Drug Discontinuation by SOC and PT

I. Incidence of AESIs by SOC, PT, and Severity

J. Incidence of Serious Adverse Events by SOC and PT

K. Incidence of SYNB1934v1 Related Serious Adverse Events by SOC and PT

L. Incidence of PPI Related Serious Adverse Events by SOC and PT.

For the DEP only:

M. Incidence of Adverse Events prior to first dose of SYNB1934v1 by SOC and PT by Dose Level

N. Incidence of Adverse Events prior to first dose of SYNB1934v1 which are PPI related by SOC and PT by Dose Level

O. Incidence of TEAEs by SOC and PT by Dose Level

P. Incidence of TEAEs by SOC, PT, and Severity by Dose Ramp Period

Q. Incidence of TEAEs by SOC, PT, and Related to SYN1934 by Dose Ramp Period.

This set of analyses will be performed for each of the 3 study periods (DEP, RWP, OLE) separately using the corresponding SAS. The following conventions will be applied:

- For the DEP, the AEs summaries will be presented by SYNB1934v1 dose group and overall. The SYNB1934v1 dose group will be determined by the dose the participant was on at the TEAE start date
- For the RWP, the AEs summaries will be presented by SYNB1934v1 (i.e., each individual iTD dose and overall) and placebo and within these 2 treatment groups by: responder, nonresponder, and overall
- For the OLE, the AE summaries will be presented by responder, nonresponder, and overall. The SYNB1934v1 dose group will be determined by the dose the participant was on at the TEAE start date.

For the presentation of TEAE incidences, the SOCs will be sorted alphabetically, and within SOC, the PT will be used and presented by decreasing total frequency based on the total population.

For the overall summary of TEAEs (i.e., item **A** above), the following information will be provided:

- Number and percent of participants with a TEAE
- Number and percent of participants with a TEAE of Grade 1

- Number and percent of participants with a TEAE of Grade 2
- Number and percent of participants with a TEAE of Grade 3
- Number and percent of participants with a TEAE of Grade 4
- Number and percent of participants with a SYNB1934v1 related TEAE
- Number and percent of participants with a PPI related TEAE
- Number and percent of participants with an AE leading to treatment interruption
- Number and percent of participants with an AE leading to treatment discontinuation
- Number and percent of participants with an AE leading to dose reduction
- Number and percent of participants with an SAE
- Number and percent of participants with a SYNB1934v1-related SAE
- Number and percent of participants who died.

Additionally, for the adolescent subgroup, TEAEs will be summarized by PT and SOC for each of the 3 study periods (DEP, RWP, and OLE).

- For the DEP, the TEAEs summaries will be presented by SYNB1934v1 dose group and overall. The SYNB1934v1 dose group will be determined by the dose the participant was on at the TEAE start date.
- For the RWP, the TEAEs summaries will be presented by SYNB1934v1 (i.e., each individual iTD dose and overall) and placebo and within these 2 treatment groups.
- For the OLE the TEAEs summaries will be presented by SYNB1934v1 dose group and overall. The SYNB1934v1 dose group will be determined by the dose the participant was on at the TEAE start date.

3.15.1.6 Adverse Events by Dose Ramp Day

For the DEP, adverse events will be analyzed relative to the Dose Ramp Schedule (see Table 8).

Specifically, TEAEs will be summarized by dose level (3×10^{11}, 6×10^{11}, 1×10^{12}) and within dose level by the dose frequency schedule.

The Dose Ramp Days will be partitioned as follows for each of the 3 doses (i.e., as described in Table 4 of the study protocol):

- 3×10^{11}: Days 1-3, 4-6, 7-9, 10-11, 12-14, and 15-21 (as well as “Overall”)
- 6×10^{11}: Days 1-3, 4-7, and 8-21 (as well as “Overall”)
- 1×10^{12}: Days 1-3, 4-7, and 8-21 (as well as “Overall”).

The SYNB1934v1 dose level and Day period will be determined by the dose and dose ramp day the participant was on at the TEAE start date. The denominator will include all participants who received at least one dose within the corresponding dose ramp day period (i.e., this calculation will be based on a participant's first and last dose day within a dose level). This will also include TEAEs which occur while a participant on at particular dose level (e.g., if a participant is on 3×10^{11} at two different times during the DEP, both of these periods will be included when assessing participants TEAEs at the 3×10^{11} dose).

These summaries will be:

- Incidence of TEAEs by PT and SOC
- Incidence of TEAEs Leading to Dose Reduction by SOC and PT
- Incidence of TEAEs Leading to Drug Discontinuation by SOC and PT.

Additionally, within each dose ramp, a "per-day" rate of adverse events will be calculated with the numerator being the number of participants with the corresponding TEAE and the denominator being the sum of the number of days the participants were in the dose ramp period. The summaries will be:

- Per-Day rate of TEAEs by PT and SOC
- Per-Day rate of TEAEs Leading to Dose Reduction by SOC and PT
- Per-Day rate of TEAEs Leading to Drug Discontinuation by SOC and PT.

3.15.1.7 Adverse Event Summaries Across Study Periods

The analyses specified in Section 3.15.1.5 will be repeated based on the data across all periods (DEP, RWP, and OLE). TEAEs will be summarized across study periods by dose group: placebo, $3x10^{11}$, $6x10^{11}$, $1x10^{12}$ and SYNB1934v1 overall. The dose group will be determined by the dose the participant was on at the TEAE start date.

3.15.1.8 GI Daily Diary Card

As discussed in Section 5.5.1 of the study protocol, participants will fill out a diary card during screening, DEP, and RWP to allow for enhanced capture of the gastrointestinal (GI) AEs. This diary will contain the GI event description start/stop date and the question "*how much did this event bother you?*" with the response being a 0–10-point numeric rating scale with 0 bothered" and 10 bothered". When the investigator records this event as an AE, the following summaries of the tolerability will be performed:

- For GI TEAEs with a start date in the Screening Period, the tolerability will be summarized by PT and overall.
- For GI TEAEs with a start date in the DEP, the tolerability will be summarized by PT and overall, for each iTD and overall.
- For GI TEAEs with a start date in the RWP, the tolerability will be summarized by PT and overall, for treatment group, and with the active arm, for each iTD and overall.

Note: the other information provided in the GI Daily Diary Card will be incorporated in the adverse event database. For AEs occurring over multiple days, the highest bothersomeness score will be recorded. Should the investigator determine that the event recorded on the daily diary card is not an adverse event, the corresponding GI Daily Diary card entry, including the bothersomeness data, will not be recorded in the adverse event database.

3.15.2 Clinical Laboratory Evaluation

Continuous laboratory data will be summarized using descriptive statistics of actual values and changes from baseline over time at each scheduled assessment timepoint (i.e., as defined in the Schedule of Events; Table 11and Table 12). These data will also be categorized as low, normal, or high based on the reference ranges of the central laboratory.

The set of clinical laboratory tests are provided in Appendix 2. Note, the lab parameter Tyr, will also be summarized as a clinical laboratory test, but in a separate table.

For each of the 3 study periods (i.e., DEP, RWP, and OLE), shift tables for hematology and serum chemistry parameters summarizing the number and percentages of participants who had both DEP baseline and at least one post baseline measurements will be displayed (i.e., using the DEP baseline as the reference for all 3 study parts). Both the lowest and highest value of post-baseline assessments will be used. For this analysis, any lab collection performed on unscheduled analysis visits will be included. The set of hematology and serum chemistry parameters which have corresponding Low/Normal/High assessments will be included.

For the RWP the results will be presented by treatment group; and presented overall for the DEP and OLE.

3.15.3 Vital Signs, Weight and Height

Semi-supine vital signs (systolic blood pressure, diastolic blood pressure, pulse, and body temperature), weight, and height will be collected as specified in the Schedule of Events (Table 11 and Table 12). Participants are required to remain in the semi-supine position for at least 5 minutes prior to obtaining vital signs. Body mass index will be calculated based on weight and height as specified in the Schedule of Events.

This data will be summarized using descriptive statistics of actual values and changes from baseline over time at each scheduled assessment timepoint.

3.15.4 ECG

Supine single 12-lead electrocardiograms (ECGs) will be performed at baseline as specified in the Schedule of Events (Table 11 and Table 12). A listing of the ECG data will be presented.

3.15.5 Physical Examination

A physical examination will be performed at screening only, these results will be presented in a listing.

3.16 Sample Size Justification

Approximately [redacted] participants will be enrolled in the DEP to ensure that approximately [redacted] are randomized into the RWP. This assumes a dropout rate of [redacted] and approximately [redacted] of participants are responders. If, during the course of the study, it becomes apparent that the assumptions were not accurate, or a dose level is dropped after the interim analysis, the number of participants enrolled into the DEP may be adjusted. The study has over [redacted] in testing that the percent mean change from baseline at the last measurement of the iTD in blood Phe level is different from 0. This assumes a mean percent reduction in blood Phe levels of [redacted] a standard deviation of [redacted] drop-out rate, and a 2-sided Type I error rate of [redacted] mean percent reduction and standard deviation in blood Phe values used in the power calculations are based on results from a similar study in participants with PKU FDA (2007).

A sample size of [redacted] in the responder RWP FAS yields approximately [redacted] power to detect a difference in the mean change in blood Phe level from baseline to Week 4 between the SYNB1934v1 and placebo treatment groups. This assumes a mean treatment group difference in blood Phe of -[redacted] between the SYNB1934v1 and placebo groups, a change from baseline standard deviation of [redacted] 1:1 randomization of SYNB1934v1 and placebo, a [redacted], and a 2-sided Type I error rate of [redacted]. The power was calculated via a t-test. The mean treatment group difference and standard deviation in blood Phe values used in the power calculations are based on results from a similar study in participants with PKU FDA (2007).

3.17 Interim Analysis

Once the first [redacted] participants have attained an iTD and completed the DEP or the [redacted] has a baseline DEP and has been in the study for [redacted], whichever occurs earlier, the DEP data will be evaluated by an independent DMC. The DMC will use the below pre-specified set of criteria based on both safety and efficacy to determine whether the dose regimen is appropriate or whether any doses should be discontinued as well as whether the study should be stopped for futility. Enrollment will not be paused during the interim analysis.

If a dose level is dropped/discontinued, participants who are in the DEP and have an iTD corresponding to a dropped dose will not be randomized into the RWP but will instead enter the OLE directly. In addition, participants who are in the OLE at a dose level that has been dropped will be transitioned to the highest dose level retained.

The size of the DEP interim analysis cohort and corresponding criteria for dropping a dose (Table 10) provide reasonable operating characteristics for proper determination if one or more doses should be dropped.

Table 10: DMC Criteria for Dose Changes and Futility

Evaluation by DMC	Criteria to Drop the Dose

1×10^{12} dose	< 20% of the participants who attain an iTD are at the 1×10^{12} dose *OR* > 75% of participants who have 1×10^{12} as their iTD had a greater Phe reduction at their 6×10^{11} dose versus their 1×10^{12} dose.
6×10^{11} dose Note: considered only if the Drop 1×10^{12} Criteria has been met	< 30% of the participants who attain an iTD have 6×10^{11} or 1×10^{12} as their iTD *OR* > 75% of participants who have 6×10^{11} or 1×10^{12} as their iTD had a greater Phe reduction at their 3×10^{11} dose versus their 6×10^{11} dose
Stop for futility	<30% of participants enrolled into the DEP attain an iTD and complete the DEP *OR* < 25% of participants who attain an iTD and complete the DEP are responders

Note: these dose-dropping criteria are only applied for the first interim analysis.

DEP = dose-escalating, open-label period; DMC = data monitoring committee; iTD = individually titrated dose; Phe = phenylalanine

In the event that, based on the interim analysis results utilizing the criteria provided in Table 10, the DMC recommends dropping either the 1×10^{12} or the 1×10^{12} and 6×10^{11} doses, the RWP FAS will be modified as described in Section 3.5.

In addition, the DMC will review interim data at defined intervals in the study (i.e., as defined in the DMC charter). The Sponsor will remain blinded to the subject-level blood Phe levels during the DEP and RWP but will have access to the DEP interim analysis results.

The data from the OLE (Part 3) will be reviewed for safety and tolerability on an ongoing basis.

3.18 Multiple Imputation Methodology

This section describes the methodology for multiple imputation for missing blood Phe data for both the DEP and RWP Periods. Based on previous studies in this indication, FDA (2007), it is anticipated that the amount of missing data will be relatively small ([redacted]).

3.18.1 Multiple Imputation of Blood Phe for the DEP

For sensitivity and supplementary analysis of the DEP primary and key secondary efficacy endpoint analyses, MI will be used to handle missing. The following is a summary of the Missing at Random imputation method being used for the DEP.

Missing Blood Phe values will be imputed via multiple imputation (MI) assuming MAR for each scheduled assessment visit (DEP Baseline and Week 3). Note that at each scheduled assessment visit there are duplicate blood Phe measurements and Week 3 refers to the participants last Week 3 blood Phe value at the iTD.

Fifty imputed datasets will be created.

The following are the set of variables to be used in the multiple imputation model:

- Screening blood Phe
- Previous DEP Baseline Blood Phe values. For the duplicate blood Phe measures occurring at each scheduled visit, they will be "ordered" by the corresponding vial number:
    - DEP BaselineA and DEP BaselineB
    - Week 3A and Week3B

Here the "A" values correspond to the duplicate blood Phe value with the smallest vial number and the "B" values correspond to the duplicate blood Phe value with the largest vial number. If there is just a single corresponding vial for an assessment it will be assigned "A". Week 3 refers to the participants last Week 3 blood Phe value at the iTD.

The imputations will be performed using monotone linear regression imputation methods which will impute the participants' blood Phe values at each of the scheduled assessment visits in the study (including the duplicate assessments at each scheduled visit).

If the missing data does not follow a monotonic pattern, a sequential approach to imputing the data via a Markov chain Monte Carlo (MCMC) method to produce a monotone missing data pattern will be applied using the MCMC impute=monotone option in Proc MI (SAS System). Then the monotone linear regression imputation methods will be applied.

Note: if the multiple imputation model does not converge or produce estimates (i.e., due to over-specification) the set of imputation variables may be modified.

Based on the multiple imputed complete blood Phe datasets, the change and percent change from baseline in blood Phe levels will be calculated and the corresponding analyses performed and combined (i.e., via MIANALYZE in SAS).

3.18.2 Multiple Imputation of Blood Phe for the RWP

For sensitivity and supplementary analysis of the RWP primary and key secondary efficacy endpoint analyses, MI methods will be used to handle missing. The following is a summary of the two imputation methods being used in the RWP, Missing at Random (MAR) and Missing Not at Random (MNAR).

The MAR and MNAR MI will each create 50 imputed datasets. The imputed datasets will be analyzed using the specified primary or key secondary efficacy endpoint analysis methodology. The results across the multiple imputed data sets will be combined using SAS Proc MIANALYZE.

3.18.2.1 Missing at Random Multiple Imputation

Missing Blood Phe values will be imputed via multiple imputation (MI) assuming MAR for each of the following assessment visits (DEP Screening, DEP Baseline, RWP Baseline, RWP Week 1, RWP Week 3, and RWP Week 4). Note that at each scheduled assessment visit there are duplicate blood Phe measurements.

Fifty imputed datasets will be created.

The following are the set of variables to be used in the multiple imputation model:

- DEP Screening blood Phe
- DEP Baseline blood Phe (i.e., using the average of the duplicate values)
- iTD Dose level
- Randomized Treatment group (SYNB1934v1 or placebo)
- Previous RWP Baseline blood Phe values. For the duplicate blood Phe measures occurring at each scheduled visit, they will be "ordered" by the corresponding vial number:
    - RWP BaselineA and RWP BaselineB
    - Week 1A and Week1B
    - Week 3A and Week3B
    - Week 4A and Week4B

Here the "A" values correspond to the duplicate blood Phe value with the smallest vial number and the "B" values correspond to the duplicate blood Phe value with the largest vial number. If there is just a single corresponding vial for an assessment it will be assigned "A".

The imputations will be performed using monotone linear regression imputation methods which will impute the participants' blood Phe values at each of the scheduled assessment visits in the study (including the duplicate assessments at each scheduled visit).

If the missing data does not follow a monotonic pattern, a sequential approach to imputing the data via a Markov chain Monte Carlo (MCMC) method to produce a monotone missing data pattern will be applied using the MCMC impute=monotone option in Proc MI (SAS System). Following the method described in Smith (2017) the non-monotonic data imputation will be performed "by" the categorical treatment group variable. Then the monotone linear regression imputation methods will be applied.

Note: if the multiple imputation model does not converge or produce estimates (i.e., due to over-specification) the set of imputation variables may be modified.

Based on the complete Blood Phe datasets, the change and percent change from baseline in blood Phe levels will be calculated.

3.18.2.2 Missing Not at Random Multiple Imputation

Missing Blood Phe values will be imputed via multiple imputation (MI) assuming MNAR for each scheduled assessment visit (RWP Baseline, Week 1, Week 3, and Week 4). Note that at each scheduled assessment visit there are duplicate blood Phe measurements.

The imputation of monotonic missing scores in the SYNB1934v1 treatment group will be based on the distribution in the placebo group. This method assumes that the trajectory of withdrawals from the SYN1934 treatment arm follows the distribution of the placebo participants. The MNAR option in Proc MI (SAS system) will be used to impute the placebo distribution as described (Yuan, 2014).

Fifty imputed datasets will be created.

The set of variables for this MNAR multiple imputation model is the same set as defined for the MAR multiple imputation (Section 3.18.2.1) with the exception of Treatment group, which is not included in the MNAR imputation (i.e., the placebo distribution is used to impute the data).

The imputations will be performed using monotone linear regression imputation methods which will impute the participants' blood Phe values at each of the scheduled assessment visits in the study (including the duplicate assessments at each scheduled visit).

If the missing data does not follow a monotonic pattern, a sequential approach to imputing the data via a Markov chain Monte Carlo (MCMC) method to produce a monotone missing data pattern will be applied using the MCMC impute=monotone option in Proc MI (SAS System). Then the monotone linear regression imputation methods will be applied.

Note: if the multiple imputation model does not converge or produce estimates (i.e., due to over-specification) the set of imputation variables may be modified.

3.18.3 Multiple Imputation Random Number Seeds

The following random number seeds will be used for the multiple imputations; these seeds were randomly generated.

- OLE MAR: 337623
- RWP MAR: 599067
- RWP MNAR: 658167

3.19 Tables Listings and Figures

The set of tables, listings, figures, and associated shells will be provided in a separate document.

3.20 Changes from the Protocol Planned Analyses

The following are the set of changes from the protocol planned analyses as specified in the study protocol.

These changes will be incorporated into the next study protocol amendment.

- In Section 2.2.2.1, the handling of the scenario when a participant in the DEP de-escalates after RWP randomization is described.
- In Section 2.5.1, the intercurrent events for the estimands were updated. In particular, participants whose dose is dropped during the interim analysis will not be excluded from the analyses.
- In Section 3.5, the DEP FAS was updated to no longer exclude participants whose dose was dropped at the interim analysis.
- In Section 3.5, the DEP FAS was updated to exclude those participants who did not take at least one dose of DEP study medication.
- In Section 3.5, the RWP FAS, responder RWP FAS, and nonresponder RWP FAS were updated to exclude those participants whose dose was dropped at the DEP interim analysis.
- In Section 3.5, the RWP FAS, responder RWP FAS, and nonresponder RWP FAS were updated to exclude those participants who did not take at least one dose of RWP study medication.
- In Section 3.14.1.2, the DEP primary efficacy analysis, iTD was removed as a covariate from the MMRM.
- In Section 3.14.2.1, the RWP primary efficacy analysis, Screening blood Phe was added as a covariate to the MMRM (i.e., as a dichotomous variable: $\leq$ / $>$ 720 µmol/L).
- In Section 3.14.2.6, the testing order of the 3 key secondary endpoints in the RWP was not consistent within the study protocol between the synopsis and Section 8.9 of protocol. The correct testing order (i.e., consistent with Section 8.9 of the study protocol) is provided in this SAP.

4 REFERENCES

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Smith, C., Kosten S. (2017). "Multiple Imputation: A Statistical Programming Story." PharmaSUG: 2017 - Paper SP2001.

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5 APPENDIX 1: SCHEDULE OF EVENTS

Table 11: Schedule of Events: Dose-escalation and Randomized Withdrawal (Parts 1 and 2)

		DEP (Part 1)			RWP (Part 2)		
Description/Week[a]	**Screen[b]**	**Study/DEP Baseline**	**iTD-Finding Period**	**End of DEP[b] (RWP Baseline)[c]**	**Wk 1**	**Wk 3**	**Wk 4 (OLE Baseline)[c]**
Study Day	**-45 to -1**	**1**	**Up to 105 days[d]**	**21 days after iTD**	**7**	**21**	**28**
Assessment[b] (Protocol Section)							
Informed consent (11.5)	●						
Medical history and prior medications (5.2)	●						
Vital signs (SBP/DBP, pulse, body temperature, height, weight[e]) (5.5.2)	●	●		●			●
Physical examination (5.2)	●						
FSH test (postmenopausal women only) (5.5.3)	●						
Pregnancy test (WOCBP only)[f] (5.5.3)	●	●		●			●
Record concomitant medications (4.3.4)	●	●	●	●	●	●	●
Adverse event reporting (5.5.1)	●	●	●	●	●	●	●
Weekly contact by site personnel (telephone, text, email)			●	●	●	●	●
Randomization (8.11)				●			
Blood Phe[g] and Tyr (5.4.1)	●	●	●	●	●	●	●
3-Day dietary intake assessment[h] (3.3)		●	●	●	●	●	●
Administer PPI once per day (4.3.4.1)	●[i]	●	●	●	●	●	●
IMP immediately after meals[j] (3.2)		●	●	●	●	●	●
Laboratory tests (hematology/CBC with differential, CRP, serum chemistry, urinalysis) (5.5.3)	●	●		●			●
Electrocardiogram (supine for 5 minutes)(5.5.2)	●						
Immunogenicity sample[k] (5.2)		●					
Optional fecal sample for microbiome analysis[l] (5.2)	●						●

AE = adverse event; CBC = complete blood count; CRP = C-reactive protein; DEP = dose-escalating period; DBP = diastolic blood pressure; FSH = follicle-stimulating hormone; IMP = investigational medicinal product; iTD = individually titrated dose; Phe = phenylalanine; PPI = proton pump inhibitor; RWP = randomized withdrawal period; SBP = systolic blood pressure; Term = termination; Tyr = tyrosine; WOCBP = women of childbearing potential.
a Weeks are relative to the study period (i.e., each part begins with Week 1). All assessments/visits may be conducted within a 3-day window.

b All visits/assessments may be performed in the clinic or by a home healthcare professional at an alternate location (e.g., home, office, etc.), except for screening and end of DEP which must either be in person or via a virtual platform capable of completing all scheduled events including examination.
c Participants who discontinue prematurely during the DEP will complete an early termination visit 30 days after the last dose of IMP as described in Table 6 of the study protocol. Participants who discontinue prematurely during RWP will complete RWP study measures as scheduled prior to the early termination visit. Early termination visits are described in Section 5.6 of the study protocol.
d Participants may titrate down to the previous dose level during the iTD period if there is difficulty tolerating a higher dose level or at the investigator's discretion. Visits and blood draws occur after a participant has been at a dose level for 3 weeks (including the ramp of that dose level as shown in Table 4 of the study protocol). Participants must remain on their iTD for 3 weeks before proceeding to Part 2. Participants cannot up-titrate again until the OLE.
e Height and weight measured only at screening.
f Serum pregnancy test at screening; urine pregnancy test at other visits.
g All blood Phe should be drawn in duplicate. Baseline samples in DEP and RWP must be taken prior to the first doses in those periods. In Part 1, blood Phe will be measured every 3 weeks after a change in dose level during the iTD-finding period. In Part 2, blood Phe will be measured at Weeks 1, 3, and 4. See Section 5.4.1 of the study protocol of the protocol for more information. Patients enrolled in the optional DEP weekly substudy will have weekly blood Phe during the DEP. See Section 5.4.2 of the study protocol for more information.
h A 3-day dietary intake assessment will occur for 3 consecutive days prior to scheduled study visits and blood Phe draws. See Diet Manual for more information.
i The first dose of a PPI will be taken on or before Day –7 and continue daily as described in Section 4.3.4.1 of the study protocol.
j Investigational medicinal product should be taken within 30 minutes of finishing a meal. See the Diet Manual for further details.
k Samples will be used for monitoring anti-drug antibodies as needed (see Section 6.3 of the study protocol).
l Fecal samples can be done at any time during the screening period, no less than 5 days prior to baseline visit.

Table 12:Schedule of Events: Part 3, Open-Label Extension Period and Follow-up

	Quarterly Visits	End of Study	Early Term.
	Every 90 days ± 5 days	at least 30 days after last dose	at least 30 days after last dose
Assessment[a] (Protocol Section)			
Vital signs (SBP/DBP, pulse, body temperature) (5.5.2)	●	●	
Pregnancy test (WOCBP only)[b] (5.5.3)	●	●	
Record concomitant medications (4.3.4)	●	●	●
Adverse event reporting (5.5.1)	●	●	●
Blood Phe[c] and Tyr (5.4.1)	●		
3-Day dietary intake assessment[d] (3.3)	●	●	
Administer PPI once per day (4.3.4.1)	●		
IMP immediately after meals (TID) (3.2.3)	●		
Laboratory tests (hematology/CBC w/differential, CRP, serum chemistry) (5.5.3)	●	●	
Optional fecal sample for microbiome analysis (5.2)		●	

AE = adverse event; CBC = complete blood count; CRP = C-reactive protein; DEP = dose-escalating period; DBP = diastolic blood pressure; IMP = investigational medicinal product; Phe = phenylalanine; PPI = proton pump inhibitor; RWP = randomized withdrawal period; SBP = systolic blood pressure; Term = termination; TID = 3 times per day; Tyr = tyrosine; WOCBP = women of childbearing potential.

a All visits/assessments may be performed in the clinic or by a home healthcare professional at an alternate location (e.g., home, office).

b Urine pregnancy test at quarterly visits.

c All blood Phe should be drawn in duplicate.

d A 3-day dietary intake assessment will occur immediately before all scheduled study visits.

6 APPENDIX 2: CLINICAL LABORATORY TESTS

Hematology (CBC with differential)	Basophils% Basophils Eosinophils% Eosinophils Hematocrit Hemoglobin Lymphocytes% Lymphocytes Mean corpuscular hemoglobin Mean corpuscular volume Monocytes% Monocytes Neutrophils% Neutrophils Platelet count Red blood cells White blood cells	**Serum chemistry**	Glucose BUN Creatinine with eGFR Sodium Potassium Chloride Calcium Total protein Albumin Fractionated bilirubin (total direct and indirect) Follicle-stimulating hormone (for postmenopausal women only)[a] Alkaline phosphatase Aspartate aminotransferase Alanine aminotransferase Pregnancy (for WOBCP only) Glucose C-reactive protein
Urinalysis	Specific gravity pH Glucose Bilirubin Ketones Occult blood Protein Nitrite Leukocyte esterase Pregnancy (for WOBCP only)		

Abbreviations: BUN = blood urea nitrogen; eGFR = estimated glomerular filtration rate; WOBCP = women of childbearing potential.

a Performed at screening only.

7 APPENDIX 3: DMC Deliverables

The analysis methodology for the DMC deliverables will be the methods as outlined in this SAP. The set of tables, figures and listings to be provided to the DMC at each of the scheduled meetings will be outlined in a separate DMC SAP document, and will follow the table, figure and listing shells document supporting this SAP. Note that some of the tables, figures, and listings may not be provided at all meetings due to a lack of data for a specific study period (e.g., there may be limited data in the OLE study period at the initial DMC meetings).